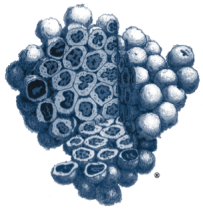

# STEM CELLS®

**Hemogenic Endothelial Progenitor Cells Isolated from Human Umbilical Cord Blood**

Xiao Wu, M. William Lensch, Jill Wylie-Sears, George Q. Daley and Joyce Bischoff

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STEM CELLS®

TISSUE-SPECIFIC STEM CELLS

# Hemogenic Endothelial Progenitor Cells Isolated from Human Umbilical Cord Blood

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**Key Words.** Endothelial progenitor cells • Hemogenic endothelium • Umbilical cord blood • AML-1 • CD133

## ABSTRACT

**Hemogenic endothelium has been identified in embryonic dorsal aorta and in tissues generated from mouse embryonic stem cells, but to date there is no evidence for such bipotential cells in postnatal tissues or blood. Here we identify a cell population from human umbilical cord blood that gives rise to both endothelial cells and hematopoietic progenitors in vitro. Cord blood CD34+/CD133+ cells plated at high density in an endothelial basal medium formed an endothelial monolayer and a nonadherent cell population after 14–21 days. AML-1, a factor required for definitive hematopoiesis, was detected at low levels in adherent cells and at high levels in nonadherent cells. Nonadherent cells coexpressed the endothelial marker vascular endothelial (VE)-cadherin and the hematopoietic marker CD45, whereas adherent cells were composed primarily of VE-cadherin+/CD45− cells and a smaller fraction of VE-cadherin+/CD45+ cells. Both nonadherent and adherent cells produced hematopoietic colonies in methylcellulose, with the adherent cells yielding more colony-forming units (CFU)-GEMM compared with the nonadherent cells. To determine whether the adherent endothelial cells were producing hematopoietic progenitors, single cells from the adherent population were expanded in 96-well dishes for 14 days. The clonal populations expressed VE-cadherin, and a subset expressed AML-1, ε-globin, and γ-globin. Three of 17 clonal cell populations gave rise to early CFU-GEMM hematopoietic progenitors and burst-forming unit-erythroid progenitors. These results provide evidence for hemogenic endothelial cells in human umbilical cord blood.** STEM CELLS *2007;25:2770–2776*

Disclosure of potential conflicts of interest is found at the end of this article.

## INTRODUCTION

Hematopoietic progenitors are known to be intimately associated with the endothelium during embryonic development. This association is seen in the blood islands of the extraembryonic yolk sac, wherein the first wave of primitive hematopoiesis is initiated. The close proximity and temporal appearance of hematopoietic and endothelial progenitors has strongly supported the concept of the hemangioblast, a cell whose existence has been demonstrated in in vitro studies of embryonic stem cells [1, 2], in postnatal bone marrow and cord blood [3], and from a brachyury+/Flk-1+ cell population within the primitive streak of the mouse embryo [4]. Hematopoietic progenitors are also formed from "hemogenic" endothelium found in a spatially and temporally restricted arrangement along the ventral aspect of the dorsal aorta and vitelline artery of the 5-week-old human embryo [5, 6]. An important distinction between the hemangioblast and hemogenic endothelium is that the hemangioblast is an undifferentiated progenitor cell that gives rise to angioblasts and hematopoietic stem cells; in contrast, hemogenic endothelium consists of endothelial cells that have assumed a morphologically mature phenotype along the vessel wall. Elegant studies by Tavian et al. revealed the appearance of rounded intra-aortic cell clusters clinging to the ventral aspect of the dorsal aorta [7]. The immunolabeling using anti-CD45, a cell surface antigen expressed exclusively on hematopoietic cells, and anti-CD34, an endothelial marker, strongly suggested that the intra-aortic cell clusters represent an intermediate between the endothelial and hematopoietic lineages. Similar budding cells positive for CD45 and vascular endothelial growth factor-receptor-2 (VEGF-R2), an endothelial marker also known as the kinase-insert domain receptor (KDR) or fetal liver kinase-1 (Flk-1), have been identified in chick embryo [8]. In the mouse, approximately 1%–2% of clonally isolated embryonic Flk-1+/VE-cadherin+/CD45− cells were shown to generate VE-cadherin-positive monolayers and rounded CD45+ cell clusters [9]. With these phenotypic markers, the study provided direct evidence for the production of hematopoietic cells from endothelial cells. In human embryos, the budding of hematopoietic progenitors from the hemogenic endothelium is restricted to day 27 to day 40 of human development and has not been detected in fetal tissues or bone marrow, the umbilical cord, or newborn human umbilical vein endothelial cells [10].

Human umbilical cord blood (HUCB) is a potentially valuable source of hematopoietic stem cells, unrestricted somatic stem cells, mesenchymal stem cells, and endothelial progenitors, that can be used for a wide array of therapeutic applications, from bone marrow transplantation to cell-based repair or replacement of diseased tissues [11–14]. Phenotypically stable

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endothelial cells with robust in vitro expansion potential have been isolated from HUCB by many different laboratories [14–18]. Because of these desirable properties, cord blood-derived endothelial progenitor cells (EPCs) are currently being developed for use in cardiovascular tissue engineering [14, 16, 19, 20], with the most likely applications being for children born with congenital heart defects. We showed previously that highly purified CD34+/CD133+ cells isolated from human umbilical cord blood differentiated into endothelial cells (ECs) with robust growth potential, a stable endothelial phenotype, and ability to form microvessels in vitro when seeded with human smooth muscle cells [15]. However, the differentiation potential of cord blood-derived CD34+/CD133+ cells, from the time of immunoselection until a sufficient number of endothelial cells have been expanded for functional studies, has not been examined. Understanding the differentiation abilities and pathways of cord-blood endothelial progenitors will be critical for maximizing the therapeutic potential and insuring long-term safety. Toward this end, we studied the morphological and cellular differentiation of CD34+/CD133+ cells from human umbilical cord blood, over 4 weeks, in culture conditions that favor growth of endothelial cells. We describe here a time period from 14 to 21 days in culture in which the adherent endothelial cells produce hematopoietic progenitor cells, much like the hemogenic endothelium in the human embryo. This is the first demonstration of hemogenic endothelium from blood-derived endothelial progenitor cells.

