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Human $CD34^{+}AC133^{+}VEGFR\text{-}2^{+}$ cells are not endothelial progenitor cells but distinct, primitive hematopoietic progenitors

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Objective. **Endothelial progenitor cells (EPCs) are used for angiogenic therapies or as biomarkers to assess cardiovascular disease risk. However, there is no uniform definition of an EPC, which confounds EPC studies. EPCs are widely described as cells that coexpress the cell-surface antigens CD34, AC133, and vascular endothelial growth factor receptor-2 (VEGFR-2). These antigens are also expressed on primitive hematopoietic progenitor cells (HPCs). Remarkably, despite their original identification, $CD34^{+}AC133^{+}VEGFR\text{-}2^{+}$ cells have never been isolated and simultaneously plated in hematopoietic and endothelial cell (EC) clonogenic assays to assess the identity of their clonal progeny, which are presumably the cellular participants in vascular regeneration.**

Methods. **$CD34^{+}AC133^{+}VEGFR\text{-}2^{+}$ cells were isolated from human umbilical cord blood (CB) or granulocyte colony–stimulating factor–mobilized peripheral blood and assayed for either EPCs or HPCs.**

Results. **$CD34^{+}AC133^{+}VEGFR\text{-}2^{+}$ cells did not form EPCs and were devoid of vessel forming activity. However, $CD34^{+}AC133^{+}VEGFR\text{-}2^{+}$ cells formed HPCs and expressed the hematopoietic lineage-specific antigen, CD45. We next tested whether EPCs could be separated from HPCs by immunoselection for CD34 and CD45. $CD34^{+}CD45^{+}$ cells formed HPCs but not EPCs, while $CD34^{+}CD45^{-}$ cells formed EPCs but not HPCs.**

Conclusions. **Therefore, $CD34^{+}AC133^{+}VEGFR\text{-}2^{+}$ cells are HPCs that do not yield EC progeny, and the biological mechanism for their correlation with cardiovascular disease needs to be reexamined.** **© 2007 International Society for Experimental Hematology. Published by Elsevier Inc.**

Since 1997, postnatal vasculogenesis has been purported to be an important mechanism for neoangiogenesis via marrow-derived circulating endothelial progenitor cells (EPCs) [1]. Based on this paradigm, EPCs have been extensively studied as biomarkers of cardiovascular disease, and as a cell-based therapy for repair of damaged blood vessels [1–10]. Circulating EPCs can be isolated from adult peripheral blood (PB), and human umbilical cord blood (CB) [6,11]. In the majority of studies to date, EPCs are typically identified and enumerated via flow cytometric identification of cells, which coexpress the cell-surface antigens CD34, AC133, and vascular endothelial growth factor receptor-2 (VEGFR-2, also know as KDR) [1,5,12–17]. As these molecules are also expressed on hematopoietic stem/progenitor cell populations [18–22], it is not surprising that EPCs have been reported to arise from the bone marrow (BM), hematopoietic stem cell (HSC), or a hemangioblast precursor [2,23–28].

Remarkably, despite their original identification and increasing use as a biomarker for vascular disease,

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$CD34^+AC133^+VEGFR\text{-}2^+$ cells have never been isolated and simultaneously plated in hematopoietic and endothelial cell (EC) clonogenic assays to assess the identity of their clonal progeny, which are presumably the cellular participants in new blood vessel formation. Therefore, identification of the cell progeny of $CD34^+AC133^+VEGFR\text{-}2^+$ cells is critical for determining the mechanism by which these cells participate in neoangiogenesis or vasculogenesis, and the rational design of cell based therapies for treating cardiovascular diseases.

Given this limitation in EPC biology, we obtained granulocyte colony–stimulating factor (G-CSF)–mobilized peripheral blood (mPB) and CB, an enriched source of both EPCs and hematopoietic progenitor cells (HPCs), to isolate and purify $CD34^+AC133^+VEGFR\text{-}2^+$ cells by fluorescence-activated cell sorting (FACS). We assayed for the presence of clonogenic EPCs and hematopoietic low proliferative potential (LPP-) and high proliferative potential colony–forming cells (HPP-CFCs) in the sorted cell population. In this study, we provide novel data to demonstrate that $CD34^+AC133^+VEGFR\text{-}2^+$ cells are not EPCs, but HPCs that express the universal hematopoietic cell-surface antigen, CD45. Further, we also identify $CD34^+$ cells, which do not express CD45, as an enriched source for EPCs. Thus, this study provides novel insights into the identity of $CD34^+AC133^+VEGFR\text{-}2^+$ cells, which offers an alternative framework for understanding the mechanisms by which these cells potentially participate in new blood vessel formation.

