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HEMATOPOIETIC DIFFERENTIATION OF UMBILICAL CORD BLOOD-DERIVED VERY SMALL EMBRYONIC/EPIBLAST-LIKE STEM CELLS

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Abstract

A population of $CD133^+lin^-CD45^-$ very small embryonic-like stem cells (VSELs) has been purified by multiparameter sorting from umbilical cord blood (UCB). In order to speed up isolation of these cells, we employed anti-CD133-conjugated paramagnetic beads followed by staining with Aldefluor to detect aldehyde dehydrogenase (ALDH) activity; we subsequently sorted $CD45^-/GlyA^-/CD133^+/ALDH^{high}$ and $CD45^-/GlyA^-/CD133^+/ALDH^{low}$ cells, which are enriched for VSELs, and $CD45^+/GlyA^-/CD133^+/ALDH^{high}$ and $CD45^+/GlyA^-/CD133^+/ALDH^{low}$ cells, which are enriched for hematopoietic stem/progenitor cells (HSPCs). While freshly isolated $CD45^-$ VSELs did not grow hematopoietic colonies, the same cells, when activated/expanded over OP9 stromal support, acquired hematopoietic potential and grew colonies composed of $CD45^+$ hematopoietic cells in methylcellulose cultures. We also observed that $CD45^-/GlyA^-/CD133^+/ALDH^{high}$ VSELs grew colonies earlier than $CD45^-/GlyA^-/CD133^+/ALDH^{low}$ VSELs, which suggests that the latter cells need more time to acquire hematopoietic commitment. In support of this possibility, real-time PCR analysis confirmed that, while freshly isolated $CD45^-/GlyA^-/CD133^+/ALDH^{high}$ VSELs express more hematopoietic transcripts (e.g., c-myb), $CD45^-/GlyA^-/CD133^+/ALDH^{low}$ VSELs exhibit higher levels of pluripotent stem cell markers (e.g., Oct-4). More importantly, hematopoietic cells derived from VSELs that were co-cultured over OP9 support were able to establish human lympho-hematopoietic chimerism in lethally irradiated NOD/SCID mice 4–6 weeks after transplantation. Overall, our data suggest that UCB-VSELs correspond to the most primitive population of HSPCs in UCB.

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Keywords

Umbilical Cord Blood; VSELs; HSCs; Oct-4

Introduction

Hematopoietic stem cells (HSCs) can be purified from bone marrow (BM), umbilical cord blood (UCB), and mobilized peripheral blood (mPB) by employing i) characteristic stem cell antigens (e.g., CD34 and CD133), ii) the absence of lineage differentiation markers (lin^-), iii) high expression of aldehyde dehydrogenase (ALDH), and iv) low accumulation of Hoe3342 ($Hoe3342^{low}$), Pyronin Y ($Pyronin\ Y^{low}$) or Rhodamine 123 ($Rh123^{low}$) dyes.[1] A combination of signaling lymphocyte activating molecules (SLAM) markers ($CD150^+$, $CD48^-$, and $CD244^-$) has also been recently proposed for discriminating primitive HSPC cell populations.[2]

Nevertheless, the phenotype of most primitive hematopoietic stem cells (HSCs), known as long-term repopulating HSCs (LT-HSCs), is not very well defined. According to their definition, LT-HSCs establish long-lasting, stable chimerism after hematopoietic transplantation and, as proposed, reside in human BM among $CD34^-CD38^-lin^-$ [3] or $CD133^+Lin^-ALDH^{high}$ cells.[4] These most primitive HSCs display only limited clonogenic potential in routine assays *in vitro*; however, they are able to engraft and establish hematopoiesis in experimental animals.[5], [6] Similar cells can be detected in UCB by employing direct intra-bone marrow transplantation and have been identified among $CD34^-flt^-lin^-$ cells. [7]

Recently, we identified in murine BM and fetal liver (FL) a population of very small embryonic/epiblast-like (VSEL) stem cells that i) are smaller than erythrocytes, ii) express $SSEA\text{-}1^+/Oct\text{-}4^+/Sca\text{-}1^+/CXCR4^+/Lin^-/CD45^-$, iii) respond to an SDF-1 gradient, and iv) have high nuclear:cytoplasm ratio and primitive euchromatin.[8] Murine VSELs do not reveal hematopoietic activity immediately after isolation, but acquire hematopoietic potential similar to stem cells from established embryonic stem cell (ESCs) lines and inducible pluripotent stem (iPS) cells following co-culture/activation over OP9 stroma.[9] Based on these findings, we hypothesized that in murine BM they fulfill the functional criteria for LT-HSCs.[9] As recently demonstrated, these cells are also early in the mesenchymal lineage hierarchy.[10] We recently identified corresponding populations of $SSEA\text{-}4^+/Oct\text{-}4^+/CD133^+/CD34^+/CXCR4^+/Lin^-/CD45^-$ cells in UCB that are i) slightly smaller than erythrocytes, ii) have high nuclear:cytoplasm ratio and euchromatin, and iii) display transcription factor signature of pluripotent stem cells (PSCs), including Oct-4 and Nanog.[11] By employing fluorescence-activated cell sorting (FACS) followed by quantitative genetic analysis, we found that $CD45^-/Lin^-/CD133^+$, $CD45^-/Lin^-/CD34^+$, and $CD45^-/Lin^-/CXCR4^+$ fractions of UCB cells are significantly enriched in VSELs.[12] However, molecular analysis revealed that the subpopulation of $CD45^-/Lin^-/CD133^+$ cells, a rare $CD45^-/Lin^-$ cell population possesses the highest expression of pluripotency markers. Based on this, we hypothesized that CD133 antigen may be the most optimal surface marker to identify the most primitive

VSELs. The presence of similar cells was recently confirmed in UCB, human BM, and mPB by other groups.[13, 14]

In the current study, to attain a faster process to avoid time-consuming multiparameter sorting procedure for isolating VSELs from UCB, we employed anti-CD133 paramagnetic beads, followed by staining with Aldefluor, a fluorescent dye to detect aldehyde dehydrogenase (ALDH), and sorted $CD45^-/GlyA^-/CD133^+/ALDH^{high}$ and $CD45^-/GlyA^-/CD133^+/ALDH^{low}$ sub-fractions of VSELs and $CD45^+/GlyA^-/CD133^+/ALDH^{high}$ and $CD45^+/GlyA^-/CD133^+/ALDH^{low}$ subfractions of hematopoietic stem/progenitor cells (HSPCs). We found that while freshly isolated VSELs did not grow colonies *in vitro*, these cells, if immediately expanded over OP9 stromal cells, acquired hematopoietic potential and grew colonies composed of $CD45^+$ cells. Furthermore, while $CD45^+$ cells gave raise to hematopoietic colonies after the first replating, the formation of colonies by $CD45^-/GlyA^-/CD133^+/ALDH^{low}$ VSELs was somewhat delayed, which suggests that they require more time to attain hematopoietic commitment. In parallel, real-time PCR analysis confirmed that while freshly isolated $CD45^-/GlyA^-/CD133^+/ALDH^{high}$ VSELs express more hematopoietic transcripts, $CD45^-/GlyA^-/CD133^+/ALDH^{low}$ VSELs exhibit higher levels of pluripotent stem cell trancription factors. Finally, in *in vivo* transplants into NOD/SCID mice we observed that both $CD45^-/GlyA^-/CD133^+/ALDH^{high}$ and $CD45^-/GlyA^-/CD133^+/ALDH^{low}$ VSELs cultured over OP9 cells give rise to human lympho-hematopoietic chimerism as assayed 4–6 weeks after transplantation. Taking all of these observations into account, we propose that, like murine BM-derived VSELs, human UCB-derived $CD45^-$ VSELs correspond to a population of the most primitive long-term repopulating HSCs (LT-HSCs).