## MATERIALS AND METHODS

### Isolation of Mononuclear Cells from Umbilical Cord Blood

HUCB was obtained from the Brigham and Women's Hospital (Boston, MA) in accordance with an Institutional Review Board-approved protocol. Two milliliters of a 10 U/ml heparin diluted in 0.9% NaCl was placed in a 60-ml syringe before obtaining cord blood. Mononuclear cells (MNCs) were isolated by density gradient sedimentation on Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden, http://www.amersham.com) using Accuspin tubes (Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com). One Accuspin tube was used for 20 ml of blood; HUCB samples ranged from 40 to 80 ml. Once the MNCs were obtained and washed, red blood cells were lysed using an ammonium chloride solution from Stem Cell Technologies (Vancouver, BC, Canada, http://www.stemcell.com).

### Isolation and Culture of CD34+/CD133+ Cells

MNCs were resuspended in endothelial basal medium (EBM-2) (CC-3156; Cambrex, Walkersville, MD, http://www.cambrex.com) that was prepared as follows: 375 ml of EBM-2, 100 ml of heat-inactivated fetal bovine serum (HyClone, Logan, UT, http://www.hyclone.com), 75 ml of human plasma obtained during the MNC isolation, 5 ml of 100× penicillin/streptomycin/fungizone (Invitrogen, Carlsbad, CA, http://www.invitrogen.com), and Single Quots (CC-4176; Cambrex; all growth factors, heparin, ascorbic acid, and antibiotics except the hydrocortisone) were combined. Each 500-ml preparation was sterile-filtered through a 0.22 $\mu$m filter and stored in aliquots at −20°C until use. This medium was used to culture the MNCs and CD34+/CD133+ cells in this study. MNCs ($2–3 \times 10^7$) were plated in 10 ml of medium on 1% gelatin-coated 100-mm dishes and placed in a 5% $CO_2$ incubator for 2 days. $CD34^+$ cells were purified from the nonadherent cell population using anti-CD34-conjugated magnetic microbeads (CD34 MultiSort Kit; Miltenyi Biotec, Auburn, CA, http://www.miltenyibiotec.com). The rationale for CD34-positive selection was based on the following factors: (a) CD34 is expressed on human ECs and EPCs, and (b) endothelial outgrowth from $CD34^+$ cells has been shown to be more homogeneous than endothelial cells derived from unselected MNCs [21]. CD34+ cells were treated with Release Reagent (Miltenyi Biotec) to release the bound anti-CD34 beads. The CD34-selected cells were then immunoselected using anti-CD133/1-conjugated magnetic microbeads (Miltenyi Biotec) according to the manufacturer's instructions; in some experiments, a second CD133 immunoselection was performed. Eluted CD34+/CD133+ cells were counted and plated at a density of 60,000–90,000 cells in 200 $\mu$l of medium in gelatin-coated 96-well plates. The yield of $CD34^+$/$CD133^+$ cells ranged from 1,500 to 5,000 cells per milliliter of HUCB, with similar numbers obtained from male and female umbilical cords. Cells were fed every 2 days by gently removing 100 $\mu$l of medium from the top of the well and adding 100 $\mu$l of fresh medium. The 100 $\mu$l removed from the well was plated in an adjacent gelatin-coated well and inspected to ensure that no cells were lost.

### Reverse Transcriptase-Polymerase Chain Reaction Detection of mRNA

RNA was isolated from adherent and nonadherent $CD34^+$/$CD133^+$ cells using the RNeasy Mini Kit and RNase-Free DNase (Qiagen, Valencia, CA, http://www1.qiagen.com). cDNA was synthesized with Superscript III RNase $H^-$ reverse transcriptase (RT) (Invitrogen). Oligonucleotide primers for each amplicon are provided in supplemental online Table 1. AML-1a primers were obtained from Choi et al. [22]. Polymerase chain reaction (PCR) was carried out using iQ Supermix (Bio-Rad, Hercules, CA, http://www.bio-rad.com) for 35 cycles. Each PCR condition cycle included 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute, followed by extension at 72°C for 10 minutes after 35 cycles. Identity of the PCR products was confirmed by DNA sequencing of the amplicon (DNA Sequencing Core Facility, Children's Hospital Boston).

### RT-PCR on Clonal Populations

Clones were grown for 10 days after single-cell plating before harvesting for RNA isolation. cDNA was synthesized with an iScript cDNA Synthesis kit (Bio-Rad) following DNase I digestion. Oligonucleotide primers for each amplicon are provided in supplemental online Table 1. Globin primers were obtained from Mahajan et al. [23] PCR was performed with Platinum Supermix High Fidelity (Invitrogen) for 35 cycles on a PT-100 machine (MJ Research, Waltham, MA, http://www.mjresearch.com). Each RT-PCR condition cycle included 95°C for 30 seconds, 56°C for 30 seconds, and 72°C for 30 seconds, with 35 cycles for each target, followed by extension at 72°C for 5 minutes after cycling.