Materials and methods

Blood samples and buffy coat cell preparation

CB samples from 14 term newborns (7 male, 7 female) and PB samples from 6 healthy adults (3 male, 3 female, ages 24 to 50 years) were collected as described previously [11]. G-CSF mPB $CD34^+$ cells were kindly provided through a Program of Excellence in Gene Therapy grant from Shelly Heimfeld at the Fred Hutchinson Cancer Research Centre, Seattle, WA. The Institutional Review Board at Indiana University School of Medicine approved all protocols, and informed consent was obtained. Mononuclear cells (MNCs) were obtained as previously described by Ficoll density gradient centrifugation [11] then washed with phosphate-buffered saline (PBS; Invitrogen, Grand Island, NY, USA) supplemented with 2% fetal bovine serum (Hyclone, Logan, UT, USA), 1% penicillin/streptomycin (Invitrogen) and 0.25 mg/mL amphotericin B (Invitrogen).

Immunomagnetic isolation of CB $CD34^+$ cells

CB MNCs expressing the CD34 antigen were immunomagnetically selected using the human indirect CD34 microbead kit and Magnetic Cell Sorting (MACS) system (Miltenyi Biotec, Auburn, CA, USA) exactly as directed by the manufacturer. Cell count and viability was assessed by Trypan blue (Sigma, St. Louis, MO, USA) staining.

Immunophenotyping and FACS

To assess cell-surface antigen expression, adult PB and CB derived MNCs, or G-CSF mPB $CD34^+$ cells were incubated with varying concentrations of the primary, secondary, or isotype control antibody, as outlined here in 250 microliter PBS supplemented with 2% fetal bovine serum. Cells were then washed and analyzed on a FACS Aria (BD Immunocytometry Systems, San Jose, CA, USA). We used primary murine monoclonal antibodies against human CD34 conjugated to fluorescein isothiocyanate (FITC, BD Pharmingen, San Diego, CA, USA), human AC133 conjugated to phycoerythrin (PE; Miltenyi Biotec) and human CD45 conjugated to PE-Cy-Chrome 5 (PE-Cy5; BD Pharmingen). We used a biotinylated primary antibody directed against VEGFR-2 (Sigma), and streptavidin conjugated to allophycocyanin (APC, BD Pharmingen) as the secondary antibody. Directly conjugated mouse immunoglobulin G_{1k} (IgG_{1k}) (BD Pharmingen) were used for isotype controls.

For isolation of specific CB- and mPB-derived cell subpopulations, MACS-enriched CB and mPB $CD34^+$ cells were stained with primary, secondary, or isotype control antibody, as outlined above. $CD34^+AC133^+VEGFR\text{-}2^+$, $CD34^+CD45^+$, or $CD34^+CD45^-$ cell subpopulations were sorted with a FACS Aria and collected into sterile 5-mL polystyrene round-bottom tubes (BD Labware) containing 2 mL complete endothelial growth medium-2, consisting of EBM-2 (Cambrex, Walkersville, MD, USA) supplemented with the entire growth factor bullet kit (Cambrex), 10% fetal bovine serum, 1% penicillin/streptomycin, and 25 mg/mL amphotericin B.