Materials and Methods

Isolation and FACS sorting of VSELs from umbilical cord blood

This study was performed in accordance with the guidelines of the local ethical and biohazard authorities at the University of Louisville School of Medicine (Louisville, Kentucky). Clinical-grade UCB research units were shipped from Cleveland Cord Blood Center and were treated with 1x BD Pharm Lyse Buffer (BD Pharmingen, San Jose, CA) for 15 min at room temperature (RT) to remove RBCs and washed twice in phosphate-buffered saline (PBS). A single-cell suspension of total nucleated cells (TNCs) obtained from clinical UCB samples was treated with antibodies against CD133 antigen-coated immunomagnetic beads and separate by using a MACS Separator (Miltenyi Biotec GMBH, Germany) to reduce cell numbers prior to cell sorting. The CD133-positive cell fraction was reacted with the Aldefluor™ Kit reagent (StemCell Tech., USA) for detecting aldehyde dehydrogenase (ALDH). After the ALDH enzyme reaction, cells were washed and resuspended in cold Aldefluor buffer (StemCell Tech.) and maintained on ice during all subsequent manipulations. Cells were incubated with phycoerythrin (PE)-conjugated murine anti-human CD235a (clone GA-R2, BD Biosciences, USA), phycoerythrin-CY7 (PE-CY7)-CD45 (clone HI30, BD Biosciences), and allophycocyanin (APC)-conjugated CD133/2 (Miltenyi Biotec GMBH, Germany). Cells were washed and resuspended in cold Aldefluor buffer and sorted by MoFlo sorter (Dako, USA) to obtain populations enriched in VSELs ($CD45^-/GlyA^-/$

CD133^{+}/ALDH^{high} and CD45/GlyA^{-}/CD133^{+}/ALDH^{low}), as well as for hematopoietic stem/progenitor cells (HSPCs, CD45^{+}/GlyA^{-}/CD133^{+}/ALDH^{high} and CD45^{+}/GlyA^{-}/CD133^{+}/ALDH^{low} cells).

Ex vivo differentiation of VSELs into hematopoietic cells in primary co-cultures over OP9 stromal cells

Freshly sorted CD45^{-}/GlyA^{-}/CD133^{+}/ALDH^{high} and CD45^{-}/GlyA^{-}/CD133^{+}/ALDH^{low} sub-fractions of VSELs and CD45^{+}/GlyA^{-}/CD133^{+}/ALDH^{high} and CD45^{+}/GlyA^{-}/CD133^{+}/ALDH^{low} subfractions of hematopoietic stem/progenitor cells (HSPCs) were plated over OP9 cells in α-MEM with 20% FBS (Molecular Probes®, Invitrogen) for 7 days and subsequently trypsinized, washed by centrifugation in α-MEM, and replated in methylcellulose-based medium (StemCell Tech, Vancouver, BC, CAN).

Evaluation of the clonogenic potential of sorted cells in methylcellulose cultures

VSELs or HSPCs freshly isolated from BM or cells harvested from OP9 cultures were plated in methylcellulose-based medium (StemCell Tech, Vancouver, BC, CAN) supplemented with murine stem cell growth factor (SCF), interleukin-3 (IL-3), granulocyte-macrophage colony-stimulating factor (GM-CSF), FLT3, thrombopoietin (TpO), erythropoietin (EpO), and insulin growth factor-2 (IGF-2). Cells were cultured for 10 days and the number of colonies formed were scored. Subsequently, methylcellulose cultures were solubilized and trypsinized and the resulting cells were washed by centrifugation in α-MEM and plated into secondary methylcellulose cultures. Cells were grown in the presence of the same growth factors and replated after 10 days into new methylcellulose cultures.

PCR analysis of gene expression in freshly sorted cells and OP9-expanded cells

Total RNA (from samples of approximately 20,000 cells each) was isolated using the RNeasy Mini Kit (Qiagen Inc., Valencia, CA) and genomic DNA removed using the DNA-free™ Kit (Applied Biosystems, Foster City, CA). Isolated messenger (m)RNA was reverse-transcribed with Taqman Reverse Transcription Reagents (Applied Biosystems), according to the manufacturer's instructions. RT-PCR was performed using Amplitaq Gold (Applied Biosystems) with 1 cycle of 8 min at 95°C; 2 cycles of 2 min at 95°C, 1 min at 62°C, and 1 min at 72°C; 38 cycles of 30 sec at 95°C, 1 min at 62°C, and 1 min at 72°C; and 1 cycle of 10 min at 72°C using sequence-specific primers. Quantitative measurement of target transcript expression was performed by RQ-PCR using an ABI Prism 7500 Sequence Detection System (Applied Biosystems). Complementary (c)DNA from indicated cells was amplified using SYBR Green PCR Master Mix (Applied Biosystems) and specific primers. All primers were designed with Primer Express software (Applied Biosystems), with at least one primer in each pair containing an exon-intron boundary. The threshold cycle (*C*t) was determined and relative quantification of the expression level of target genes was obtained with the $2^{-\Delta\Delta Ct}$ method, using β2-microglobulin (β2mg) as an endogenous control gene and mononuclear cell (MNC) genes as calibration controls. All primers used in RT- and RQ-PCR are listed in Table I and Table II, respectively.

FACS analysis of OP9-expanded cells

Cells cultured over OP9 were plated in methylcellulose to grow hematopoietic colonies. Subsequently, colonies were solubilized and evaluated by FACS (LSRII, BD Biosciences) for expression of CD45, CD14, GlyA, CD3, CD19, and CD41. For detection of antigens, we employed phycoerythrin-CY7 (PE-CY7)-conjugated murine anti-human antibody for CD45 (clone HI30) and fluorescein-conjugated anti-human lineage marker antibodies for CD14 (clone M5E2), GlyA (clone GLA-R2), CD3 (clone UCHT1), CD19 (clone HIB19), and CD41 (clone HIP2). All antibodies were obtained from BD Biosciences (USA).

Hematopoietic transplantation studies

For transplantation experiments, NOD/SCID mice were irradiated with a sub-lethal dose of γ-irradiation (350 cGy). After 24 hours, freshly isolated VSELs, HSCs, or OP9-primed/expanded cells were transplanted into mice by tail vein. Anesthetized transplanted mice were sacrificed 6 weeks after transplantation to evaluate chimerism in BM, PB, and spleen. For this analysis we used the same anti-human antibodies listed above.

Statistical analysis

All data in quantitative (q)ChIP and gene expression analyses were analyzed using one-factor Analysis of Variance (ANOVA) with Bonferroni's Multiple Comparison Test. We used the Instat1.14 program (GraphPad Software, La Jolla, CA) and statistical significance was defined as $p<0.05$ or $p<0.01$.

Results

ALDH activity as a new strategy to purify CD133$^+$ GlyA$^-$ CD45$^-$ UCB VSELs

UCB-VSELs were initially purified from erythrocyte-depleted UCB by multiparameter sorting for a population of CD133$^+$CD45$^-$Lin$^-$ cells. This procedure, however, is time consuming and the multiparameter sorting time required to process one entire cord blood unit (~50–100 ml) to isolate rare VSELs from UCB MNCs would take up to 3–4 days.