### Indirect Immunofluorescence

Adherent cells were replated onto 1% gelatin-coated glass coverslips for 24 hours, fixed with −20°C methanol, and incubated with primary antibody diluted 1:1,000 followed by fluorescein isothiocyanate (FITC)-conjugated secondary antibody at 5 $\mu$g/ml. von Willebrand factor (vWF) was detected using rabbit anti-human vWF (DAKO, Glostrup, Denmark, http://www.dako.com). VE-cadherin was detected using a mouse monoclonal antibody against human VE-cadherin (clone TEA1/31; Immunotech, Luminy, France, http://www.immunotech.com).

### Flow Cytometry

Nonadherent cells were removed from the culture with a pipette. The adherent cells were washed with phosphate-buffered saline (PBS) and removed from the well with an enzyme-free PBS-based Cell Dissociation Buffer (Invitrogen) for 3 minutes. Adherent and nonadherent cells were centrifuged at 2,000 rpm for 2 minutes, resuspended in 0.5 ml of PBS/0.5% bovine serum albumin (BSA)/2 mM EDTA, centrifuged again, and resuspended in PBS/0.5% BSA/1.26 mM $CaCl_2$/0.8 mM $MgSO_4$. Prior to addition of antibody conjugates, cells were incubated with Fc Blocking Reagent (Miltenyi Biotec) for 15 minutes at 4°C. Cells in a 0.1-ml volume were incubated singly or in combination with 2 $\mu$l of FITC-conjugated mouse $IgG_1$, phycoerythrin (PE)-conjugated mouse $IgG_{2a}$, PE-conjugated anti-human VE-cadherin (R&D Systems Inc., Minneapolis, http://www.rndsystems.com), or FITC-conjugated anti-CD45 (BD

Biosciences, San Diego, http://www.bdbiosciences.com) for 45 minutes at room temperature, washed twice with 1.0 ml of PBS/0.5% BSA/1.26 mM $CaCl_2$/0.8 mM $MgSO_4$, and resuspended in 0.25 ml of PBS/0.1% paraformaldehyde. Flow cytometry was performed on a BD Biosciences FACSCalibur flow cytometer. Cells labeled with PE-conjugated anti-VE-cadherin alone and with FITC-conjugated anti-CD45 alone were used to set compensation for the double-label analyses.

### Hematopoietic Colony Formation Assay

Nonadherent cells were removed from the attached endothelial monolayers, which were then washed with PBS, treated for 10 seconds with trypsin:EDTA, and washed again to remove loosely adherent cells. Both the nonadherent and adherent fractions were then independently plated in methylcellulose to determine their hematopoietic colony-forming potential. Individual specimens were mixed with 1.0 ml of methylcellulose containing stem cell factor, granulocyte-macrophage colony-stimulating factor, interleukin-3, and erythropoietin (catalog no. 4434; Stem Cell Technologies) and plated in 35-mm nontreated tissue culture dishes at concentrations ranging from 100 to 100,000 cells per dish (each concentration in duplicate). When the number of input cells was limiting, such as following clonal isolation of EPCs, colony-forming assays were scaled down to a total volume of 300–500 $\mu$l/well and plated in an Ultra Low Cluster 24-well flat-bottomed plate (Corning, Corning, NY, http://www.corning.com) at concentrations of 100 and 1,000 cells per milliliter (in duplicate). Assays were incubated at 37°C in a humidified incubator (5.0% $CO_2$), visually inspected at 7 days, and scored at 14 days for the presence of hematopoietic colonies. Cytospins were prepared from input cells as well as harvested hematopoietic colonies and stained by Wright/Giemsa stain (Richard-Allan Scientific, Kalamazoo, MI, http://www.rallansci.com). In addition, freshly isolated CD34+/CD133+ cells were plated in 1.0 ml of methylcellulose in 35-mm dishes to determine hematopoietic colony-forming potential of the initial cell population. From two different cord blood preparations, each plated in duplicate, we found that for every 100 CD34+/CD133+ cells plated, 6 colony-forming units (CFU)-Mac colonies, 14 CFU-GM colonies, 3 CFU-GEMM colonies, and 19 blast-forming unit-erythroid (BFU-E) colonies were formed.

## RESULTS

### CD34+/CD133+ Cells Differentiate into Adherent Endothelial Cells and Nonadherent Cells

CD34+/CD133+ cells isolated from HUCB are initially nonadherent to gelatin or fibronectin-coated dishes and do not proliferate significantly for the first 14 days in culture [15]. We monitored expression of the stem cell marker CD133, which is known to be rapidly downregulated when progenitor cells are placed in culture [24], and VEGF-receptor-2, over the first 14 days in vitro (Fig. 1A). CD133 mRNA was expressed at day 1 and day 7 but not at day 14, whereas VEGF-R2, a mesodermal progenitor marker that is also expressed on EPCs and mature ECs, was detected at all three time points. The retinoblastoma cell line WERI-RB-1 (American Type Culture Collection, Manassas, VA, http://www.atcc.org) was used as a positive control for detection of CD133 [25] and a negative control for VEGF-R2, whereas human dermal microvascular ECs [26] were used as a positive control for VEGF-R2 and a negative control for CD133. Coinciding with the downregulation of CD133, adherent cells with an endothelial-like cobblestone morphology became visible by day 14 (Fig. 1B). Numerous round cells with a healthy and homogeneous appearance were seen above the monolayer of cells. At high power, features that suggest a progenitor phenotype such as round nuclei and scant cytoplasm were seen in the nonadherent cells (Fig. 1C). The endothelial phenotype of the adherent cells was confirmed by immunostaining the cells at day 27. As expected, the expression of VE-cadherin was localized to the cell-cell borders (Fig. 1D), and the expression of vWF was intracellular and punctate. We showed in a previous study that

