Supplementary Data

Supplementary Materials and Methods

Human very small embryonic-like cell isolation

Human very small embryonic-like (HuVSEL) cells were isolated from peripheral blood mononuclear cells following an established mobilization and leukapheresis process. Apheresis products were collected under an IRB approved protocol at NeoStem's laboratory in Cambridge, MA, or were purchased commercially (AllCells, Alameda, CA). Healthy Caucasian males were recruited as donors and screened for metabolic or infectious diseases, use of drugs and tobacco, and obesity. For 3 days before cell collection, each donor received daily subcutaneous injections (480 μg/day) of granulocyte colony-stimulating factor (NEUPOGEN®; Amgen, Thousand Oaks, CA) to facilitate mobilization of VSEL cells from the bone marrow to the blood stream. Apheresis mononuclear cell collection was performed on the fourth day, processing two to three total blood volumes.

Apheresis products were processed at NeoStem. Products were first fractionated by elutriation on an Elutra cell separation system (Terumo BCT, Lakewood, CO), enriching for small cells (35 to 70 mL/min counterflow). Elutriated cells were further enriched by positive selection on an AutoMACS Pro cell separator using pooled anti-CD34 and anti-CD133 microbeads (Miltenyi Biotec, Auburn, CA). The enriched cells were then purified by fluorescence activated cell sorting (FACS) using a high speed Moflo XDP cell sorter (Beckman Coulter, Miami, FL) equipped with four lasers (488, 642, 405, and 355 nm). The forward scatter (FSC) threshold was set low to ensure inclusion of small cells. Cells were analyzed and sorted using a sequential gating strategy. An initial gate was set on side scatter (SSC) versus 7-AAD. All live (7-AAD negative) cells were then displayed on a FSC versus SSC plot to exclude objects under 2 μm such as platelets. Live cells larger than 2 μm were then gated for Lin^- Glycophorin A $(CD235a)^-$ $CD41^-$ cells, which were then applied to a fourth plot of CD34/CD133 versus CD45 (CD34 and CD133 were both APC conjugated, to label cells that are $CD34^+$ and/or $CD133^+$). From this fourth plot, $CD34/CD133^+$ $CD45^-$ VSEL cells were selected and then displayed on a SSC versus FSC plot to reveal their location compared with standardized beads (2, 5, and 10 μm) to confirm their size. Unstained cells and isotype-negative control antibodies were used to set photomultiplier tube voltages for baseline fluorescence and to set quadrant statistics for analyzing positive fluorescence above baseline. FACS-purified VSEL cells were typically over >90% pure. Cells were frozen and shipped by overnight courier to the University of Michigan without any demographic information attached.

Murine very small embryonic-like cell isolation

Murine very small embryonic-like (MuVSEL) cells ($Lin^-Sca\text{-}1^+CD45^-$ cells) were isolated from C57BL/6-Tg(CAG-EGFP)131Osb/LeySopJ mice (Jackson Laboratory, Bar Harbor, ME) using a BD FACSAria™ (BD Biosciences, San Jose, CA) as reported previously [1]. The following antibodies (BD Biosciences) were used to stain these cells: biotin-conjugated rat anti-mouse Ly-6A/E (Sca-1) (clone E13-161.7) and streptavidin-PE-Cy5-conjugated secondary antibody, anti-CD45R/B220-PE (clone RA3-6B2), anti-Gr-1-PE (clone RB6-8C5), anti-TCRαβ PE (clone H57-597), anti-TCRγζ PE (clone GL3), anti-CD11b PE (clone M1/70), anti-Ter-119 PE (clone TER-119), and anti-CD45-APC-Cy7 (clone 30-F11). FACS-purified MuVSEL cells were typically over >90% pure.