In order to establish a more efficient method for VSEL purification from UCB, we employed a three-step isolation strategy (Figure 1) based on removal of erythrocytes by hypotonic lysis (1st step), followed by immunomagnetic separation of CD133$^+$ cells (2nd step), followed by FACS-based isolation of CD133$^+$GlyA$^-$CD45$^-$ cells after exposure to Aldefluor™ (3rd step). The resulting cells may be either ALDH^{high} or ALDH^{low}. The rationale for using lysis buffer to remove erythrocytes was based on our observation that it leads to a higher yield of VSELs than removal of erythrocytes by centrifugation over a Ficoll-Paque gradient.[12] On the other hand, the selection of CD133 antigen for VSEL isolation was based on our previous observation that CD133$^+$ VSELs are highly enriched for PSC transcription factor expression (e.g., Oct-4 and SSEA-4).[12] Inclusion of anti-GlyA antibody was based on the known fact that 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.

Figure 2 panel A shows the isolation strategy of immunomagnetic-separated UCB $CD133^+$ cells based on ALDH activity and CD133, GlyA, and CD45 expression. By employing this strategy, we have enriched for VSELs ($CD45^-/GlyA^-/CD133^+/ALDH^{high}$ and $CD45^-/GlyA^-/CD133^+/ALDH^{low}$) as well as for HSPCs ($CD45^+/GlyA^-/CD133^+/ALDH^{high}$ and $CD45^+/GlyA^-/CD133^+/ALDH^{low}$). Figure 2 panel B shows these cell fractions as a percentage of total $CD133^+/GlyA^-$ UCB cells. The fractions of $CD133^+$ cells enriched for $CD45^-/GlyA^-/CD133^+/ALDH^{low}$ and $CD45^-/GlyA^-/CD133^+/ALDH^{high}$ VSELs are the smallest ones (~5% and ~10%, respectively). The majority of $CD133^+/GlyA^-$ cells were $CD45^+/GlyA^-/CD133^+/ALDH^{high}$ HSPCs (~70%) followed by $CD45^+/GlyA^-/CD133^+/ALDH^{low}$ HSPCs (~15%). The total number of these rare cells per 100 ml of UCB is shown in Table III. Both $CD45^+/GlyA^-/CD133^+/ALDH^{high}$ and $CD45^+/GlyA^-/CD133^+/ALDH^{low}$ VSELs are 2–3 orders of magnitude less numerous than the $CD45^+$ fractions of HSPCs.

Molecular characterization of sorted cells

The presence of Oct-4, Nanog, and SSEA-1 proteins in UCB-sorted $CD45^-/Gly\text{-}A^-/CD133^+/ALDH^{low}$ VSELs was confirmed by immunohistochemical staining (Figure 3A). Next, RT-PCR analysis of gene expression confirmed that $CD45^-/GlyA^-/CD133^+/ALDH^{low}$ VSELs have the highest expression of Oct-4, Scl-2, and HoxB4 (Figure 3B and C). In contrast, LMO2 expression was highest in $CD45^+/GlyA^-/CD133^+/ALDH^{high}$ HSPCs. Interestingly, freshly sorted human VSELs and HSCs expressed similar levels of c-myb.

Clonogeneic potential *in vitro* of UCB-derived VSELs

After sorting by FACS from UCB, $CD45^-/GlyA^-/CD133^+/ALDH^{high}$, $CD45^-/GlyA^-/CD133^+/ALDH^{low}$, $CD45^+/GlyA^-/CD133^+/ALDH^{high}$, and C D 4 $5^+/GlyA^-/CD133^+/ALDH^{low}$ cells were subsequently plated in MethoCult medium supplemented with a cocktail of cytokines and growth factors promoting growth of clonogenic colonies (CFU-C). We observed that while $CD45^+/GlyA^-/CD133^+/ALDH^{high}$ cells freshly isolated from UCB had the highest clonogenic potential, $CD45^-/GlyA^-/CD133^+/ALDH^{low}$ cells did not grow colonies at all. The clonogenic potential of $CD45^-/GlyA^-/CD133^+/ALDH^{high}$ and $CD45^+/GlyA^-/CD133^+/ALDH^{high}$ cells was very low (Figure 4 panel A).

Because in our previous work we observed that, like ESCs and induced iPS cells, murine VSELs must be co-cultured/primed over OP9 stroma in order to acquire hematopoietic commitment[9], we employed a similar strategy for human VSELs. We found that after 7 days UCB-purified VSELs ($CD45^-/GlyA^-/CD133^+/ALDH^{low}$, and $CD45^-/Gly\text{-}A^-/CD133^+/ALDH^{high}$) plated over OP9 cells formed colonies resembling cobblestones (Figure 4 panel B). Of note, the ability to form cobblestone areas was lower for $ALDH^{low}$ VSELs.

In the next step, cells from these cultures were trypsinized on day 7 and subsequently tested for clonogenic potential in MethoCult cultures. Figure 4 panel C (left panel) shows the number of colonies formed by cells isolated from OP9 stroma cultures that were initiated by all four fractions of sorted UCB cells. The number of plated cells was adjusted to have the same numbers of human cells. We observed a significant increase in the clonogenic potential of UCB-VSELs, in particular, in a population of cells that was initiated by $CD45^-/GlyA^-/CD133^+/ALDH^{high}$ VSELs; however, colonies were also formed by cells initiated

from $CD45^{-}/GlyA^{-}/CD133^{+}/ALDH^{low}$ VSELs. The clonogenic potential of cells derived from cultures initiated by UCB VSELs additionally increased 10 days after they were plated into secondary methylcellulose cultures (Figure 4 panel C – right panel), which suggests that these cells need more time to differentiate along the hematopoietic lineage. Of note, colonies grown in these cultures from VSEL-derived $CD45^{+}$ cells were 2–3 times larger than those initiated by HSPC-expanded cells.

FACS analysis of cells derived from aspirated methylcellulose hematopoietic colonies initiated by VSEL-derived cells revealed that cells in these colonies highly express CD45, CD14, CD3, CD19, and CD41 antigens (Figure 4 panel D). Interestingly, cells expanded from $CD45^{-}/CD133^{+}/ALDH^{high}$ VSELs were significantly enriched for $GlyA^{+}$ erythroid precursors.

UCB-VSELs expanded over OP9 cells are able to engraft in NOD/SCID immunodeficient animals

Finally, the *in vivo* hematopoietic potential of UCB-derived VSELs expanded over OP9 cultures was tested after transplantation into immunodeficient, sublethally irradiated NOD/SCID mice. However, because no chimerism was observed when freshly sorted, non-OP9-cultured freshly purified VSELs were transplanted (data not shown), we transplanted OP9 primed/expanded cells.

Accordingly, OP9-primed cultures initiated by the same number of sorted cells were trypsinized after 7 days and cells were injected intravenously. After 6 weeks mice were sacrificed and human-murine chimerism was evaluated in all major hematopoietic lineages in BM, spleen, and peripheral blood by FACS using human-specific antibodies. The highest level of chimerism in BM and spleen in all hematopoietic lineages was achieved after transplantation of $CD45^{+}/CD133^{+}/ALDH^{high}$ HSPCs (Figure 5). However, we also observed significant chimerism after transplantation of OP9-cultured VSELs.