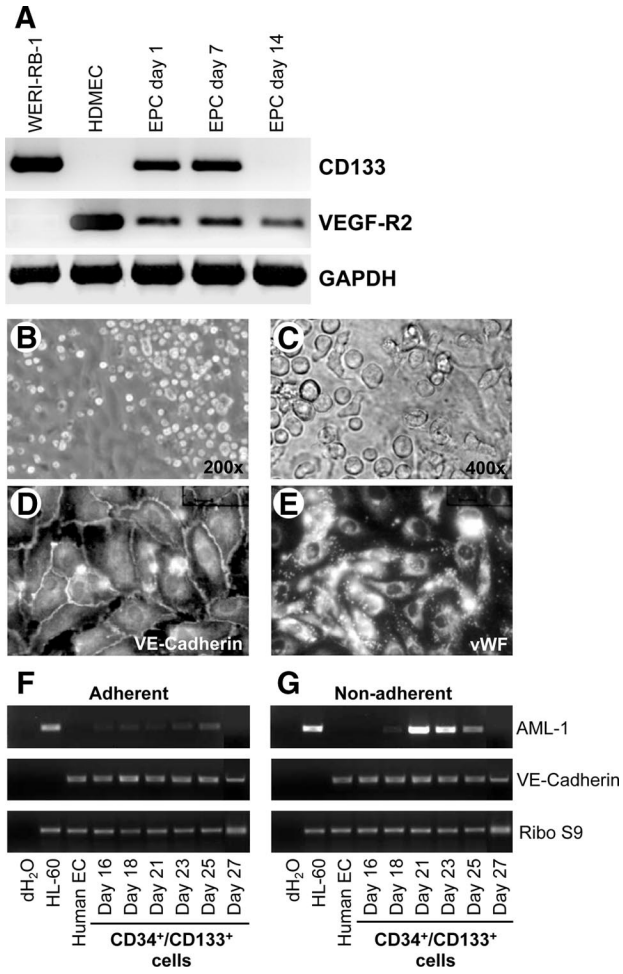


**Figure 1.** Differentiation of CD34+/CD133+ cells into adherent and nonadherent cells. **(A):** CD133, VEGF-R2, and GAPDH mRNA transcript levels were measured by reverse transcriptase-polymerase chain reaction (RT-PCR). RNA was isolated from CD34+/CD133+ cells cultured for 1, 7, and 14 days, from the retinoblastoma tumor cell line WERI-RB-1, and from normal HDMECs. **(B, C):** Phase-contrast micrographs CD34+/CD133+ cells at day 21 at magnifications of ×200 **(B)** and ×400 **(C)**. **(D, E):** Indirect immunofluorescence staining of adherent cells at day 27 using anti-VE-cadherin **(D)** and anti-vWF **(E)**. **(F, G):** AML-1, VE-cadherin, and Ribo S9 mRNA transcript levels in adherent **(F)** and nonadherent **(G)** cells measured by RT-PCR at time points from day 16 to day 27. HL-60, a human promyelocytic leukemia cell, mRNA served as a positive control for AML-1 and negative control for VE-cadherin; mature human ECs served as a negative control for AML-1 and a positive control for VE-cadherin. PCRs were carried out for 35 cycles. Day 27 mRNAs from adherent and nonadherent cells were isolated from a different CD34+/CD133+ preparation of cells. Abbreviations: AML-1, acute myeloid leukemia-1; $dH_2O$, distilled water; EC, endothelial cells; EPC, endothelial progenitor cells; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HDMEC, human dermal microvascular endothelial cells; Ribo S9, ribosomal S9; VEGF-R2, vascular endothelial growth factor-receptor-2; vWF, von Willebrand factor.

these EPC-derived ECs maintain a stable and functional endothelial phenotype when expanded in vitro [15].

### AML-1 Expression in Adherent and Nonadherent Cells

On the basis of the morphology of the nonadherent cells, we postulated that these cells might be hematopoietic progenitors produced by the underlying endothelial monolayer, much like what occurs in the hemogenic endothelium of the dorsal aorta during embryonic development. To test this, we examined ex-

pression of AML-1, a DNA binding subunit that has been shown to have a critical role in definitive hematopoiesis [27]. AML-1 is also known as Runx1 in mice, as well as by two other names: CBFA2 and PEBP2αB. Runx1 is expressed in the cell clusters localized at sites of hemogenic endothelium, and more recently it has been shown that Runx1 expression in the endothelium is needed for hematopoiesis [28]. We isolated RNA from adherent and nonadherent cells at time points ranging from day 16 to day 27 after plating the CD34+ /CD133+ cells and analyzed expression of AML-1 and the endothelial-specific marker VE-cadherin by semiquantitative RT-PCR. The adherent monolayer was washed vigorously to ensure that nonadherent cells were removed. In the adherent cells, a low level of AML-1 mRNA was detected in cells from day 16 to day 25 (Fig. 1F). PCR products for AML-1 were subjected to DNA sequencing to verify identify of the reaction products. As expected, VE-cadherin was detected in the adherent cells at all time points. In the nonadherent cells, AML-1 expression peaked dramatically at day 21, with strong expression at days 23 and 25 but little to no detectable expression at day 27 (Fig. 1G). This temporal pattern of expression suggests the potential for hematopoietic differentiation is restricted to a limited period of time. VE-cadherin was detected in the nonadherent cells at all time points, consistent with an endothelial origin for these cells.