Culture of colony-forming unit endothelial cells

Colony-forming unit endothelial cells (CFU-ECs) were cultured using the EndoCult Liquid Medium Kit (StemCell Technologies, Vancouver, BC, Canada) per manufacturer's protocol. CB MNCs, mPB $CD34^+$ cells, or sorted $CD34^+AC133^+VEGFR\text{-}2^+$, $CD34^+CD45^+$ or $CD34^+CD45^-$ cell subpopulations were resuspended in complete EndoCult medium. Cells were seeded onto 6-, 12-, or 24-well fibronectin-coated tissue culture plates (BD Biosciences, Bedford, MA, USA) at $1 < 5 \times 10^6$ cells/well for CB MNCs and mPB $CD34^+$ cells, and between 2000 and 200,000 cells/well for the sorted CB and mPB cell subpopulations. After 48 hours, wells were washed with media and nonadherent cells were collected. Nonadherent cells were plated in their existing media in 24-well fibronectin-coated tissue culture plates for 3 days, and colonies were enumerated by visual inspection with an inverted microscope (Olympus, Lake Success, NY, USA) at 40× magnification on days 5 to 10.

Culture of endothelial colony-forming cells

CB MNCs, mPB $CD34^+$ cells, or sorted $CD34^+AC133^+VEGFR\text{-}2^+$, $CD34^+CD45^+$ or $CD34^+CD45^-$ cell subpopulations were resuspended in endothelial growth medium-2. Cells were seeded onto 6-, 12-, or 24-well tissue culture plates precoated with type I rat tail collagen (BD Biosciences) at 2×10^7 cells/well for CB MNCs, 1×10^6 cells/well for mPB $CD34^+$ cells and between 2000 and 200,000 cells/well for the sorted CB and mPB cell subpopulations. Endothelial colony-forming cells (ECFC) were cultured as described previously [11], and colonies were enumerated by visual inspection with an inverted microscope at 40× magnification.

Hematopoietic colony assays
CB and mPB cells were assayed for HPC-forming ability by suspending 2×10^4 CB MNCs, 1.25×10^5 mPB CD34$^+$ cells, or 1000 sorted CD34$^+$AC133$^+$VEGFR-2$^+$, CD34$^+$CD45$^+$ or CD34$^+$CD45$^-$ cells in 0.66% to 1.0% agar (Becton Dickinson, Franklin Lakes, NJ, USA) in the presence of 50 ng/mL human stem cell factor (1000 U/mL human interleukin-1α), and 100 U/mL human interleukin-3 (all from PeproTech Inc., Rocky Hill, NJ, USA) as described previously [29,30]. Cells were plated in a 35-mm Petri dish in triplicate. Cultures were scored for LPP- and HPP-CFCs on days 14 and 21, respectively.

Matrigel assay
Matrigel assays were performed as described previously [11]. Early passage (two to three) ECFCs and freshly sorted CB- and mPB-derived CD34$^+$AC133$^+$VEGFR-2$^+$ cells were seeded onto 96-well tissue culture plates coated with 30 μL Matrigel (BD Biosciences) at a cell density of 5000 to 20,000 cells/well. Cells were observed every 2 hours by visual microscopy with an inverted microscope (Olympus) at 40× magnification for capillary-like formation.

Reverse transcriptase-polymerase chain reaction
Total cellular RNA was isolated from CB MNCs or freshly sorted CB or mPB derived CD34$^+$AC133$^+$VEGFR-2$^+$, CD34$^+$45$^+$ and CD34$^+$45$^-$ cell subpopulations using the RNeasy Micro kit (Qiagen, Valencia, CA) per the manufacturer's instructions. First-strand complementary DNA (cDNA) synthesis was performed using the SuperScript III First-Strand Synthesis System for reverse transcriptase polymerase chain reaction (RT-PCR; Invitrogen) according to the manufacturer's instructions. PCR was performed using a gene-specific primer pair for human CD45 and VEGFR-2 (Invitrogen). CD45 forward 5′-AATGAGAATGTGGAATGTGG-3′, reverse 3′-TTGCGTTAGTAAACTTGTGG-5′ and VEGFR-2 forward 5′-TAGTTGTCGTTGTAGGGTATAGG-3′, reverse 3′-AAATGTGCTGTTCTTCTTGGTC-5′. Sequences were obtained from Genebank. PCR reaction conditions consisted of one cycle at 94°C for 2 minutes, 30 cycles at 94°C for 30 seconds, 30 seconds at 55°C, and 45 seconds at 72°C. PCR products were imaged under ultraviolet fluorescence of ethidium bromide in 2% agarose gels using the Kodak Electrophoresis Documentation and Analysis System 120 (Eastman Kodak, Rochester, NY, USA).