Discussion

The phenotype of the most primitive human LT-HSCs is still not very well defined and several potential candidate cells have been proposed based on the expression of cell-surface antigens (e.g., $CD133^{+}$, $CD34^{+}$, $CD38^{-}$, and Lin^{-}), SLAM markers, and low levels of staining by some fluorescent dyes (e.g., $Rh123^{dull}$, Pyronin Y^{low}, and Hoechst 33342^{low}). [1], [2] A useful detection system for identification of primitive HSPCs is exposure of cells to Aldefluor, which detects aldehyde dehydrogenase (ALDH) activity.[15] Aldefluor is a substrate for ALDH, a cytosolic enzyme highly expressed in less-differentiated hematopoietic cells and implicated in resistance to some alkylating agents.[15] Aldefluor in the presence of ALDH becomes modified to the fluorescent molecule that marks ALDH-expressing cells. The Aldefluor-based staining may be combined with other stem cell markers, for example, CD133 antigen. It was reported that human $ALDH^{high}/Lin^{-}$ cells, when transplanted into immunodeficient NOD/SCID mice, robustly reconstitute hematopoiesis. [16] Similar cells were recently also found among populations of human BM-derived $CD133^{+}/lin^{-}/ALDH^{high}$ cells. [4]

On the other hand, evidence is accumulating that human hematopoietic tissues (BM or UCB) may contain some rare, primitive hematopoietic stem cells that do not match the phenotype of classical HSCs and do not exhibit *in vitro* hematopoietic activity immediately after purification.[6] Accordingly, we have demonstrated that human BM contains a population of rare $CD34^{-}Lin^{-}CD38^{-}$ HSCs that show poor clonogenic activity *in vitro*, but *in vivo* engraft robustly in immunodeficient mice. [6] Similarly, it has been reported that human UCB contains rare, primitive $CD34^{-}flt^{-}Lin^{-}$ cells that, in contrast to normal adult HSCs, do not engraft after intravenous injection and exhibit hematopoietic potential only after intra-bone delivery. [17] More recently, these cells were found to be highly enriched in a $CD34^{-}$ fraction of UCB cells that were depleted of differentiated cells by a cocktail of antibodies against 18 different lineage markers. [18] Moreover, since all of the phenotypic markers for LT-HSC were proposed and established by employing uncultured cells, one of the recent reports employing *ex vivo* expansion system combined with *in vivo* transplants demonstrated that a population of UCB $CD133^{+}/CD38^{-}$ cells resembles the most primitive HSPCs. [19] Thus, the CD133 antigen seems to be a very good marker for the most primitive HSPCs.

Recently, we identified a population of VSELs in murine BM that do not exhibit hematopoietic activity immediately after isolation, but later acquire full hematopoietic potential, including the ability to reconstitute long-term hematopoiesis in lethally irradiated recipients following co-culture/activation over OP9 stroma.[8, 9] Based on this, we proposed that in murine BM these cells fulfill the functional criteria for LT-HSCs.[9] Interestingly, these cells were also identified as precursors of MSCs indicative of multipotentiality.[10] A corresponding population of $SSEA\text{-}4^{+}/Oct\text{-}4^{+}/CD133^{+}/CD34^{+}/CXCR4^{+}/Lin^{-}/CD45^{-}$ cells was identified recently by us [11, 12] and others [13, 14] in human UCB, BM, and mPB, and thus we became interested in their potential hematopoietic specification. Because human VSELs are very rare, the processing of one UCB unit by a standard procedure employing multiparameter sorting requires up to 4 days. This is, of course, unacceptable taking into consideration cell viability, the time of sorter usage, and the time commitment of a sorter operator.

Thus, our first goal was to develop a faster large-scale isolation protocol. To speed up the procedure for VSEL sorting, we employed i) hypotonic lysis removal of erythrocytes to obtain UCB nucleated cells, ii) enrichment for $CD133^{+}$ VSELs by immunomagnetic beads, and, as a final step, iii) sorting of VSELs from erythrocyte-depleted/immunomagnetic paramagnetic bead-enriched $CD133^{+}$ mononuclear cells by employing staining with Aldefluor combined with anti-CD133 (different epitope) and fluorochrome-conjugated anti-CD45 and anti-GlyA antibodies. By employing this strategy, all four populations of cells i) $CD45^{-}/GlyA^{-}/CD133^{+}/ALDH^{high}$, ii) $CD45^{-}/GlyA^{-}/CD133^{+}/ALDH^{low}$, iii) $CD45^{+}/GlyA^{-}/CD133^{+}/ALDH^{high}$, and iv) $CD45^{+}/GlyA^{-}/CD133^{+}/ALDH^{low}$ were sorted and subsequently tested for hematopoietic activity.

We observed that UCB-purified $CD45^{-}/GlyA^{-}/CD133^{+}/ALDH^{high}$ and $CD45/GlyA^{-}/CD133^{+}/ALDH^{low}$ VSELs, like their murine counterparts, do not exhibit hematopoietic potential immediately after isolation. However, based on our observations that murine $CD45^{-}$ VSELs may become HSCs after co-culture over OP9 stroma cells we became

interested in whether human CD45$^-$ VSELs may become specified into the hematopoietic lineage as well. In fact, we observed that UCB-derived CD45$^-$ VSELs, like murine VSELs, human ESCs, or iPS cells, become specified into the hematopoietic lineage in co-cultures over an OP9 feeder layer. [20] This robust specification of human UCB-derived CD45$^-$ VSELs is supported by their ability to form hematopoietic colonies *in vitro* that express pan-hematopoietic CD45antigen as well as several major lympho-hematopoietic lineage markers (CD41, GlyA, CD14, CD3, and CD19). We assume that the fact that human UCB-VSELs, like their murine BM-derived counterparts [9], can be more efficiently expanded into the lympho-hematopoietic lineage than ESCs or iPS cells demonstrates that they are already more committed to hematopoiesis, and thus may correspond to a population of UCB-derived LT-HSCs. These small cells isolated from human UCB highly express Oct-4, Nanog, and SSEA-4 at both the mRNA and protein levels. Moreover, what is highly relevant for their ability to specify into the hematopoietic lineage, freshly isolated VSELs already express the HoxB-4 gene, which is not expressed in ESCs, but is ultimately required for their hematopoietic expansion. [21], [22]

More importantly, we found that UCB-derived VSELs not only differentiate over OP9 stroma into clonogenic hematopoietic progenitors but also into HSCs, which are able to establish human-murine chimerism in immunodeficient NOD/SCID animals. It is well known that the level of human-murine chimerism depends on several factors, such as phenotype, the number of transplanted cells, or the severity of immunodeficiency in the mice employed as recipients.[23] In the case of our experiments, we transplanted trypsinized OP9 co-cultures whose activity could be influenced by the presence of the infused OP9 cells. Further studies, however, are needed to address whether murine OP9 cells home to the hematopoietic organs of immunodeficient mice and thus may provide more favorable conditions in which human cells could expand.

Thus, our data unequivocally demonstrate that the UCB-derived population of CD45$^-$VSELs may differentiate into CD45$^+$ HSPCs and that these extremely rare cells (~10^3/100 ml of UCB for CD45$^-$/GlyA$^-$/CD133$^+$/ALDH^{low} cells and ~4×10^3/100 ml of UCB for CD45$^-$/GlyA$^-$/CD133$^+$/ALDH^{high} cells) show significant hematopoietic potential.

Taking into consideration the hematopoietic potential of UCB-derived VSELs, it would be interesting to see whether the number of these cells changes in BM in aplastic anemia patients. If VSELs would be protected from damage, they might offer an alternative source of autologous HSPCs for transplantation in aplastic patients. Furthermore, we have demonstrated that murine VSELs are highly resistant to irradiation [9], which suggests that human VSELs could also survive myeloablative conditioning therapy for transplantation and could stimulate hematopoiesis in the recipient after unsuccessful or partial engraftment of transplanted cells. This however, will also require further study.

