Clonal Analysis Reveals a Common Progenitor for Endothelial, Myeloid, and Lymphoid Precursors in Umbilical Cord Blood

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Rationale: Several studies demonstrate that hematopoietic tissues are a source of endothelial progenitor cells, which contribute to newly formed blood vessels during tissue repair in adults. However, it is not clear which cell type in these hematopoietic tissues gives rise to endothelial progenitor cells.

Objective: To identify the origin of endothelial progenitors within the hematopoietic hierarchy and to assess their in vivo revascularization potential.

Methods and Results: Using a single-cell sorting approach and in vitro multilineage differentiation assays, here we show that individual CD34+/CD45−CD133+/CD38− cells from cord blood uniquely have the ability to differentiate into T- and B-lymphoid, myeloid, and endothelial cells. The latter were characterized by the expression of VE-cadherin, KDR, von Willebrand factor, endothelial nitric oxide synthase, the lack of CD45, CD133, and c-fms (colony stimulating factor-1 receptor). Unexpectedly when transplanted into hindlimb ischemic NOD-scid IL2Rγnull mice, freshly isolated CD34+/CD45+/CD133+/CD38− cells maintained their hematopoietic identity and were rarely found to integrate into host blood vessels. Nevertheless, they significantly improved perfusion, most likely through a paracrine mechanism. On the other hand, endothelial cells derived in vitro from this fraction were able to form vessels in vivo in both Matrigel plug and hindlimb ischemia transplantation assays.

Conclusions: These findings indicate that the CD34+/CD45+/CD133+/CD38− cell fraction contains a common progenitor for the hematopoietic and vascular lineages and may represent a valuable cell source for therapeutic applications. (Circ Res. 2010;107:00-00.)

Key Words: cord blood ■ clonal analyses ■ endothelial progenitors ■ ischemia ■ engraftment

It has been hypothesized that endothelial progenitor cells (EPCs) reside in adult bone marrow (BM) and are mobilized into peripheral circulation by cytokines or tissue ischemia.1,2 Transplantation of either culture-expanded EPCs or freshly isolated cells from adult hematopoietic sources results in enhanced blood-flow3 and improved function of ischemic tissues.4–10 However, engraftment levels vary significantly from laboratory to laboratory. This variability could be attributable to the heterogeneity of vascular precursor populations identified in hematopoietic tissues, as well as differences in experimental design. To date, controversy still exists with respect to the identification and the origin of these precursors in hematopoietic tissues. For instance, a number of cell types obtained using different strategies have been referred to as EPCs, including differentiated endothelial cells with more limited proliferation ability,11–13 and cells associated with the myelomonocytic lineage.14–16

Although several investigators still use the whole mononuclear cell fraction to study postnatal revascularization,14,17 a number of surface markers have been shown to be useful to identify endothelial progenitors in hematopoietic tissues. EPCs were first isolated from peripheral blood using antibodies to VEGFR-2 (KDR) or CD34.4 Although expression of KDR in hematopoietic tissues is controversial, CD34 has found common use as a marker for isolating EPCs,3,7,9 Although it is clear that CD34 purification enriches for EPCs, CD34 by itself is not a particularly good marker because it is also expressed in hematopoietic stem cells (HSCs),18 multiple hematopoietic progenitor cells,19 and mature circulating endothelial cells (ECs).20 The hematopoietic stem cell marker
CD133\textsuperscript{21} has been suggested to provide better enrichment for endothelial progenitors because it is expressed on EPCs but downregulated in mature endothelial cells.\textsuperscript{22} Consistently, a number of studies support the premise that the CD133\textsuperscript{+} cell fraction is enriched for EPCs\textsuperscript{23} and provide evidence for superior perfusion following their transplantation in animal models of ischemia.\textsuperscript{24,25} However, because CD133 is also present in early hematopoietic progenitors,\textsuperscript{21} phenotypic distinction between hematopoietic and endothelial progenitors is not possible. To make things more complicated, 2 recent studies argue that CD133\textsuperscript{+} cells do not possess the ability to generate endothelial cells.\textsuperscript{26,27} Therefore, to date, the origin of endothelial progenitors within the hematopoietic hierarchy remains controversial.

This study was designed to assess whether hematopoietic progenitors, in particular CD133\textsuperscript{+}, have the ability to differentiate into endothelial cells. To rule out the existence of independent progenitors for hematopoietic and endothelial lineages within the CD133 compartment, we tested for the presence of a common precursor at the single cell level. In this way, one can characterize both the single starting cell and its specific progeny. By using single-cell sorting assays in combination with replating studies in multiple cord blood samples, here we show the existence of a cell endowed with the ability to differentiate toward the endothelial, myeloid, and lymphoid lineages.

### Methods

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

### Sample Collection and Purification

Samples of umbilical cord blood (UCB) from healthy term newborns were obtained according to procedures approved by the institutional review boards of University of Texas Southwestern Medical Center and University of Minnesota. Mononuclear cells (MNCs) were isolated by Ficoll–Hypaque density-gradient centrifugation. In most cases, before fluorescence-activated cell (FACS), cells were pre-enriched for CD34 using MACS magnetic beads. Detailed purification approach is described in the Online Data Supplement.