Results

Culture of CD34$^+$AC133$^+$VEGFR-2$^+$ cells yields hematopoietic progenitor and not EC colonies
CD34$^+$AC133$^+$VEGFR-2$^+$ cells are widely considered to be the most primitive precursor of EPCs [7,9,14]. However, the identity of the progeny derived from CD34$^+$AC133$^+$VEGFR-2$^+$ cells is currently unknown because these cells have never been isolated and simultaneously plated in hematopoietic and EC clonogenic assays to determine their specific colony-forming potential. To address this question, we initially determined the frequency of this cell population in MNCs isolated from both adult PB and CB utilizing stringent gating strategies and appropriate isotype controls (Fig. 1 A–D). The frequency of the CD34$^+$AC133$^+$VEGFR-2$^+$ cells in mPB CD34$^+$ cells was also determined (Fig. 1 E and F). In adult PB, the CD34$^+$AC133$^+$VEGFR-2$^+$ cells are extremely rare cells, which constitute a frequency of only 84 per 10^6 MNCs (0.0084 ± 0.0052%, n = 6). In CB, we determined that the frequency of CD34$^+$AC133$^+$VEGFR-2$^+$ cells per 10^6 MNCs (0.021% ± 0.009%, n = 6) was enriched by approximately 2.5-fold. This observation is consistent with previous studies, which demonstrate that both HPCs and EPCs are enriched in CB [11,31,32]. Furthermore, in mPB, the frequency of the CD34$^+$AC133$^+$VEGFR-2$^+$ cells per 10^6 CD34$^+$ cells (2.07% ± 0.15%, n = 3) was enriched by approximately 246- and 98-fold compared to that of adult PB and CB MNCs, respectively. This large increase in frequency is not surprising considering the starting cell population of mPB CD34$^+$ cells is homogeneous as compared to the heterogeneous starting populations when utilizing adult PB and CB MNCs.

In order to obtain sufficient cell numbers for hematopoietic and EC colony-plating experiments, we isolated CD34$^+$AC133$^+$VEGFR-2$^+$ cells from CB or mPB utilizing the FACS gating strategy as outlined in Figure 1 C–F. CB and mPB derived CD34$^+$AC133$^+$VEGFR-2$^+$ cells were then cultured separately utilizing previously described culture methods for identifying CFU-ECs, ECFCs and hematopoietic LPP and HPP-CFCs [11,30]. The rationale for assaying for CFU-ECs, ECFCs, LPP-, and HPP-CFCs is straightforward. First, two populations of EPCs have been described, CFU-ECs and ECFCs. CFU-ECs, also referred to as early outgrowth EPCs, are hematopoietic-derived cells, which express both endothelial and hematopoietic cell-surface antigens and secrete angiogenic growth factors to facilitate angiogenesis [33,34]. ECFCs, which are also referred to as blood outgrowth ECs or late outgrowth EPCs, are nonhematopoietic-derived EPCs with robust proliferative potential and in vivo vessel-forming capacity [11,34,35]. Second, in the hematopoietic cell system, the most proliferative progenitor cell types, which have variable expression of the cell-surface antigens CD34, AC133, and VEGFR-2 that can be cultured in vitro in the absence of a stromal cell monolayer are LPP- and HPP-CFCs [18–22].

Remarkably, while plating unfractionated CB MNCs in CFU-EC and ECFC culture medium consistently yielded CFU-ECs and ECFCs at a frequency reported previously, CB and mPB derived CD34$^+$AC133$^+$VEGFR-2$^+$ cells never formed CFU-ECs or ECFCs in the same assay (Table 1). In contrast, plating of CB- and mPB-derived CD34$^+$AC133$^+$VEGFR-2$^+$ cells in HPC assays consistently produced numerous LPP- and HPP-CFCs (Table 2). Importantly, consistent with a primitive HPC, which expresses the cell-surface antigens CD34 and AC133, CB derived CD34$^+$AC133$^+$VEGFR-2$^+$ cells yielded approximately 48- and 36-fold more LPP- and HPP-CFCs than