## Nonadherent Cells Coexpress the Hematopoietic Marker CD45 and the Endothelial Marker VE-Cadherin

To identify cells in the process of differentiating from endothelial to hematopoietic cells, we analyzed the nonadherent and adherent cells for coexpression of the hematopoietic cell marker CD45 and the endothelial-specific membrane protein VE-cadherin (Fig. 2). CD45, also known as leukocyte common antigen, is a high molecular weight cell surface protein expressed on all hematopoietic cells with the exception of red cells. VE-cadherin, also known as cadherin-5 and CD144, is 135-kDa calcium-dependent adhesion molecule expressed on vascular endothelial cells. It has been shown to function in the assembly of endothelial intercellular junctions, endothelial cell survival, and angiogenesis [29–31]. Double labeling of CD34+/CD133+ cells after 17 days in culture is shown in Figure 2. Isotype-matched $IgG_1$ conjugated to FITC and $IgG_{2a}$ conjugated to PE binding to the nonadherent (Fig. 2A) and the adherent (Fig. 2B) cells are shown to establish levels of nonspecific staining in the two-color analysis. The nonadherent cells derived from CD34+/CD133+ cells were found to coexpress CD45 and VE-cadherin (Fig. 2C). Adherent cells were predominantly VE-cadherin-positive/CD45-negative (67%), with a small fraction, 21%, co-expressing VE-cadherin and CD45 (Fig. 2D). In experiments not shown, we found similar results with CD146, a cell adhesion molecule that is often used as a marker for endothelial cells [32, 33] although recent reports show it is not restricted to endothelial cells [34, 35]. In these experiments, nonadherent cells were CD146+/CD45+, whereas the adherent cells were predominantly CD146+/CD45−, with a small percentage CD146+/CD45+ (range, 1%–8%) (data not shown). In experiments using either VE-cadherin or CD146 as endothelial markers, we found that nonadherent CD45-expressing endothelial cells were produced from as early as day 17 until day 23 but no longer detected at day 28. The underlying adherent cells were healthy and could be expanded as phenotypically stable ECs [15].

## Hematopoietic Progenitors in the Nonadherent and Adherent Cells

The presence of VE-cadherin+/CD45+ cells, which we hypothesized might represent hemogenic endothelial cells,

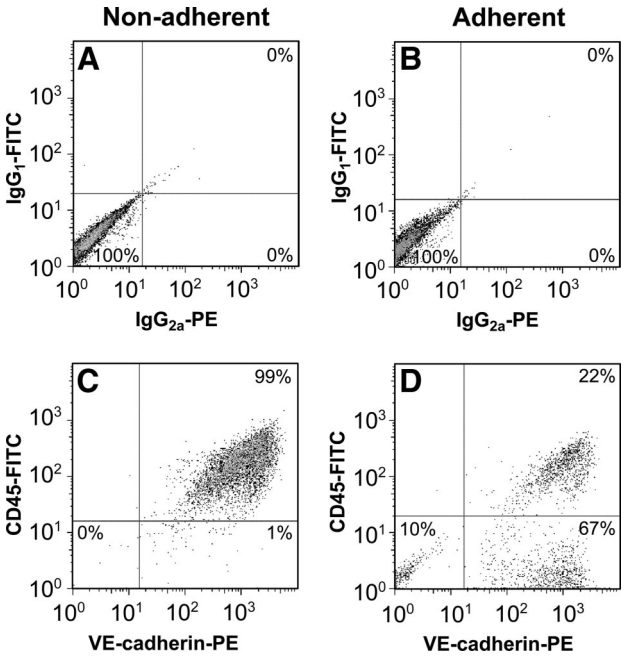


**Figure 2.** Nonadherent cells coexpress CD45 and VE-cadherin. Nonadherent **(A)** and adherent **(B)** cells from day 17 were harvested and analyzed by flow cytometry using isotype-matched control antibodies conjugated to FITC and PE, respectively. Gates were set to include >90% of the cells. Cross-bars were set such that more than 99% of the gated cells were in the lower left quadrant. Nonadherent **(C)** and adherent **(D)** cells were incubated with anti-CD45-FITC and anti-VE-cadherin-PE and analyzed under the same conditions used for the controls. Abbreviations: FITC, fluorescein isothiocyanate; IgG, immunoglobulin; PE, phycoerythrin; VE, vascular endothelial.