### Clonal Analysis and Evaluation of Multipotentiality

Single CD34\textsuperscript{+}CD45\textsuperscript{+}CD133\textsuperscript{+}CD38\textsuperscript{−} cells were seeded in proliferation medium containing VEGF, SCF, and TPO. After 1 week, resulting colonies were divided into 4 subfractions and then subcultured in specific conditions to assess their ability to differentiate into endothelial, myeloid, T- and B-lymphoid lineages. Differentiation and characterization procedures are described in detail in the Online Data Supplement.

### Matrigel Plug Assay and Hindlimb Ischemia Model

NOD-scid IL2R\textsuperscript{null} mice (The Jackson Laboratory) were used as recipients. Animal care and all procedures were performed according to University of Minnesota Institutional Animal Care & Usage Guidelines. Detailed information regarding these procedures, as well as analyses of engraftment and laser Doppler perfusion imaging, are described in the Online Data Supplement.

### Results

#### Endothelial Potential Is Restricted to the CD34\textsuperscript{+}CD45\textsuperscript{+}CD133\textsuperscript{+}CD38\textsuperscript{−} Cell Fraction

We began by subfractionating the CD34\textsuperscript{+}CD133\textsuperscript{+} fraction from UCB based on CD38 and CD45 expression. As observed in Figure 1A (and Online Figure I), the large majority of CD34\textsuperscript{+}CD45\textsuperscript{+}CD133\textsuperscript{+} cells coexpress CD38. The ability of this early hematopoietic cell population to generate endothelial precursors in vitro was assessed side-by-side with more committed hematopoietic cell types, including CD33\textsuperscript{+} (myeloid progenitors) (Figure 1B) and CD14\textsuperscript{+} (monocytes/macrophages) (Figure 1C). CD33\textsuperscript{+} and CD14\textsuperscript{+} cells adhered to the plastic dish when cultured under endothelial conditions, displayed a spindle endothelial-like phenotype by week 2, but failed to expand under these culture conditions (Figure 1D). On the other hand, the CD34\textsuperscript{+}CD45\textsuperscript{+}CD133\textsuperscript{+}CD38\textsuperscript{−} cell fraction proliferated considerably and eventually gave rise to an endothelial-like cell population, which emerged after 4 to 6 weeks in culture (Figure 1D and 1E), that resembled human umbilical vein endothelial cells (HUVECs) (Figure 1F). The endothelial phenotype of these cells was confirmed by the expression of VE-cadherin, von Willebrand factor (vWF), KDR, and to a lesser extent endothelial nitric oxide synthase (Figure 1H). These endothelial markers were first detected by week 3, and were abundant by week 6 (Figure 1H), whereas hematopoietic markers were significantly downregulated by this time (Figure 1G).

#### CD34\textsuperscript{+}CD45\textsuperscript{+}CD133\textsuperscript{+}CD38\textsuperscript{−} Clones Possess Endothelial, Myeloid, and Lymphoid Potential

To dissect the multi-lineage differentiation potential of this cell fraction, clonal analysis was performed by FACS single cell sorting (Figure 2). Clones derived from CD34\textsuperscript{+}CD45\textsuperscript{+}CD133\textsuperscript{+}CD38\textsuperscript{−} single cells (85 from a total of 1024 wells, cloning efficiency of 8.2%) were expanded for 1 week, and each divided into 4 fractions for endothelial, myeloid as well as T- and B-lymphoid cell growth (Figure 2).

Our results showed that 49.5% of obtained clones (42 from a total of 85 clones) derived from the CD34\textsuperscript{+}CD45\textsuperscript{+}CD133\textsuperscript{+}CD38\textsuperscript{−} population were able to produce all 4 lineages (Figure 3A), as confirmed by detailed characterization of these clones. Several myeloid colonies were detected following the replating of one-fourth of the cells from each clone in complete hematopoietic methylcellulose medium (Figure 3B). To determine the
capacity of these clones to differentiate into T and B lymphocytes in vitro, the second and third parts of these colonies were cultured on OP9-DL128 and MS-529 stromal cells, respectively. The presence of B lymphocytes was demonstrated by expression of CD19 and IgM after culture on MS-5 for 45 days (Figure 3C). Lineage-specific differentiation was further confirmed by a PCR assay for DJ and VDJ recombination at the immunoglobulin heavy chain locus (Figure 3C). Likewise, double positive CD4+H11001CD8+H11001 T lymphocytes were observed after 45 days in culture on OP9-DL1 (Figure 3D). To assess whether these clonal-derived lymphocytes were capable of undergoing T-cell activation, day 45 expanding CD4+H11001CD8+H11001 T lymphocytes were stimulated with IL-2, anti-CD3 and anti-CD28 antibodies for 5 days.28 At this time, cells became CD27+H11001CD69+H11001, a phenotype characteristic of activated T-cells, whereas before stimulation, these markers were absent (Figure 3D).