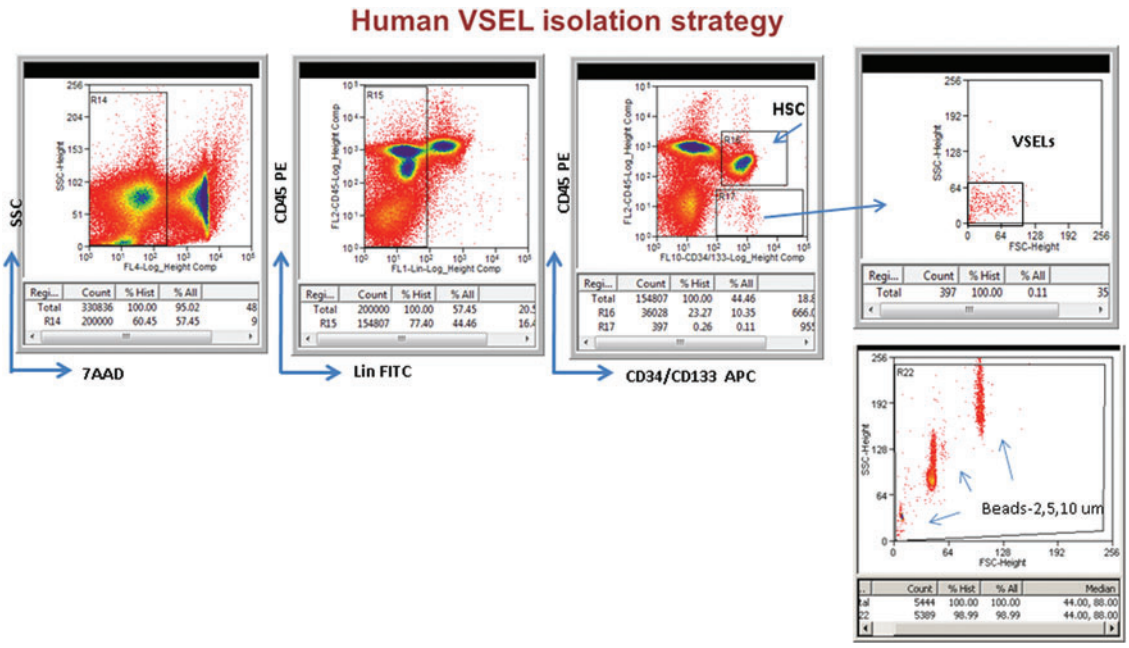
Mouse and human VSEL cell culture and differentiation in vitro

Human and mouse VSEL cells were cultured and differentiated as previously described [2] with some modifications. Briefly, C2C12 cells were cultured in a growth medium (HG-DMEM; Invitrogen, Grand Island, NY) containing 10% fetal bovine serum (FBS; Invitrogen) until almost 100% confluent and subsequently treated with 10 μg/mL mitomycin-C (Sigma, St. Louis, MO) for 8 h to arrest cell proliferation. Human or mouse VSEL cells were plated at 300–500 cells per well (eight-well chamber slide, Lab-tek).

Cells were allowed to grow for 1 week in the growth medium. Later, the cells were switched to a differentiation medium: For nerve differentiation (ectoderm), cells were cultured in the NeuroCult Basal Medium containing neural stem cell differentiation supplements (Stem Cell Technologies, Vancouver, BC, Canada). For insulin producing cell differentiation (endoderm), VSEL cells on feeder layers were cultured in the DMEM/F-12 medium (Invitrogen) with 1% FBS, and 50 ng/mL activin A (R &D Systems, Minneapolis, MN). After 2 days, the medium was replaced with the DMEM/F-12 containing 4 mM glutamine, 4.5 g/L glucose, and 5% FBS with N2 supplement-A (1%; Invitrogen), B27 supplement (2%; Invitrogen), and 10 mM nicotinamide (StemCell Technologies). For osteoblast differentiation (mesoderm), the cells were induced by the α-MEM (Invitrogen) supplemented with 10% FBS, 50 μg/mL L-ascorbic acid (Sigma), 10 mm glycerophosphate (Invitrogen), 100 nM dexamethasone (Sigma), and penicillin/streptomycin (Invitrogen). The medium was changed every 3 days, and cells were analyzed after 21 days.