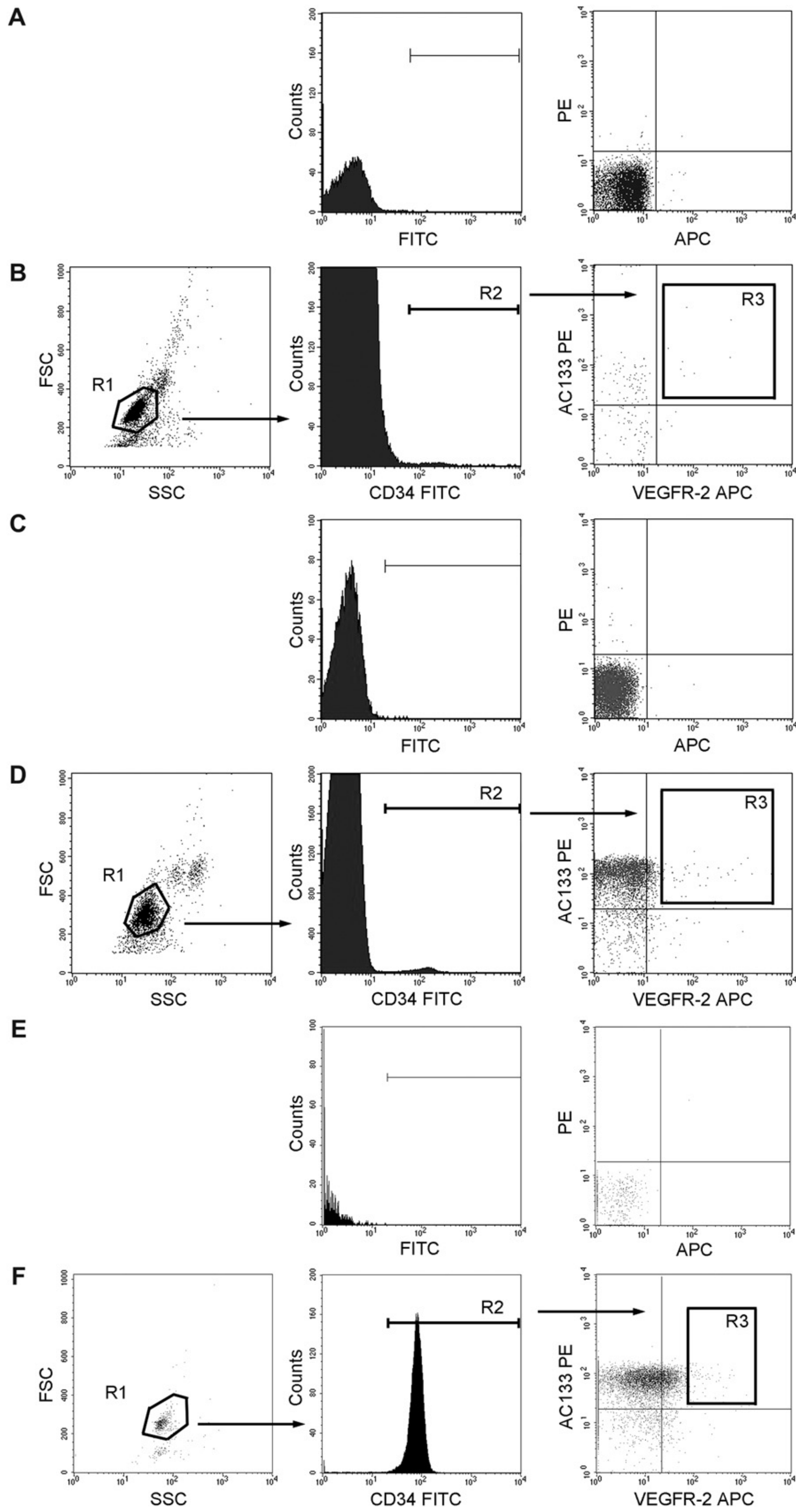
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Table 1. CFU-EC and ECFC colony frequency in CB-derived MNCs, CB-derived CD34$^+$AC133$^+$VEGFR-2$^+$ cells, mPB-derived CD34$^+$ cells, and mPB-derived CD34$^+$AC133$^+$VEGFR-2$^+$ cells