Consistent with the initial results (Figure 1), CD34+CD45+CD133+CD38− derived clones yielded a monolayer of proliferating cells in the presence of endothelial cell growth medium (resulting from one fourth of original clone; Figure 2). The endothelial nature of these cells expanded for 4 to 5 weeks was confirmed by FACS analysis which revealed the expression of CD146, CD144 (VE-cadherin), and KDR, as well as the lack of CD45 and CD14 (Figure 4A; a total of 42 endothelial cultures were analyzed and all displayed similar results). The majority of these cells coexpressed KDR and CD146 (Figure 4B), confirming their endothelial phenotype. Further immunostaining analysis demonstrated coexpression of vWF and VE-cadherin in expanded endothelial cells (Figure 4C). When cultured in Matrigel, these clonal-derived monolayer cells were capable of generating extensive capillary-like structures (Figure 4D).
Additionally, we performed quantitative gene expression analysis to further confirm the endothelial, and nonhematopoietic, nature of these clones. Accordingly, high levels of KDR, vWF, VE-cadherin and endothelial nitric oxide synthase were detected in CD34+/CD45−CD133+/CD38−-derived endothelial clones, similarly to control HUVECs (Figure 4E). On the other hand, hematopoietic markers, including CD45, CD133 and c-fms, the receptor for macrophage colony-stimulating factor, were absent (Figure 4F), confirming the nonhematopoietic nature of these cells. Because CD41 and Runx-1 have been associated with the emergence of hematopoietic cells from endothelial cells30–33 during development, a process known as hemogenic endothelium, we also investigated the expression levels for these genes in CD34+/CD45−CD133+/CD38−-derived endothelial clones and their freshly isolated hematopoietic counterparts.

CD34+/CD45−CD133+/CD38− cells, before EC differentiation, were found to express much higher levels of CD41 and Runx-1 (Online Figure II) than their respective endothelial cell clones. We also followed the expansion potential of CD34+/CD45−CD133+/CD38−-derived endothelial progenitor clones (a total of 20 clones). These cells were amenable to expansion, reaching their growth peak at ~50 days, at which point, senescence occurred (Online Figure II).

**CD34+/CD45−CD133+/CD38− Cells Improve Perfusion in Ischemic Mice**

To examine whether transplantation of CD34+/CD45−CD133+/CD38− cells would increase vascularization in the ischemic hindlimb of NOD-scid IL2Rγnull mice that had been subjected to femoral artery ligation, freshly purified CD34+/CD45−CD133+/CD38− cells (3×10^5) were locally transplanted into...
the ischemic thigh muscle area immediately after surgery at 3 different injection points. Control groups consisted of mice receiving the same number of MNCs or simply PBS. Serial analyses with Laser Doppler Perfusion Imaging (LDPI) revealed superior vascularization in mice that had been treated with CD34+CD45+CD133+CD38+ cells (Figure 5A). Accordingly, the ratio of the ischemic/nonischemic hindlimb blood flow in the CD34+CD45+CD133+CD38+ group increased when compared with PBS- or MNC-treated mice at postoperative days 21 and 28 (Figure 5B).

CD34+CD45+CD133+CD38+ Cells Rarely Differentiate Into Endothelial Cells in Ischemic Mice

One month after the transplantation, mice were analyzed for the presence of human cells by immunofluorescence staining. We performed double staining with human and mouse specific anti-CD31 antibodies to best discriminate recipient-versus donor-derived vessels, as well as to identify chimeric vessels. As expected, all control mice injected with PBS (n=7) stained only with mouse CD31, similar to the control muscle staining (Online Figure III). In the group of mice that had received MNCs, we were able to detect human cells in 4 of 7 mice; however most of these mice presented only a few CD31+ cells in 1 or 2 areas of the injected muscle (Online Figure IV), a finding that supports the lack of perfusion improvement in mice transplanted with MNCs (Figure 5A and 5B). Although human CD31+ cells were easily detected in most of the mice transplanted with CD34+CD45+ CD133+CD38+ cells (7 of 8), we found only one chimeric vessel (one chimeric vessel in 1 of 7 engrafted mice) (Figure 5C). The majority of engrafted human CD31+ cells were found surrounding the recipient vessels (Figure 5D), a pattern observed in all transplanted mice. We found that the majority of the human CD31+ cells surrounding the recipient vessels maintained expression of CD34+ (Figure 5E). These results indicate that the endothelial differentiation of this progenitor is compromised in the xenogeneic environment, and that contribution of human cells to improved perfusion was not attributable to donor-derived vasculogenesis, but possibly to a paracrine mechanism.

Endothelial Cells Derived From the CD34+CD45+CD133+CD38+ Cell Fraction Participate in Neovascularization In Vivo

To assess whether the ischemic model does not favor the in vivo differentiation of CD34+CD45+CD133+CD38+ cells into endothelial cells, we differentiated them first in vitro into endothelial cells, we differentiated them first in vitro into endothelial cells (Figure 6A and 6B), and injected these into mice using the Matrigel plug assay. Two weeks after implan-
tation, plugs were removed and analyzed by immunofluorescence staining for human and mouse CD31. In the case of freshly isolated cells, we observed again the presence of human CD31+ cells surrounding the mouse-derived vessels or alternatively, as clusters of hematopoietic cells (Online Figure V). In contrast, plugs that were injected with CD34+CD45+CD133+CD38+ cells pre-
sented both donor- and recipient-derived vessels (Figure 6C and 6D), as indicated by staining with human- and mouse-specific anti-CD31 antibodies (Figure 6E and 6G [human] and 6F [mouse], respectively). These newly formed vessels were functional as evidenced by the presence of erythrocytes in the lumen (Figure 6C through 6G).

Based on these findings, we investigated the ability of CD34+CD45+CD133+CD38+ -derived endothelial cells to contribute to new vessels in the ischemic hindlimb model. To confirm the functionality of human-derived endothelial cells, biotinylated tomato lectin, which specifically binds to perfused endothelial cells, was injected intravenously into transplanted mice just before euthanasia. As observed in Figure 7, functional human-derived blood vessels were integrated into the host circulatory system, as demonstrated by costaining for
Lectin and CD31. These results confirm the endothelial nature of the endothelial cells that were differentiated from the common progenitor, the CD34⁺CD45⁻CD133⁺CD38⁻ cell fraction.