Based on the foregoing, we propose that VSELs are the most primitive UCB-residing population of stem cells that possess the potential to become specified into the hematopoietic lineage and thus may share some of the characteristics of LT-HSCs. A crucial requirement for their application in the clinic is optimization of their hematopoietic specification in an OP9-free system. We also need to ascertain whether human VSELs show

the same molecular signature (e.g., hypomethylation of the Oct-4 promoter, modification of somatic imprinting, or the presence of bivalent domains) as their murine counterparts. Furthermore, future studies are needed to compare several populations of putative UCB-derived LT-HSCs in parallel experiments to determine the similarities and differences between these cell populations.

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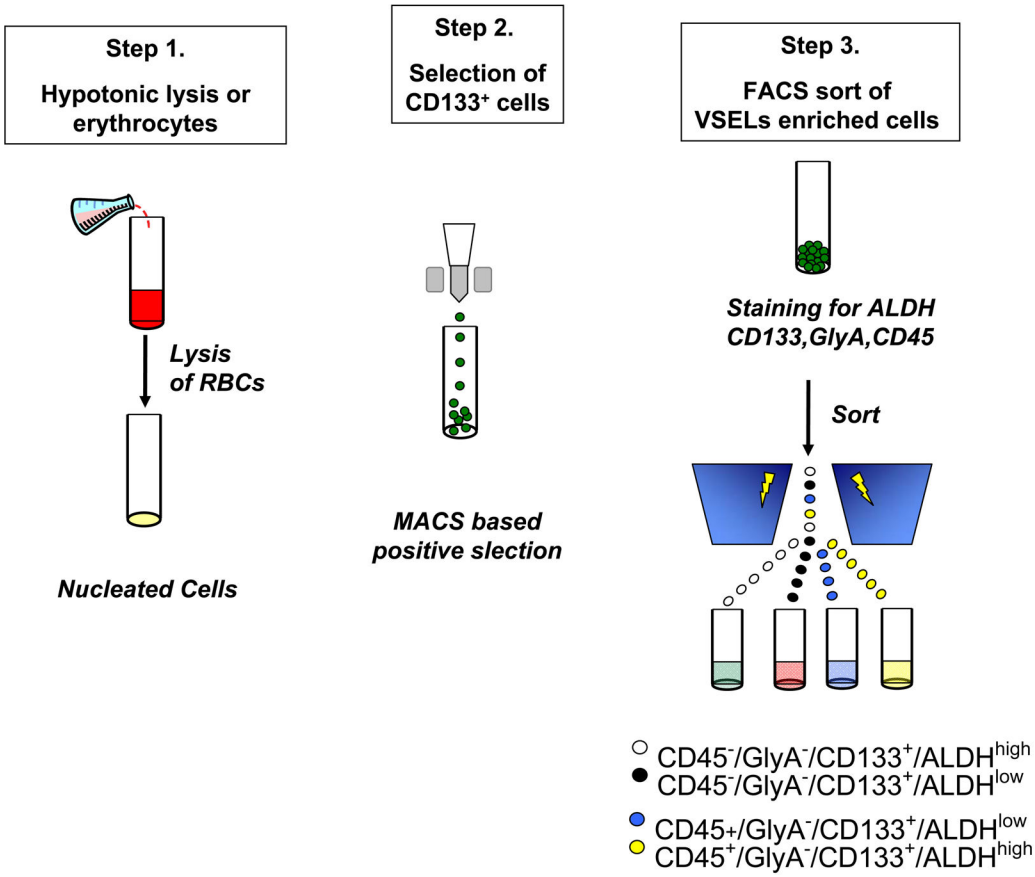
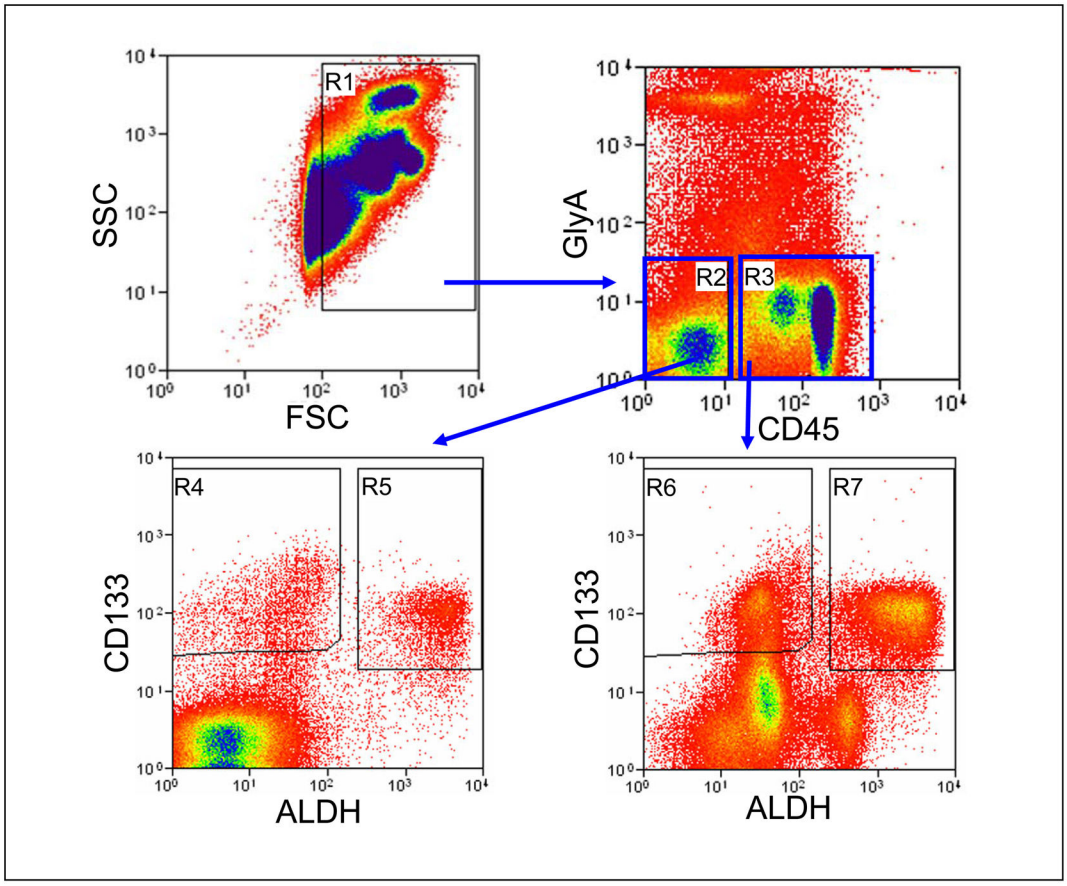


Figure 1. Three-step strategy for larger-scale preparation of VSELs from UCB

We employ a three-step isolation strategy based on removal of erythrocytes by hypotonic lysis (1st step) followed by immunomagnetic separation of $CD133^+$ cells (2nd step) followed by their exposure to Aldefluor and FACS-based isolation of $CD133^+GlyA^+CD45^-$ or $CD133^+GlyA^+CD45^+$ cells that are $ALDH^{high}$ or $ALDH^{low}$ (3rd step). By employing this strategy, we can isolate VSELs ($CD45^-/GlyA^-/CD133^+/ALDH^{high}$ and $CD45/GlyA^-/CD133^+/ALDH^{low}$), as well as HSPCs ($CD45^+/GlyA^-/CD133^+/ALDH^{high}$ and $CD45^+/GlyA^-/CD133^+/ALDH^{low}$).