prompted us to assay the nonadherent and adherent cells for morphological features of hematopoietic cells (Fig. 3A, 3B) and, second, for the ability to generate hematopoietic colonies in vitro in a methylcellulose hematopoietic colony formation assay [36]. Wright/Giemsa-stained cytospin preparations of nonadherent day 21 cells revealed the presence of hematopoietic cells, including eosinophils and polymorphonuclear leukocytes (Fig. 3A). The adherent cells did not contain identifiable hematopoietic cells but instead consisted of cells with a uniform appearance (Fig. 3B). In the methylcellulose hematopoietic colony formation assay, the nonadherent cells produced erythrocyte progenitors (BFU-E) (Fig. 3C), granulocyte/monocyte progenitors CFU-GM (Fig. 3E), and CFU-M (Fig. 3G), but early progenitors of both erythrocyte and myeloid lineages (CFU-GEMM) were rarely detected. Notably, the adherent cells produced BFU-E (Fig. 3D) and CFU-GEMM (Fig. 3F). The quantification of the number and types of colonies formed in one of three experiments is shown in Table 1. Although BFU-E, CFU-GM, and CFU-M colonies were generated from both nonadherent and adherent cells, CFU-GEMM colonies were most often detected in the adherent cells. In this experiment, two CFU-GEMM were formed from 10,000 adherent cells (Table 1; Fig. 3F) but none from 10,000 nonadherent cells. In another experiment, three CFU-GEMM were formed from 6,000 adherent cells, whereas one CFU-GEMM formed from 10,000 nonadherent cells, suggesting that the adherent endothelial monolayer harbored more immature progenitors compared with the nonadherent cells. These functional assays firmly establish the presence of hematopoietic progenitor cells in the nonadherent and adherent cells.

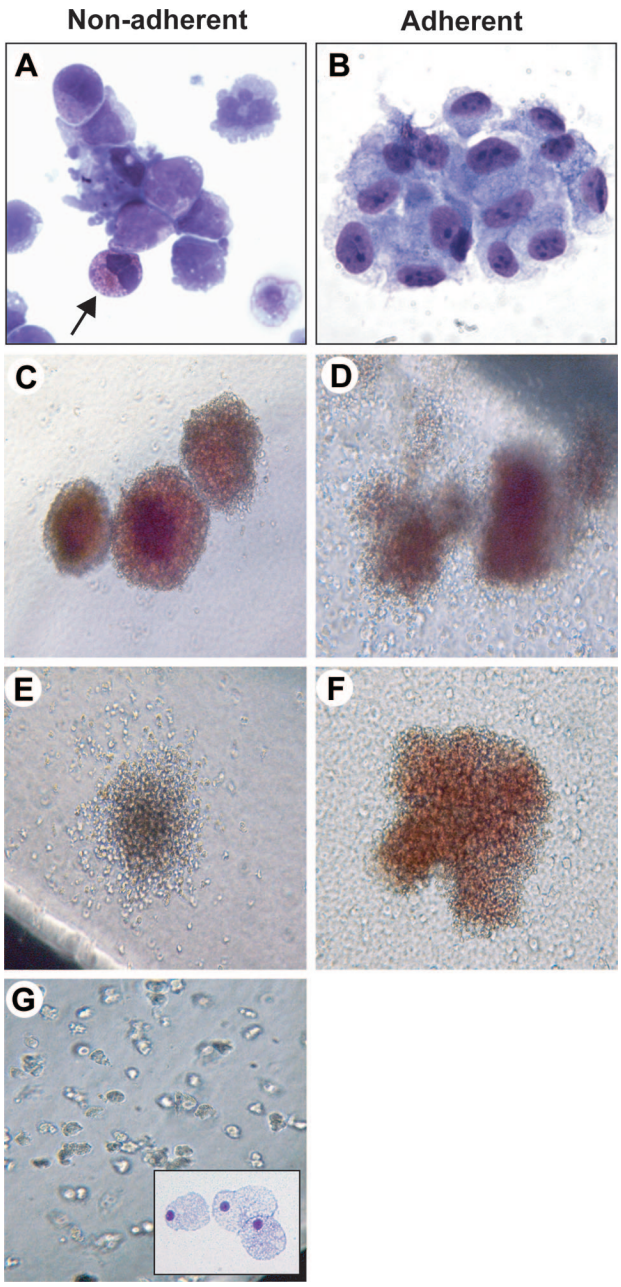


**Figure 3.** Nonadherent and adherent cells form hematopoietic colonies in methylcellulose. Nonadherent **(A)** and adherent **(B)** cells from day 21 cultures were stained with Giemsa to assess cellular morphology. The arrow in **(A)** indicates an eosinophil. Nonadherent cells formed blast-forming unit-erythroid (BFU-E) **(C)**, colony-forming units (CFU)-GM **(E)**, and CFU-GM(M) **(G)** colonies in methylcellulose. In **(G)**, a high-magnification inset shows the macrophage morphology of the cells in this colony. Adherent cells formed BFU-E **(D)** and CFU-GEMM **(F)**. Colony formation was photographed and quantified (Table 1) after 14 days.