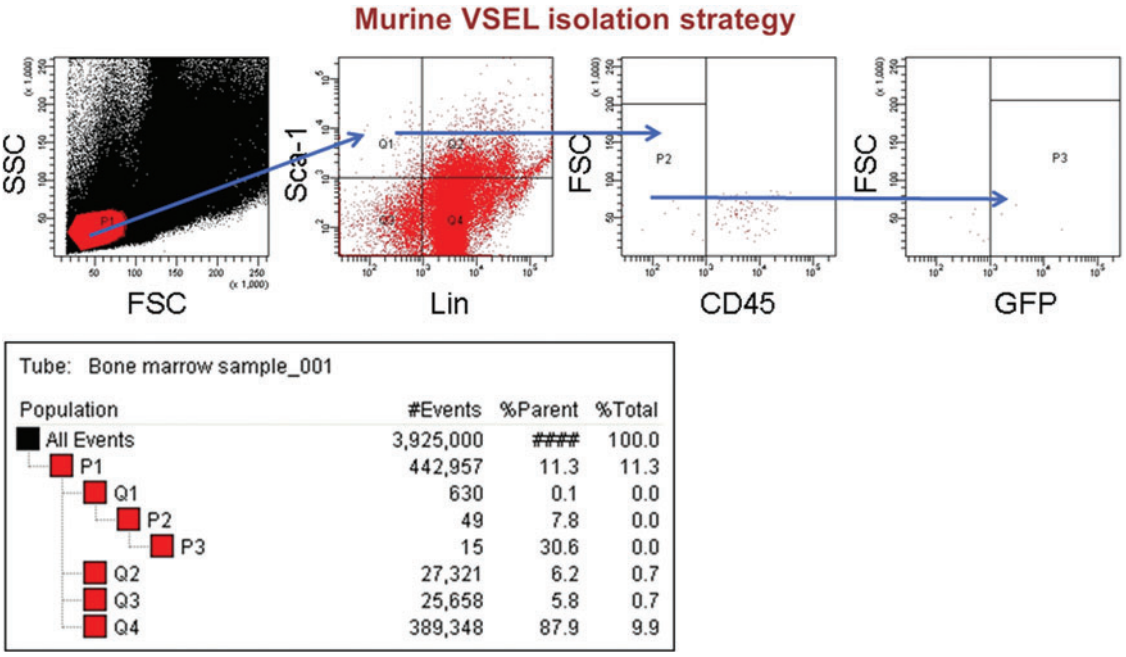
Immunofluorescence microscopy

Pepsin treatment of paraffin sections was performed at 37°C for 15 min before application of Image-iT FX signal enhancer (Invitrogen, San Diego, CA) (30 min) and fluorescence-labeled primary antibodies. Poststaining fixation was performed with 10% formalin (Sigma). Slides were mounted with ProLong Gold anti-fade reagent with DAPI (Invitrogen). Images were taken with the Olympus FV-500 confocal microscope. The anti-human HLA-A,B,C antibody

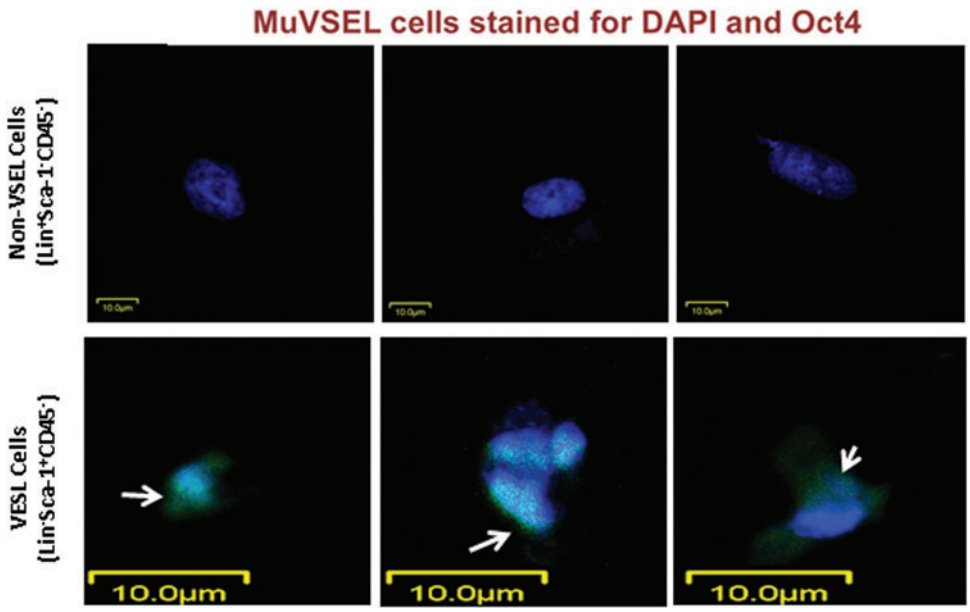


SUPPLEMENTARY FIG. S1. Human VSEL cells were sorted by using a sequential gating strategy, distinct population of CD34/133^{+} CD45^{-} cells were then further confirmed using sized beads with the same FSC setting, but decreased side scatter to have beads on scale. FSC, forward scatter; HSC, hematopoietic stem cell; SSC, side scatter; VSELs, very small embryonic-like cells.