R4: $CD45^-/GlyA^-/CD133^+/ALDH^{low}$
R5: $CD45^-/GlyA^-/CD133^+/ALDH^{high}$
R6: $CD45^+/GlyA^-/CD133^+/ALDH^{low}$
R7: $CD45^+/GlyA^-/CD133^+/ALDH^{high}$

Figure 2 A

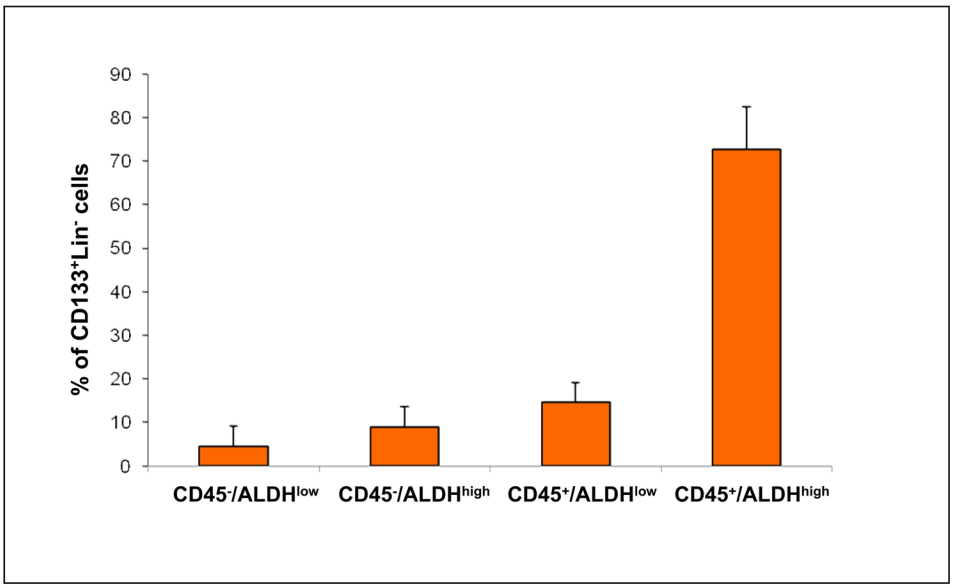


Figure 2 B

Figure 2. Gating strategy for FACS sorting of UCB VSELs and HSCs based on expression of CD133, CD45, and ALDH activity

Panel A. UCB nucleated cell populations are stained using monoclonal antibodies against human CD235a (GlyA), CD45, and CD133 and exposed to Aldefluor. Sort gates are established by sequentially gating on FSC vs. SSC in region R1, followed by gating to define $CD45^{-}/GlyA^{-}$ (region R2) and $CD45^{+}/GlyA^{-}$ (region R3) populations. Cell populations are also defined based on their ALDH activity. Accordingly, cells are sorted as $CD45^{-}/GlyA^{-}/CD133^{+}/ALDH^{low}$ (region R4) and $CD45^{-}/GlyA^{-}/CD133^{+}/ALDH^{high}$ (region R5) subfractions of VSELs and as $CD45^{+}/GlyA^{-}/CD133^{+}/ALDH^{low}$ (region 6) and $CD45^{+}/GlyA^{-}/CD133^{+}/ALDH^{high}$ (region R7) hematopoietic stem cell (HSC) populations. **Panel B.** The percentage of all fractions of sorted VSELs ($CD45^{-}$) and HSPCs ($CD45^{+}$) $ALDH^{high/low}$ among UCB-derived $CD133^{+}GlyA^{-}$ cells. The data shown represent the combined results from six independent experiments.