## Analysis of Clonal Populations from the Adherent Cells

The coexpression of VE-cadherin and CD45 on individual cells (Fig. 2) suggests the presence of a transient cellular intermediate generated from the endothelial monolayer that gives rise to the hematopoietic progeny, as this would not occur if the hematopoietic cells in Figure 3 were generated from a hematopoietic stem cell (HSC) present in the nonadherent or adherent cell populations. To investigate further, we plated single cells isolated from the adherent endothelial monolayer as follows: CD34+/CD133+ cells were grown for 14 days, at which time nonadherent cells were removed and the monolayer was briefly trypsinized for 10 seconds and washed to remove loosely adherent cells. The remaining adherent cells were harvested with trypsin, plated manually as single cells in 96-well dishes, and grown for 10 days under the same conditions. The cloning efficiency was 15% in the experiment shown in Figure 4, with cell numbers sufficient in some to produce colonies that covered the well. Cloning efficiency ranged from 15% to 30% in four separate experiments (data not shown). Clonal populations were analyzed for AML-1, embryonic ($\varepsilon$), fetal ($\gamma$), and adult ($\beta$) globin transcripts as well as VE-cadherin and ribosomal S9 by RT-PCR (Fig. 4A). Of the 14 clonal populations with sufficient cells for analysis, two strongly expressed AML-1, embryonic ($\varepsilon$) and fetal ($\gamma$) globins (lanes 3 and 10) but not $\beta$-globin (not shown), whereas four others were weakly positive for AML-1 and $\varepsilon$-globin (lanes 5, 6, 11, and 12). All clones expressed VE-cadherin; none had detectable $\beta$-globin. One clone was analyzed for CD146 expression by indirect immunofluorescence and found to be positive (data not shown). The cellular morphology of one clone, 6 days after plating as a single cell, is shown in Figure 4B and 4C. Arrows in Figure 4C point to potentially budding cells seen as round translucent cells. These images are consistent with the round nonadherent cells budding from the adherent endothelial cells—a feature of hemogenic endothelium.

**Table 1.** Hematopoietic colony formation

| Input Cells | BFU-E | CFU-GM | CFU-M | CFU-GEMM |
|---|---|---|---|---|
| Nonadherent | 80 | 1030 | 190 | 0 |
| Adherent | 17 | 26 | 60 | 2 |

CD34+/CD133+ cells were cultured for 14 days, separated into nonadherent and adherent cells, and plated in methylcellulose as described in Materials and Methods. Colonies were evaluated and counted after 14 days. Numbers indicate the average from duplicate plates normalized to 10,000 cells. The range from three experiments was 0–1 CFU-GEMM colonies/10,000 nonadherent cells and 2–5 CFU-GEMM colonies/10,000 adherent cells. Abbreviations: BFU-E, burst-forming unit-erythroid; CFU, colony-forming unit.

To confirm that the clonal derivatives were functional hemogenic endothelial cells, cells were placed in methylcellulose to assess the production of hematopoietic progenitors. Adherent cells were prepared on day 14 and plated as single cells in 96-well dishes for 7 days. Twenty clonal populations were harvested by trypsinization; half of the cells from each well were used for RNA isolation, and the other half were used to assay for hematopoietic colony formation. The number of cells plated in methylcellulose ranged from 125 to 625, with an average of 237. Three of the clonal populations gave rise to colonies; three BFU-E, three CFU-GEMM (Fig. 4D), and four unidentified colonies were observed. In three experiments, 15%, 27%, and 30% of clones gave rise to colonies in methylcellulose. These relatively low percentages are not unexpected given the length of time the cells were cultured in vitro.

## DISCUSSION

In these studies, we discovered a transient window of time when EPC-derived ECs appeared to be hemogenic; that is, when the endothelium produced hematopoietic progeny. The window of time occurred after CD133, the stem cell marker, was downregulated and a cobblestone monolayer of endothelial cells (VE-cad-

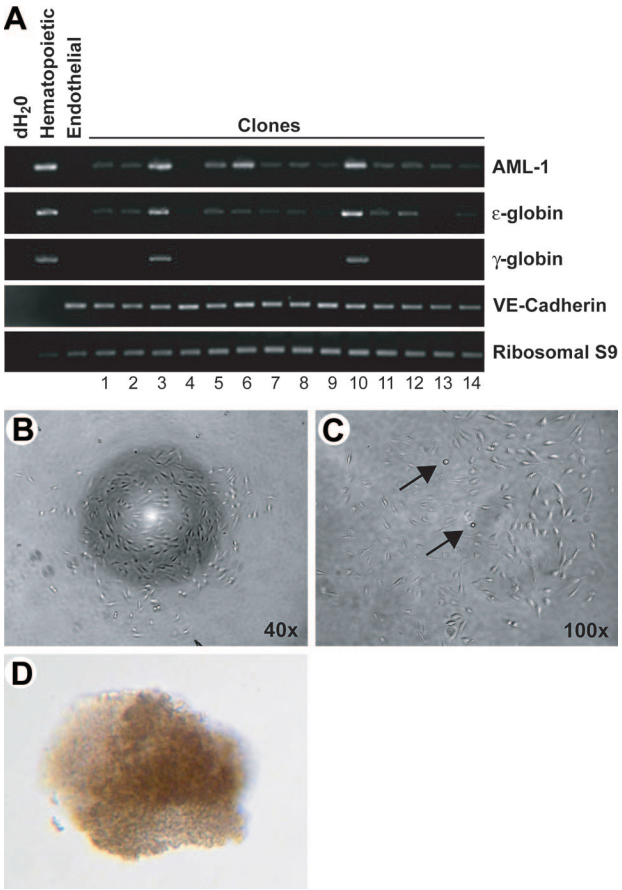


**Figure 4.** Clonal cells from the adherent monolayer express endothelial and hematopoietic markers and produce budding cells and hematopoietic progenitors. **(A):** Clonal populations were analyzed for expression of AML-1, embryonic ($\varepsilon$), fetal ($\gamma$), and adult ($\beta$) globins, VE-cadherin, and ribosomal S9 by reverse transcriptase-polymerase chain reaction (PCR). Hematopoietic cells (HL-60 for AML-1 and K562 cells for globins) and human ECs served as positive and negative controls. Ribosomal S9 served as an internal control for the PCR and gel loading. **(B, C):** Phase-contrast micrographs of a clone 6 days after plating as a single cell, photographed at $\times$40 **(B)** and $\times$100 **(C)**. Arrows in **(C)** indicate budding cells. **(D):** Colony-forming units-GEMM colony formed from clonal cells harvested after 14 days of growth and plated in methylcellulose for an additional 14 days. Abbreviations: AML-1, acute myeloid leukemia-1; $dH_2O$, distilled water; VE, vascular endothelial.