	CB MNCs		CB CD34$^+$AC133$^+$ VEGFR-2$^+$		mPB CD34$^+$		mPB CD34$^+$AC133$^+$ VEGFR-2$^+$	
Experiment no.	CFU-EC	ECFC	CFU-EC	ECFC	CFU-EC	ECFC	CFU-EC	ECFC
1	3.20*	0.65*	0*	0*	0*	0*	0*	0*
2	10.00	0.55	0	0	0	0	0	0
3	1.00	0.45	0	0	0	0	0	0
Average	4.73	0.55	0.00	0.00	0.00	0.00	0.00	0.00
SEM	2.71	0.06	0	0	0	0	0	0

CB = cord blood; CFU-EC = colony-forming unit endothelial cells; ECFC = endothelial colony-forming cells; MNC = mononuclear cells; mPB = mobilized peripheral blood; SEM = standard error of mean; VEGFR-2 = vascular endothelial growth factor receptor-2.
*Frequency of ECFC or CFU-EC colony formation per 10^6 cells plated.

plating of unfractionated MNCs, respectively (Table 2). Further, mPB-derived CD34$^+$AC133$^+$VEGFR-2$^+$ cells yielded approximately 9- and 26-fold more LPP- and HPP-CFCs respectively, than plating of mPB CD34$^+$ cells only (Table 2).

As the CD34$^+$AC133$^+$VEGFR-2$^+$ cell subpopulation was devoid of CFU-EC and ECFC activity, but enriched for primitive hematopoietic CFC activity, we next tested whether adult PB-, CB-, or mPB-derived CD34$^+$AC133$^+$ VEGFR-2$^+$ cells expressed the hematopoietic specific cell-surface antigen CD45, to confirm the hematopoietic identity of this cell population. We isolated MNCs cells from both adult PB and CB and CD34$^+$ cells from mPB and tested for the expression of the CD45 antigen in CD34$^+$AC133$^+$VEGFR-2$^+$ cells utilizing stringent gating strategies and appropriate isotype controls (Fig. 2 A–F). Consistent with restricted hematopoietic CFC activity, $>99\%$ of CD34$^+$AC133$^+$VEGFR-2$^+$ cells coexpress the CD45 antigen ($n = 6$ for adult PB, $n = 4$ for CB and $n = 3$ for mPB). Importantly, mRNA for CD45 was detected by RT-PCR in CD34$^+$AC133$^+$VEGFR-2$^+$ cells and control MNCs but not in human umbilical vein endothelial cells, which were used as a negative control (Fig. 2G). Furthermore, mRNA for VEGFR-2 was also detected by RT-PCR in CD34$^+$AC133$^+$VEGFR-2$^+$ cells and human pulmonary artery endothelial cells, which were used as a positive control (Fig. 2H). Finally, we previously demonstrated that ECFCs form capillary-like structures in Matrigel in vitro and chimeric blood vessels in vivo when transplanted into immunodeficient mice [34]. Consistent with a hematopoietic cell phenotype, CD34$^+$AC133$^+$ VEGFR-2$^+$ cells derived from both CB and mPB failed to form capillary-like structures in Matrigel in vitro, despite plating varying concentrations of cells (5000–20,000 cells/well) (Fig. 2I). Collectively, these data demonstrate that CD34$^+$AC133$^+$VEGFR-2$^+$ cells are not EPCs but HPCs that express the hematopoietic antigen, CD45.

ECFCs and hematopoietic CFCs can be efficiently separated in CB-derived CD34$^+$ cells by immunoselection for CD45

Based on the above results and previously published studies that demonstrate that CD34 is coexpressed on freshly isolated EPCs and HPCs [1,13,14,18–21], we hypothesized that immunoselection of CB-derived CD34$^+$ cells by cellular expression of CD45 would facilitate dissection of endothelial from hematopoietic CFC activity. To test this hypothesis, we FACS-sorted MACS-enriched CB-derived CD34$^+$ cells into either CD34$^+$CD45$^+$ or CD34$^+$CD45$^-$ cell subpopulations utilizing a stringent gating strategy and appropriate isotype controls (Fig. 3A and B). Most CB-derived CD34 expressing cells coexpress CD45 (Fig. 3B). However, there are rare cells that are CD34$^+$CD45$^-$ (Fig. 3B). Absence or presence of CD45 expression was confirmed by detection of mRNA for CD45 by RT-PCR in the sorted cell populations (Fig. 3C).