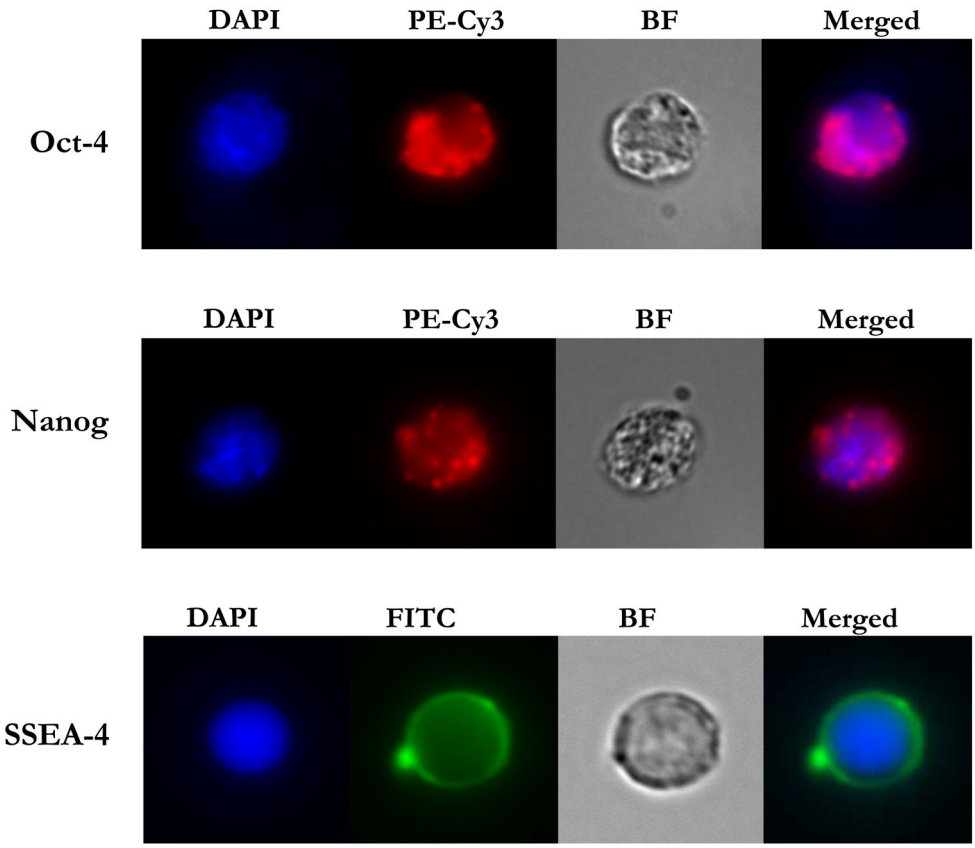


Figure 3 A

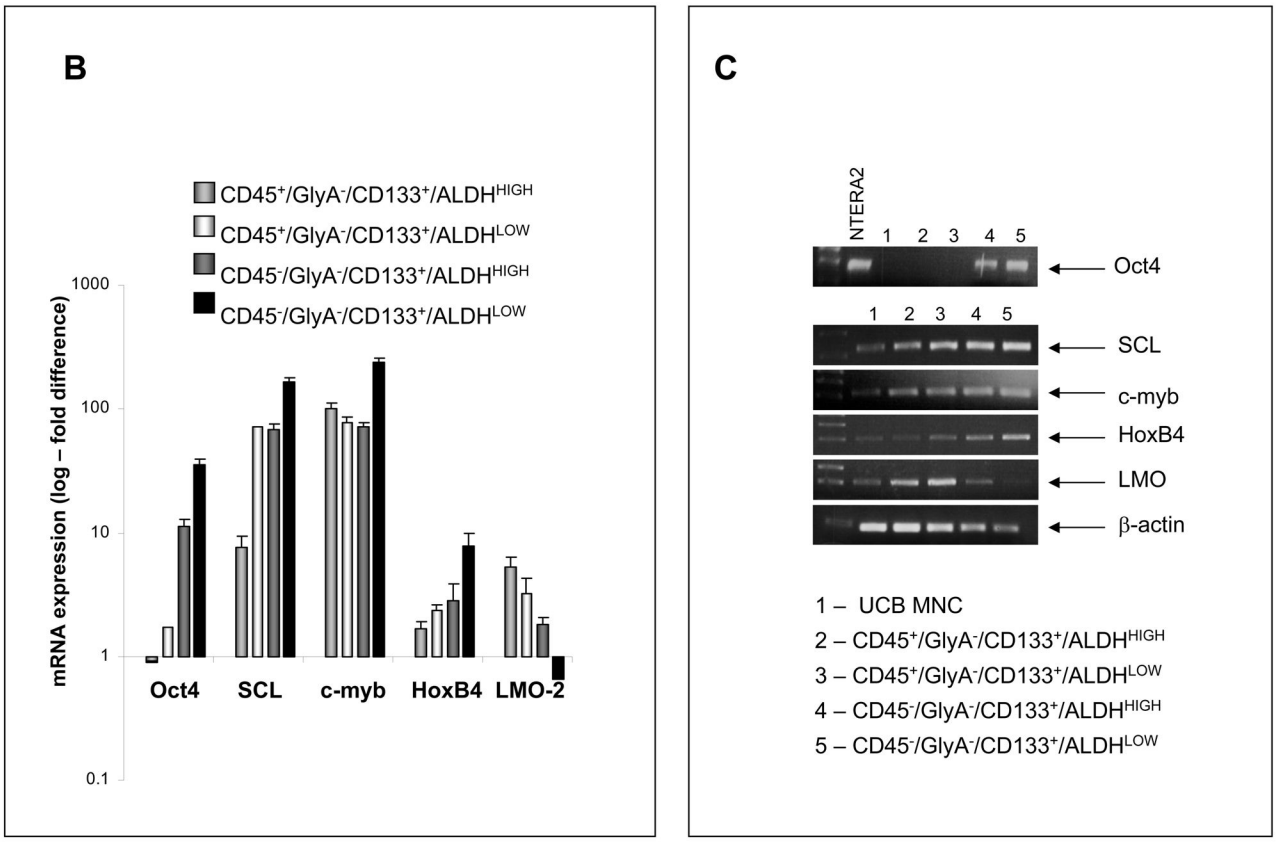


Figure 3 B C

Figure 3. Hematopoietic differentiation of VSELs

Panel A. UCB-derived $CD45^{-}/GlyA^{-}/CD133^{+}/ALDH^{low}$ express SSEA-4, Oct-4, and Nanog. Cells isolated by FACS were evaluated for expression of Oct-4, Nanog, and SSEA-4. All images were taken under Plan Apo 60XA/1.40 oil objective (Nikon, Japan). Nuclei are visualized after DAPI staining. Staining was performed on cells isolated from four independent sortings. Representative data are shown. **Panel B**. RT-PCR analysis of the expression of hematopoietic genes in freshly isolated VSELs and HSCs. The data shown represent the combined results from four independent experiments carried out in triplicate per group (n=12). **Panel C.** A representative gel out of the three independent experiments performed is shown.

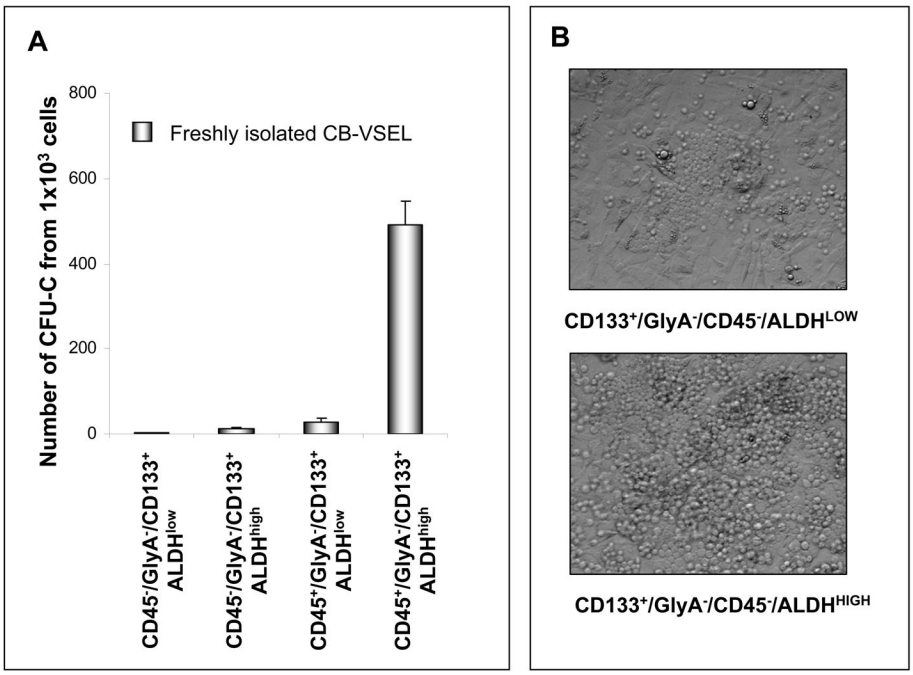
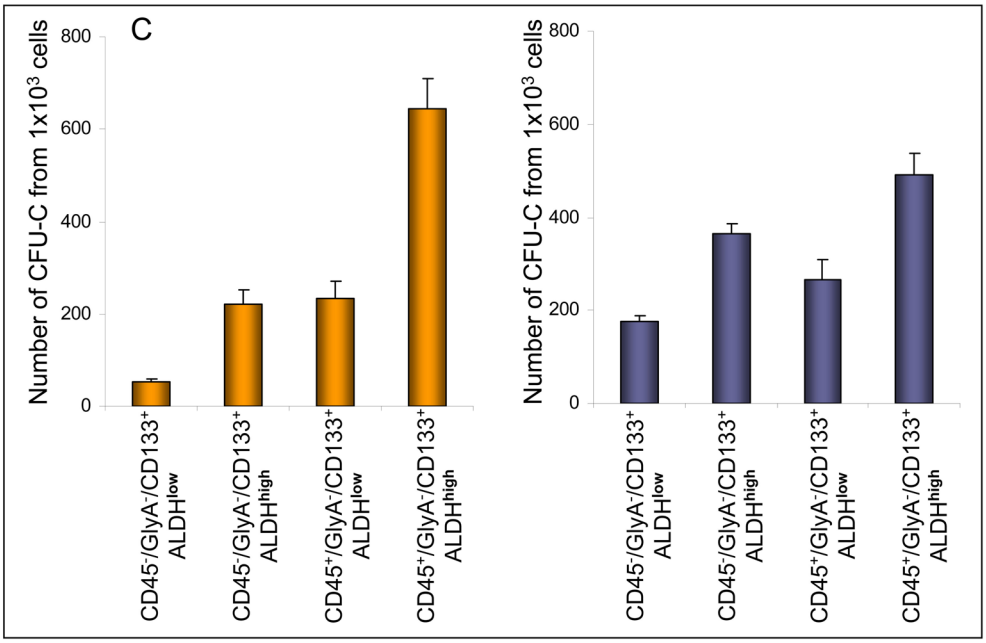