Discussion
The identification of endothelial progenitors in the adult circulation by Asahara et al⁴ has challenged the dogma that vasculogenesis is restricted to embryogenesis. Adult endothelial progenitors are found in association with the hematopoietic compartment.¹² However whether endothelial and hematopoietic progenitors have a common origin or represent independent lineages within the bone marrow remains a topic of debate. Interestingly, a study involving the analysis of blood and bone marrow samples obtained from chronic myeloid leukemia revealed the presence of BCR/ABL, the oncoprotein encoded by the Philadelphia chromosome, in both hematopoietic and endothelial cells.³⁴ This is particularly relevant because detection of the Philadelphia chromo-

some in all blood lineages of individuals with chronic myeloid leukemia has been considered as direct evidence for the existence of the HSC in humans.³⁵ Stronger evidence for an association between these 2 lineages in postnatal life was provided by Grant and colleagues.³⁶ By transplanting individual Sca-1⁺c-Kit⁺Lin⁻ BM HSCs from GFP transgenic mice into irradiated recipients, these authors demonstrated that on hematopoietic engraftment, donor-derived GFP⁺ cells contributed to the endothelial lineage when mice were subjected to retinal ischemia.³⁶ Similar results were observed following the transplantation of cord blood CD34⁺ cells into NOD/scid mice. These results were corroborated by another study demonstrating that infusion of individual Sca-1⁺c-Kit⁺Lin⁻ BM HSCs resulted in broad incorporation of donor-derived cells into the endothelial compartment even in the absence of additional vascular injury, which was not a result of cell fusion.³⁷ Further studies by this group suggest that endothelial engraftment resulted from the stem cell pool or from a common myeloid progenitor but not from a...
precursor resides within the CD34 progenitors in human cord blood. Unexpectedly, this bipotent common precursor for endothelial, myeloid, and lymphoid demonstrate for the first time, the existence of an adult sorting assays, and subsequent replating studies, here we lineage.

in the hematopoietic hierarchy gives rise to the endothelial hematopoietic compartment, it is still unclear which cell type the hypothesis that endothelial progenitors arise within the multipotent progenitor, not the hematopoietic stem cell, is the point of divergence of the endothelial and hematopoietic lineages.

Despite the evident ability of CD34⁺CD45⁺CD133⁺CD38⁺ cells to differentiate into hematopoietic and endothelial cells in vitro, hematopoietic differentiation predominates in ischemic transplantation. This could be attributed to 2 major issues: (1) hemangioblasts may not be endowed with in vivo vascularization potential because, to date, this ability has not been investigated in ES- or embryo-derived hemangioblasts; (2) an ischemic xenogeneic mouse environment is not appropriate or sufficient to induce the differentiation of this human precursor into endothelial cells.

Because our goal here was to investigate whether hematopoietic cells, in particular CD133⁺, have the ability to differentiate into endothelial cells, we did not address the existence of other angiogenic cells outside of the hematopoietic compartment, as suggested by other investigators. It might be the case that these endothelial progenitors are endowed with robust in vivo revascularization potential, although this has yet to be determined in animal models of ischemia. Mouse-to-mouse transplantation experiments involving the analyses of mice that had been subjected to unilateral femoral artery occlusion following the engraftment of BM cells isolated from transgenic mice expressing enhanced green fluorescent protein (GFP) revealed that donor GFP⁺ cells fail to incorporate into the adult growing vasculature, but were detectable around growing collateral arteries. A similar outcome was obtained following the transplantation of hematopoietic stem cells isolated from GFP transgenic mice directly into ischemic myocardium of wild-type mice. Interestingly here we observed a similar engraftment pattern following the transplantation of freshly isolated CD34⁺CD45⁺CD133⁺CD38⁺ cells into hindlimb ischemic mice. In these engrafted mice, human CD31⁺CD45⁺ cells were found in large quantities surrounding recipient’s vasculature, suggesting that the improved perfusion observed in transplanted mice may be mostly attributable to a paracrine proangiogenic function of these cells. This would be in agreement with recent studies involving the transplantation of human CD133⁺ cells or EPCs, which indicate that these cell types secrete angiogenic factors. On the other hand, when CD34⁺CD45⁺CD133⁺CD38⁺ cells are allowed to differentiate into endothelial cells in vitro, and then injected into mice using the Matrigel plug assay or the ischemia model, these cells give rise to functional human-derived and chimeric mouse–human blood vessels. Taken together, our data reveal that the CD34⁺CD45⁺CD133⁺CD38⁺ cell fraction, which per se may have an angiogenic effect in vivo, is endowed with the ability to differentiate in vitro into myeloid, lymphoid, and endothelial lineages. The endothelial cells generated from this common precursor are able to participate in new blood vessel formation, providing a rationale for the use of this cell population.

Figure 7. In vivo revascularization potential of CD34⁺CD45⁺CD133⁺CD38⁺-derived endothelial clones in hindlimb ischemic mice. A, High magnification of a newly formed human-derived capillary vessel (white arrow), which coexpresses human CD31 (in red) and lectin (in green). This vessel sits close to a recipient-derived vessel, which is Lectin− and negative for human CD31 (green arrow). B, High magnification of a hybrid blood vessel. CD31 staining reveals the incorporation of human CD31⁺ cells in a vessel that homogeneously express lectin (in green) and vWF (in purple), which labels both mouse and human cells. Merged images show coexpression of human CD31 with lectin and vWF, as indicated by white arrows.
as well as their progenitor, for therapeutic applications in ischemia conditions.