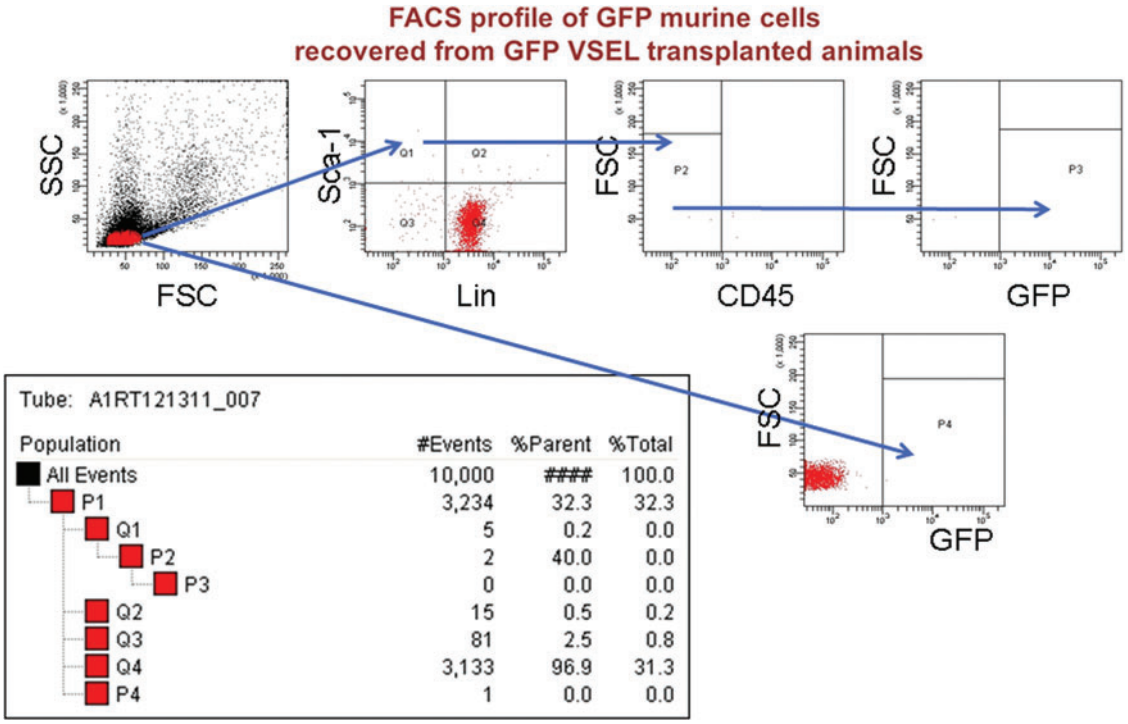
(BioLegend, San Diego, CA) was used with the Zenon Alexa Fluor488 mouse IgG2a labeling kit. A monoclonal antibody (OCG2) to osteocalcin (Abcam, Cambridge, MA) was labeled with a Zenon Alexa Fluor 555 Mouse IgG2a labeling kit (Invitrogen). Antibody (2C1.3A11) to nestin (Abcam) was used with the Zenon Alexa Fluor 555 Mouse IgG1 labeling kit (Invitrogen). In some experiments, a rabbit polyclonal antibody to insulin (Abcam) was labeled with a Zenon Alexa Fluor 555 Rabbit IgG labeling kit (Invitrogen). Additional sections were stained with the anti-Runx2 antibody (1:250, rabbit polyclonal, ab 48811-100; Abcam) or an IgG isotype-matched control (Sigma) to validate osteoblast staining.



SUPPLEMENTARY FIG. S2. MuVSEL cells were sorted by using a sequential gating strategy. The table represents the entire population. To demonstrate single cells in Q1, we had to reduce the % of events displayed (reduced to 25%).



SUPPLEMENTARY FIG. S3. Small $Lin^{-}Sca\text{-}1^{+}CD45^{-}$ cells (VSEL cells) or $Lin^{+}Sca\text{-}1^{-}CD45^{-}$ (non-VSEL cells) from mononuclear cells (1×10^{8} cells/mL) in C57/BL6 mice were isolated by FACS sorting (FACSAria II; Becton Dickinson, Mountainview, CA). After cytospin, the small $Lin^{-}Sca\text{-}1^{+}CD45^{-}$ cells (VSEL cells) were stained for OCT4 and DAPI. $Lin^{+}Sca\text{-}1^{-}CD45^{-}$ (non-VSEL cells) were used as a negative control for OCT4 expression. Cells were fixed and then blocked with the Image-iT FX signal enhancer for 30 min and incubated for 2 h at room temperature in the dark with 10 µg/mL anti-OCT4 antibody (cat. Ab19857; Abcam, Cambridge, MA) (Green; *arrows*) combined with reagents of Zenon Alexa Fluor 488 labeling kit (*green*). The cells were postfixed with 10% formalin for 10 min followed by processing with the ProLong Gold antifade reagent with DAPI medium (*blue*). Images were acquired with an Olympus FV500 confocal microscope.



SUPPLEMENTARY FIG. S4. We collected GFP-positive cells (P4) from P1 (very small size), since no GFP VSEL cells were found after Passage 1.

Supplementary References

1. Kucia M, R Reca, FR Campbell, E Zuba-Surma, M Majka, J Ratajczak and MZ Ratajczak. (2006). A population of very small embryonic-like (VSEL) CXCR4(+)SSEA-1(+)Oct-4+ stem cells identified in adult bone marrow. Leukemia 20:857–869.

2. Liu Y, L Gao, EK Zuba-Surma, X Peng, M Kucia, MZ Ratajczak, W Wang, V Enzmann, HJ Kaplan and DC Dean. (2009). Identification of small Sca-1(+), Lin(−), CD45(−) multipotential cells in the neonatal murine retina. Exp Hematol 37:1096–1107, 1107.e1.