Figure 4 A, B



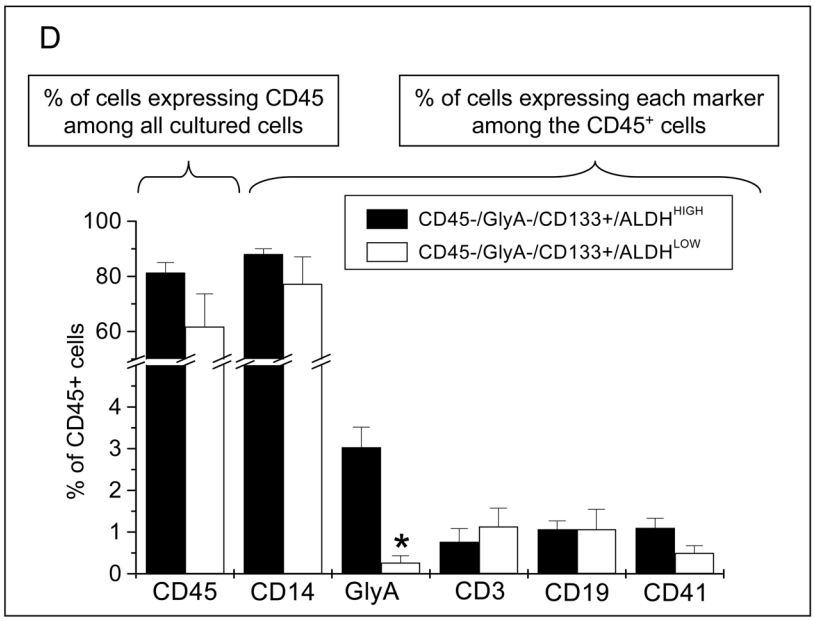


Figure 4 C D

Figure 4. VSELs are specified into HSCs in co-cultures over OP9 stromal cells

Panel A. In contrast to CD45^{+}/GlyA^{-}/CD133^{+}/ALDH^{high} HSPCs, VSELs freshly isolated from murine BM do not grow hematopoietic colonies. **Panel B.** Day 7 cultures over OP9 stromal cells initiated by UCB-derived CD45^{-}/GlyA^{-}/CD133^{+}/ALDH^{high} and CD45^{-}/GlyA^{-}/CD133^{+}/ALDH^{high} VSELs (representative pictures are shown; magnification × 20). **Panel C.** The number of colonies formed in methylcellulose by OP9-primed VSELs and HSCs (left), as well as VSEL- and HSC-derived cells replated after 10 days in secondary methylcellulose cultures (right). The data shown in Panels A and C represent the combined

results from four independent experiments carried out in triplicate for each group (n=12). * $p<0.05$, ** $p<0.0001$. **Panel D.** FACS analysis of hematopoietic gene expression on cells isolated from colonies formed in methylcellulose by OP9-cultured UCB-derived $CD45^{-}/GlyA^{-}/CD133^{+}/ALDH^{high}$ and $CD45^{-}/GlyA^{-}/CD133^{+}/ALDH^{high}$ VSELs. The data shown represent the combined results from three independent experiments carried out in duplicate per group (n=6).

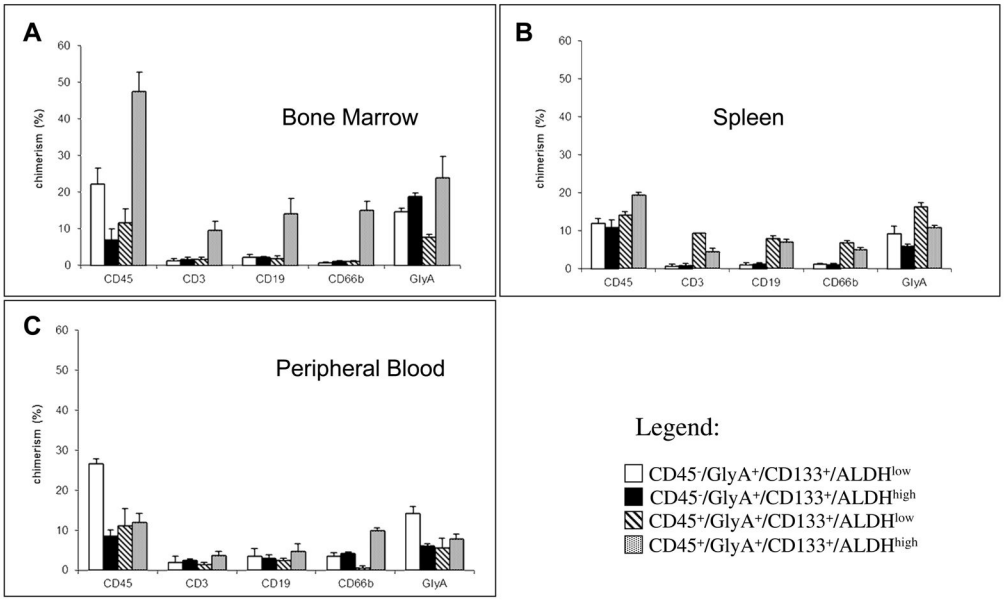


Figure 5. ***In vivo*** **transplants of freshly sorted UCB-derived VSELs and HSPCs**

Panel A. Analysis of cells expressing human CD45, CD3, CD19, CD66b, and GlyA was performed 6 weeks after transplantation in BM (**Panel A**), spleen (**Panel B**), and PB (**Panel C**). The data shown represent the combined results from 6 mice per group (n=6). Mice were transplanted with 10^6 $CD45^+$ OP9-cultured cells.

Table I

Sequences of primers employed for (q)RT-PCR analysis

Gene	Forward	Reverse
SCL	GGC TTT GTG TGA AGG CAG AGA	TCG CCA GCA TGA ACA GTG AT
C-myb	TGC CAATTA TCT CCC GAA TCG	AAC AGA CCA ACG TTT CGG ACC
HOXB-4	ACG GTA AAC CCC AAT TAC GC	TGT CAG GTA GCG GTT GTA GTG A
LMO-2	GTG TGC GAA CAG GAC ATC TAC G	AGA CGG CGT CTT CAG TGA ACA
Oct-4	GAG CCC TGC ACC GTC ACC	TTG ATG TCC TGG GAC TCC TCC
B-Actin	CGA TCC ACA CGG AGT ACT TG	GGA TGC AGA AGG AGA TCA CTG

Table II

Sequences of primers employed for RT-PCR analysis

Gene	Forward	Reverse
SCL	GTT CAC CAC CAA CAA TCG AGTG	GAT ATA CTT CAT GGC CAG GCG
c-myb	GCC AAT TAT CTC CCG AAT CGA	TTC GTC CAG GCA GTA GCT TTG
HOXB-4	ACG GTA AAC CCC AAT TAC GCC	TTT TCC ACT TCA TGC GCC G
LMO-2	CCC TTC AGA GGA ACC AGT GGA T	CTT TCA CCC GCA TTG TCA TCT C
Oct-4	GAT GGC GTA CTG TGG GCC C	TTG ATG TCC TGG GAC TCC TCC
B-Actin	CGA TCC ACA CGG AGT ACT TG	GGA TGC AGA AGG AGA TCA CTG

Table III

Total number of VSELs per 100 ml of UCB.

Phenotype of cells	Total number of cells per 100 ml of UCB
$CD45^{-}/GlyA^{-}/CD133^{+}/ALDH^{high}$	~ 10^3 (±0.3)
$CD45^{-}/GlyA^{-}/CD133^{+}/ALDH^{low}$	~3.6×10^3 (±0.8)
$CD45^{+}/GlyA^{-}/CD133^{+}/ALDH^{low}$	~46×10^3(±2.0)
$CD45^{+}/GlyA^{-}/CD133^{+}/ALDH^{high}$	~375×10^3 (±12.0)