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Disclosures
None.

References
Novelty and Significance

What Is Known?

- Endothelial progenitor cells (EPCs) are not restricted to embryogenesis.
- In postnatal life, EPCs are present in hematopoietic tissues and are endowed with in vivo regenerative potential.
- Origin of endothelial progenitors within hematopoietic tissues is still unknown.

What New Information Does This Article Contribute?

- Identification of a multipotent hematopoietic progenitor in cord blood that gives rise to endothelial precursors.
- This multipotent progenitor can be isolated based on the expression of CD34, CD45, CD133, and CD38 antigens.
- Transplantation of freshly isolated CD34+CD45+CD133+CD38+ cells into ischemic hindlimbs results in improved perfusion, possibly through a paracrine effect.
- Transplantation of endothelial cells differentiated in vitro from the CD34+CD45+CD133+CD38+ cell fraction gives rise to functional human-derived and chimeric mouse–human blood vessels.

Recent investigations have highlighted the potential of EPCs for therapeutic applications in vascular medicine. Hematopoietic tissues from adults have been the primary cell source in these laboratory studies and early clinical trials. Most previous investigations have involved the infusion of heterogeneous populations of cells. To date, it is not clear which cell type within these hematopoietic tissues is responsible for vascular regeneration. We demonstrate here through clonal analysis that at least one source of endothelial progenitor cells in human cord blood can be traced back to a common progenitor of lymphoid and myeloid hematopoietic cells, a cell marked by the antigens CD34, CD45, CD133, and CD38. Although injection of this freshly isolated progenitor cell into ischemic hindlimbs of immunodeficient mice results in improved recovery from ischemia, engrafted cells do not adopt an endothelial fate directly in this in vivo environment but rather adopt a proangiogenic hematopoietic fate. However, when this progenitor is first differentiated in vitro into endothelial cells, these cells generate functional human-derived and chimeric mouse–human blood vessels. These findings provide insights into the identity of EPCs and their positioning with regard to the hematopoietic hierarchy, giving scientific rationale for future clinical applications in patients with ischemic vascular diseases.
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SUPPLEMENT METHODS

Sample collection and purification

Samples of umbilical cord blood from healthy term newborns were obtained according to procedures approved by the institutional review boards of UT Southwestern Medical Center and University of Minnesota. Mononuclear cells (MNCs) were isolated by Ficoll-Hypaque density-gradient centrifugation and CD34⁺ enrichment was obtained by positive selection with the MACS magnetic beads separation method following the manufacturer’s instructions (Miltenyi). For sorting experiments, CD34⁺ enriched samples were incubated with allophycocyanin (APC)-conjugated monoclonal anti-CD34 (Pharmingen), fluorescein-5-isothiocyanate (FITC)-conjugated monoclonal anti-CD45 (Pharmingen), phycoerythrin (PE)-conjugated monoclonal anti-CD133 (Miltenyi), and phycoerythrin-Cy7 (PE-Cy7)-conjugated monoclonal anti-CD38 (eBioscience). FITC-conjugated monoclonal anti-CD33 and APC-conjugated anti-CD14 antibodies were also used (both from eBioscience). Cells were washed, resuspended in PBS containing 0.5% BSA (Fisher Scientific) and 2mM EDTA (Fisher Scientific), and sorted on a FACS Aria (Becton-Dickinson) after addition of propidium iodide (Pharmingen) to exclude dead cells. Isotype controls of APC, PE, FITC, PE-Cy7, as well as the forward-scatter and side-scatter properties of normal live cord blood cells were used to establish sorting gates.

Clonal analysis and evaluation of multi-potentiality

Single CD34⁺CD45⁺CD133⁺CD38⁺ cells isolated from cord blood were seeded in round bottom 96 well-plates in growth medium containing IMDM, 15% FBS, iron-saturated transferrin (200 µg/m), human VEGF (5 ng/ml), human SCF (100 ng/ml), and human
TPO (25 ng/ml), which we refer as proliferation medium. After one week in culture, resulting colonies were collected, divided into four sub-fractions, and then sub-cultured in specific conditions to assess their multi-potentiality (endothelial, myeloid, T and B lymphoid). Endothelial potential was studied by plating the cells in fibronectin-coated dishes with EBM-2 Basal medium (Clonectics, Lonza) plus standard EGM-2 SingleQuotes (Clonectics, Lonza) that includes 2% FBS, VEGF, hFGF, R3-IGF-1, hEGF, hydrocortisone, ascorbic acid, heparin and GA-1000. For the first 10 days, medium was changed twice a week. After this period, cultures were passaged every 4 days when subconfluent (60-70%). At the end of 4 weeks, endothelial characterization was performed. In some experiments, cell growth was monitored until cells reached senescence. Myeloid activity was studied by plating the cells into secondary methylcellulose medium containing IL-3, GM-CSF, SCF, and Epo (H4434; Stem Cell Technologies). The number of colony-forming cells (CFCs) was enumerated after 2 weeks in culture. Differentiation towards the T lymphoid lineage was evaluated by plating cells onto 24-well dishes pre-plated with a confluent monolayer of OP9-DL1 stromal cells in medium consisting α-MEM supplemented with 20% FBS, human IL-7 (5 ng/ml; Peprotech) and human Flt3-ligand (5 ng/ml) (Peprotech) ¹. B lymphoid differentiation was investigated by plating cells onto 24-well dishes pre-plated with a confluent monolayer of murine bone marrow MS-5 stromal cells in medium consisting of α-MEM supplemented with 10% FBS, human IL-7 (5 ng/ml) and human SCF (100 ng/ml) ². Medium was changed every week. After 6 weeks in culture, cells were harvested using trypsin-EDTA (Invitrogen) and subjected to phenotypic analysis.