herin+ vWF+, CD146+ and VEGF-R2+ cells) had formed. From day 16 to day 25 after plating CD34+/CD133+ cells, AML-1 was expressed and round nonadherent cells with hematopoietic morphologies were produced. The adherent cells were clearly endothelial in morphology and expression of endothelial markers, yet these cells gave rise to early hematopoietic progenitors. The identification of cells coexpressing the hematopoietic marker CD45 and the endothelial marker VE-cadherin provides direct evidence for an endothelial/hematopoietic intermediate. Finally, clonally derived cells from the adherent endothelial cells express hematopoietic transcripts (AML-1, $\varepsilon$-globin, and $\gamma$-globin) and the endothelial transcript VE-cadherin and produce early hematopoietic progenitors (Fig. 4), strongly suggesting that the adherent endothelial cells are hemogenic. These results provide the first demonstration of hemogenic endothelial cells in perinatal tissue or blood and indicate an expanded differentiation potential for cord blood-derived EPCs.

This interpretation relies on the exclusive expression of VE-cadherin in the endothelial lineage, which has come under question from studies in which the murine VE-cadherin promoter was engineered to drive expression of Cre-recombinase: evidence for VE-cadherin promoter-driven Cre recombinase activity was found in a subset of hematopoietic organs [37]. To address the possibility of VE-cadherin expression in human hematopoietic progenitor cells from umbilical cord blood, we tried to detect VE-cadherin by RT-PCR in hematopoietic colonies formed from the CD34+/CD133+ cells. No VE-cadherin transcripts were detected in two different preparations of RNA from pooled colonies from two different cord bloods (supplemental online data), indicating that human VE-cadherin is not expressed at detectable levels in human hematopoietic progenitor cells.

A CD34+/CD133+ population of cells from blood would likely contain both HSCs and EPCs. Therefore, the production of hematopoietic progenitors in our culture system could be due to undefined paracrine factors produced by EPCs that nourish and promote proliferation of HSC, as has been described [38]. Although this possibility remains (i.e., that our findings describe a low-level contaminating fraction of hematopoietic progenitors cocultured with our initial EPC isolates), three complementary sets of data argue against this. First, individual cells express phenotypic markers of both hematopoietic and endothelial lineages; that is, the hematopoietic marker CD45, which has never been described on EPCs or ECs, and the endothelial marker VE-cadherin. Second, we prepared clonal populations by a rigorous single-cell plating technique and showed that progeny cells coexpress AML-1, $\varepsilon$-globin, $\gamma$-globin, and VE-cadherin. Third, clonal VE-cadherin-positive cells produced hematopoietic progenitors. Moreover, based on the predominance of adherent cell types with homogeneous expression of VE-cadherin and von Willebrand factor in our cloned cells, it is highly unlikely that our results can be explained by the inadvertent plating of multiple cell types into a single well. We attempted to test this possibility by performing mixing experiments with male and female CD34+/CD133+ cells, prior to single-cell plating, and genetic analyses for donor-specific markers, but the levels of genomic DNA obtained were too low.

A previous study has identified CD34+/VEGF-R2+ cells from HUCB with hemangioblast properties [3]. This was shown by dispersing single CD34+/KDR+ cells into wells and then transferring single cells into semisolid medium designed to support hematopoietic, endothelial, or mixed hematopoietic/endothelial cell growth. Colonies of cells expressing hematopoietic and endothelial markers were reported, but outgrowth of a monolayer of endothelial cells, as would be expected from endothelial progenitor cells, was not described [3]. Our results here differ in important ways. First, the cells were selected for CD133, a stem cell marker, and CD34, a hematopoietic progenitor/endothelial cell marker, and shown to express VEGF-R2 transcripts. Second, we were able to grow endothelial monolayers from single cells and show that these same cells could generate early hematopoietic progenitor cells, CFU-GEMM, in methylcellulose (Fig. 4). Finally, we show temporal expression of AML-1 coinciding with hemogenic activity. Furthermore, we know from this study and our previous work that CD34+/CD133+ cells from HUCB produce EPC-derived ECs with the ability to proliferate as phenotypically stable endothelial cells and to exhibit endothelial functions [15]. Hence, our data build upon previous work and support the existence of bipotential hemogenic endothelial cells in postnatal life.

The function of bipotential hemogenic endothelial cells in peri- or postnatal life is important to consider and will require additional experiments. Are these extraneous cells that have remained through fetal development but whose function is no longer needed? Or do the cells implicate a store of hemogenic endothelial cells on reserve for use during the rapid growth and development of infancy? Perhaps the cells serve as endothelial progenitors for the expanding vasculature of the child, or for vascular repair throughout life, but

do not contribute to hematopoiesis. Our in vitro culture conditions may have uncovered a differentiation potential that is no longer needed or used in postnatal life. It is interesting to speculate, however, that the in vitro conditions could be exploited and even manipulated further, to produce a source of hematopoietic stem/progenitor cells for bone marrow transplantation.

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## DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

G. Daley owns stock in, has acted as a consultant to, has served as an office or member of the Board for, and has a financial interest in Viacell, Inc.

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# Hemogenic Endothelial Progenitor Cells Isolated from Human Umbilical Cord Blood

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