**Human T-cell activation**
Experiments to induce T-cell activation were carried out on day 45 of co-culture. Co-cultures maintained with recombinant human IL-7 and Flt3-ligand (Peprotech) were treated with exogenous human IL-2 (6 ng/ml) (Peprotech) and stimulated with anti-CD3 (1 μg/ml) and anti-CD8 (1 μg/ml) monoclonal antibodies (both from Pharmingen) for 5 days \(^1\). The expression of T-cell activation markers was analyzed before and 5 days after stimulation.

**Flow cytometry analyses**

Live cells were gated based on forward- and side-scatter and lack of propidium iodide uptake. For endothelial characterization, we used FITC- or APC-conjugated anti-KDR, PE-conjugated anti-human CD146, and non-conjugated goat anti-human VE-cadherin (CD144) antibodies (all from R&D). For secondary staining, a PE-conjugated rabbit anti-goat antibody was applied (Pharmingen). FITC-conjugated monoclonal anti-CD45 and anti-CD14 antibodies were also utilized (both from Pharmingen). In the lymphoid cultures, the large OP9-DL1 and MS-5 stromal cells could be easily discriminated from their forward- and side-scatter profile. For T cells, FITC-conjugated monoclonal anti-CD4 and biotinylated monoclonal anti-CD8 antibodies were used (both from Pharmingen). For B cells, FITC-conjugated monoclonal anti-CD19 (eBioscience) and biotinylated monoclonal anti-IgM (Pharmingen) were used. Secondary staining for CD8 and IgM was performed with Streptavidin-APC (Pharmingen). To assess T-lymphocyte activation, FITC-conjugated anti-human CD69 and PE-conjugated anti-human CD27 antibodies were used (Pharmingen).

**Endothelial cell differentiation into capillary-like structures**
CD34+CD45+CD133+CD38+ single cell-derived endothelial cells that had been cultured for a month in endothelial expansion medium were harvested with trypsin-EDTA, washed with growth medium, and then seeded at a density of 5,000 to 20,000 cells per well of a 96-well plate pre-coated with 30 µl Matrigel (Becton-Dickinson) and incubated at 37° C for 30 minutes to allow polymerization. Tube-like structures were documented after 12 hours of culture.

**DNA preparation and PCR analysis of immunoglobulin gene rearrangement**

Cells were harvested with trypsin and resuspended in lysis buffer supplemented with 500 µg/ml proteinase K (Invitrogen). After 90 minutes of incubation at 56° C, proteinase K was subsequently inactivated by heating at 95° C for 10 minutes. PCR was performed in a volume of 50 µl containing 100 µM deoxynucleotide triphosphate (dNTPs), 1 µM each primer, and 0.3 U AmpliTaq polymerase (Applied Biosystems). PCR conditions consisted of 10 minutes at 94 °C and 38 cycles of 45 seconds at 94 °C, 60 seconds at 65°C, 60 seconds at 72 °C. Amplification of complete VDJH rearrangement was performed using a 5’ consensus framework 3 (FR3) primers (5’-GACACGCGCCGTGTATTACTGTGC-3’) and a 3’ consensus JH primer (5’-AACTGCAGAGGAGACGGTGAC-3’). For detection of incomplete DJH rearrangement, a mixture of consensus 5’ primers for each of the 7 main DH gene families was used (Online Table 1) with the same 3’ consensus JH primer used for detection of complete VDJH rearrangement.

**Quantitative real time polymerase chain reaction (qPCR) analysis**

Total RNA was extracted using Trizol (Invitrogen) as recommended by the manufacturer. cDNA synthesis was performed using random primers and ThermoScript™ RT
Quantitative PCR was performed using the TaqMan Universal Master Mix (Applied Biosystems). The samples were amplified for 40 cycles on the 7900HT Fast Real-Time PCR System (Applied Biosystems). Target gene transcripts were amplified, as well as GAPDH, for a housekeeping control gene. All reactions were performed in triplicate. The ΔΔCt method was used to compare and quantify fold induction between samples from all time points, MNC and HUVEC, normalizing to GAPDH. We performed quantitative PCR for hematopoietic and endothelial specific genes with probe sets from Applied Biosystems.

**Matrigel plug assay and hind-limb ischemia model**

Male 6-8 weeks old NOD-scid IL2Rgamma<sup>null</sup> mice (Jackson laboratories) were used as recipients for these *in vivo* experiments. Animal care and all procedures were performed according to University of Minnesota Institutional Animal Care & Usage Guidelines. For the Matrigel plug assay, freshly sorted CD34<sup>+</sup>CD45<sup>+</sup>CD133<sup>+</sup>CD38<sup>+</sup> cells (2-3 x 10<sup>5</sup>) or endothelial cell clones (3 x 10<sup>5</sup>) that had been differentiated from this fraction, as described above, were resuspended in 250 or 500ul Matrigel (Becton Dickinson) supplemented with 500ng/mL FGF (Peprotech), 200ng/mL VEGF (Peprotech and 50μg/mL heparin (APP Pharmaceuticals) and injected subcutaneously into the groin area of NOD-scid IL2Rgamma<sup>null</sup> mice (4 mice for each experimental group). Controls consisted only of Matrigel plus growth factors (n=3). After 15 days, matrigel plugs were harvested, fixed in 10% Zinc Formalin for 48h at 4°C, processed for paraffin embedding and 5 to 8μm serial sections were collected for immunostaining analyses.

For the hind-limb ischemia model, NOD-scid IL2Rgamma<sup>null</sup> mice were anesthetized with intraperitoneal injection of a combination of anesthetics Ketamine (80
mg/kg) and xylazine (10 mg/kg). The femoral artery of left hind-limb was completely excised from its proximal origin as a branch of the external iliac artery to the point distally where it bifurcates into the saphenous and popliteal arteries. For the first set of transplantations, immediately after the surgery, animals were transplanted with $3 \times 10^5$ of UCB CD34$^+$CD45$^+$CD133$^+$CD38$^+$ cells (n=10) or MNCs (n=10) intramuscularly in three sites of the ischemic thigh. Another control group consisted of mice-injected with PBS (n=10). Four weeks after surgery, mice were euthanized, the chest was opened and the vasculature was perfused with saline (8 min) and 4% paraformaldehyde (8 min). Immediately the limb muscles were dissected, fixed in 4% paraformaldehyde overnight at 4°C, processed for paraffin embedding and 5 to 8µm serial sections were collected for immunostaining analyses. For the second set of transplantations, immediately after the surgery, animals were transplanted with $6 \times 10^5$ CD34$^+$CD45$^+$CD133$^+$CD38$^+$ single cell-derived endothelial cells (n=3) intramuscularly in three sites of the ischemic thigh. Four weeks after surgery, mice were injected in the tail vein with 100 µl of Biotinylated-*Lycopersicon esculentum* (tomato) Lectin (200µg/mouse; Vector laboratories), after 10 min the animals were euthanized, the chest was opened and the vasculature was perfused with saline (8 min) and 10% Zinc Formalin (8 min). Immediately the limb muscles were dissected, fixed in 10% Zinc formalin for 48h at 4°C, processed for paraffin embedding and 5 to 8µm serial sections were collected for immunostaining analyses.

**Immunohistochemistry of cultured cells and tissue sections**

For Immunofluorescent staining, cells cultured on slides were fixed using 4% paraformaldehyde, permeabilized with 1% Triton X-100 (Sigma), blocked with 10% goat serum, and then incubated with primary antibodies which included rabbit polyclonal anti-
human von Willebrand Factor (vWF; DAKO) and mouse monoclonal anti-human VE-cadherin (Pharmingen). For secondary staining, Cy2-conjugated donkey anti-mouse and Cy3- donkey anti-rabbit antibodies were used (Jackson Immunoresearch Laboratories). For immunofluorescence staining of tissues, sections were dewaxed and rehydrated; antigen was retrieved using Target Retrieval Solution (DAKO) or Proteinase K (Dako) according to the manufacturer’s protocols. Non-specific binding sites were blocked with 3% BSA, and the cell permeabilization was done by adding 0.2% Tween® 20 (Sigma) to the washing buffer (Tris-Buffered Saline 1x). The tissue sections were incubated overnight at 4°C with primary antibodies which included rat monoclonal anti-mouse CD31 (1:25; Pharmigen), mouse monoclonal anti-human CD31 (1:25; DAKO), rabbit polyclonal anti-human Von Willebrand Factor (1:300; DAKO) and rat monoclonal anti-human CD45 (1:20; Santa Cruz). For secondary staining, Alexa Fluor 647 goat anti-rabbit, Alexa Fluor 555 goat anti-mouse, and Alexa Fluor 488 donkey anti-rat (Invitrogen Molecular Probes) were used. For Lectin staining, we used Fluorescein Streptavidin (1:200; Vector laboratories). Cell nuclei were stained using DAPI (Fluka). Isotype control antibodies were used as negative control staining. The images were acquired using Zeiss Axio Imager M1 Upright Microscope and Zeiss Confocal Microscope Zeiss (LSM 510 Meta).

Laser Doppler Perfusion Imaging (LDPI)

A laser Doppler perfusion imager (Moor Instruments) was used for serial noninvasive physiological evaluation of neovascularization, as previously described. Mice were monitored by serial scanning of surface blood flow of hind-limbs on days 0 (before and immediately after surgery), 7, 14, 21 and 28 after treatment. The digital color-coded
images were analyzed to quantify the blood flow in whole leg, and mean values of perfusion of the ischemic and non-ischemic legs were calculated. To avoid data variations due to ambient light and temperature, hind-limb blood flow was expressed as the ratio of left (ischemic) to right (non-ischemic) legs.

**Statistical analysis**

Differences between samples were assessed by using the Student’s two-tailed t test for independent samples.

**References**


Online Figure I. FACS gates CD34/CD133, CD45/CD38 and control gates. (A) CD34+CD133+CD45+CD38+ cells isolated from UCB (upper panel) and controls (lower panel), (B) Controls for FACS gates in Figure 1A (upper panel), Figure 1B (left lower panel) and Figure 1C (right lower panel). (C) Representative FACS profiles for CD45 expression in UCB mononuclear cells. Histogram plots show isotype control staining profile (thin line) versus specific antibody staining profile (thick line). Percentages represent the fraction of cells that express CD45.

Online Figure II. Presence of bipotent precursors in the CD34+CD45+CD133+CD38+ cell fraction of human bone marrow. Human bone marrow MNCs were pre-enriched for CD34 with magnetic beads and further purified by FACS based on CD34, CD45, CD133 and CD38 expression. (A) Frequency distribution of human bone marrow clones (n=30) in regard to differentiation potential towards the endothelial (E), myeloid (M), and lymphoid (L) lineages. (B) Myeloid colony activity. CD34+CD45+CD133+CD38+ single cell derived colonies were plated for myeloid colony forming activity. Data represent the number of colonies produced by one-fourth of total cells from each well. Data are expressed as mean ± SEM of a total of 30 clones isolated from 2 independent BM samples. (C) Lymphoid differentiation of a representative clone obtained from the CD34+CD45+CD133+CD38+ cell fraction. The presence of B lymphocytes is demonstrated by the expression of CD19 and IgM after 45 days in culture. Fluorescence intensity for APC-labeled IgM is indicated on the y axis and FITC-labeled CD19 on the x axis (left panel). Differentiation into T lymphocytes is evidenced by the presence of double positive CD4+CD8+ cells after 45 days in culture. Fluorescence intensity for APC-
labeled CD8 is indicated on the y axis and FITC-labeled CD4 on the x axis. (right panel).

(D) CD34⁺CD45⁺CD133⁺CD38⁺ single cell-derived clones have the ability to differentiate into endothelial cells as demonstrated by the expression of CD146, VE-cadherin (CD144), KDR, and absence of CD45. Plots show isotype control IgG staining (gray histogram) versus specific antibody staining (black histogram). Far right, representative FACS plot showing that the majority of these clone-derived adherent cells co-express CD146 and KDR. Percentages in C-D represent the fraction of cells that express a given surface antigen. Right panel, representative FACS plot shows co-expression of KDR and CD146 in these cells. (E) Growth kinetics of endothelial cell clones derived from CD34⁺CD45⁺CD133⁺CD38⁺ cells.

**Online Figure III.** Control staining for human and mouse antibodies. Staining of human (thymus) and mouse (muscle) vessels using human or mouse specific CD31 antibodies reveals specific staining of each antibody without any cross reactivity between the two species. vWF antibody is non specific and labels both human and mouse vessels. (B) Images show lectin staining (Strep-FITC) of a control mouse vessel after systemic perfusion with biotinylated lectin. Vessel is negative for human CD31, and positive for lectin and vWF. (C) Control staining for human CD45 (green) in human thymus (upper panel) and mouse spleen (lower panel) paraffin sections. The absence of staining with human CD45 in mouse spleen shows that this antibody is specific for human tissue. DAPI is showed in blue.
**Online Figure IV.** *In vivo* results following the transplantation of MNCs into ischemic mice. Micrographs show representative images of transplanted muscle sections stained with human CD31 (red) and human CD45 (green) antibodies. DAPI staining is shown in blue.

**Online Figure V.** *In vivo* revascularization potential of freshly isolated CD34⁺CD45⁺CD133⁺CD38⁺ cells using the Matrigel Plug Assay. Human CD31⁺ cells (in red) are detected in Matrigel plugs, but they are not part of new vessels. They are found as clusters of cells (white arrow in upper panel) or lining up mouse-derived vessels (lower panel). Mouse CD31⁺ endothelial cells (green) migrate into the matrigel from peripheral connective tissue surrounding it to form capillary vessels. Lower panel shows capillary vessels derived from mouse endothelial cells. White arrow indicates a human CD31 positive cell (in red) lying beside a mouse capillary vessel. Red arrow indicates RBCs inside in the lumen of the capillary vessel.

**(G)** qPCR analysis for CD41 and Runx-1 in freshly sorted CD34⁺CD45⁺CD133⁺CD38⁺ cells (6 independent biological samples, a-f) and after differentiation of these cells into the endothelial lineage (8 independent clones, A-H). HUVECs and MNCs were used as reference. Transcripts are normalized to GAPDH in **E-G.** (I) Growth kinetics of 20 endothelial cell clones derived from CD34⁺CD45⁺CD133⁺CD38⁺ cells collected from 3 independent cord blood samples. ♦indicates the mean of cell number at each passage. Results are presented as Mean ± SEM.
Online Figure I

A

CD34 vs CD133

CD45 vs CD38

APC vs PE

FITC vs PECy7

B

APC vs FITC

PE vs PECy7

PE vs FITC

APC vs PE
CD45 expression in UCB Mononuclear Cells

MNC-1: 99.8%
MNC-2: 99.9%
MNC-3: 98.3%
Online Figure II

A

CD41

Fold change to GAPDH

Before EC differentiation

After EC differentiation

HUVEC

B

Runx1

Fold change to GAPDH

Before EC differentiation

After EC differentiation

HUVEC

C

Number of cells (x10^5)

Time in culture (days)
Online Figure III

Control staining for human and mouse antibodies

A

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Online Figure III

C

Human CD45  DAPI  Merge

Human thymus

Mouse spleen
Online Figure IV

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Online Figure V
### Online Table I. Sequence of DH primers

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