CB-derived CD34$^+$CD45$^+$ or CD34$^+$CD45$^-$ cell subpopulations were plated in the CFU-EC, ECFC, and HPC assays as outlined in Materials and Methods. ECFC activity is greatly enriched in the CD34$^+$CD45$^-$ cell subpopulation, but this fraction is devoid of CFU-EC and

Figure 1. Fluorescence-activated cell sorting (FACS) gating strategy for isolation of CD34$^+$AC133$^+$ vascular endothelial growth factor receptor-2 (VEGFR-2)$^+$ cells. Representative flow-cytometric analysis of adult peripheral blood (PB) mononuclear cells (MNCs) stained with isotype controls (**A**) or monoclonal antibodies against human CD34, AC133, and VEGFR-2 (**B**). FACS plots are representative of six independent analyses using cells from different adult donors. Representative FACS plots of CB MNCs (**C**–**D**) or mobilized peripheral blood (mPB)–derived CD34$^+$ cells (**E**–**F**) stained with isotype controls (**C**,**E**) or CD34, AC133 and VEGFR-2 (**D**,**F**) for isolation of CD34$^+$AC133$^+$VEGFR-2$^+$ cells. First, the lymphocyte population (R1) was gated on a forward (FSC) vs side (SSC) scatter plot to exclude dead cells and debris. Cells were then sequentially gated based on CD34 expression (R2) and AC133 and VEGFR-2 coexpression (R3). CD34$^+$AC133$^+$VEGFR-2$^+$ cells were isolated by sorting cells, which were contained in gates R1, R2 and R3. Analysis represents independent experiments using cells from three different CB donors and three different mPB donors.

Table 2. LPP- and HPP-CFC colony frequency in CB-derived MNCs, CB-derived CD34$^+$AC133$^+$VEGFR-2$^+$ cells, mPB-derived CD34$^+$ cells, and mPB-derived CD34$^+$AC133$^+$VEGFR-2$^+$ cells

	CB MNCs		CB CD34$^+$AC133$^+$ VEGFR-2$^+$		mPB CD34$^+$		MPB CD34$^+$AC133$^+$ VEGFR-2$^+$	
Experiment no.	LPP	HPP	LPP	HPP	LPP	HPP	LPP	HPP
1	1.3*	1.2*	410*	240*	3.04*	8.42*	29.6*	205.6*
2	7.8	4.7	420	280	3.37	5.12	29.6	157.6
3	19	16	473	277	4.21	7.34	31.4	173.8
Average	9.37†	7.3†	434.33	265.67	3.54‡	6.96‡	30.2	179
SEM	5.17	4.47	19.55	12.86	0.60	1.68	1.04	24.42

CB = cord blood; CFC = colony-forming cells; HPP = high proliferative potential; LPP = low proliferative potential; MNC = mononuclear cells; mPB = mobilized peripheral blood; SEM = standard error of mean; VEGFR-2 = vascular endothelial growth factor receptor-2.
*Frequency of LPP- or HPP-CFC colony formation per 10^4 cells plated.
†For LPP- and HPP-CFC frequency of CB MNCs vs. CB CD34$^+$AC133$^+$VEGFR-2$^+$, $P < 0.0001$ by unpaired Student's *t*-test.
‡For LPP- and HPP-CFC frequency of mPB CD34$^+$ vs. mPB CD34$^+$AC133$^+$VEGFR-2$^+$, $p < 0.0001$ by unpaired Student's *t*-test.

hematopoietic activity (Tables 3 and 4). In fact, the number of ECFCs was enriched 368-fold compared to plating unfractionated MNCs in EC colony culture conditions (Table 3). In contrast, CD34$^+$CD45$^+$ cells are greatly enriched in hematopoietic LPP- and HPP-CFC activity but not CFU-EC or ECFC activity (Tables 3 and 4). Thus, CB-derived EPCs (i.e., ECFCs) are CD34$^+$CD45$^-$ restricted, and hematopoietic CFCs can be efficiently separated from ECFCs in CD34$^+$ cell populations by immunoselection for CD45.

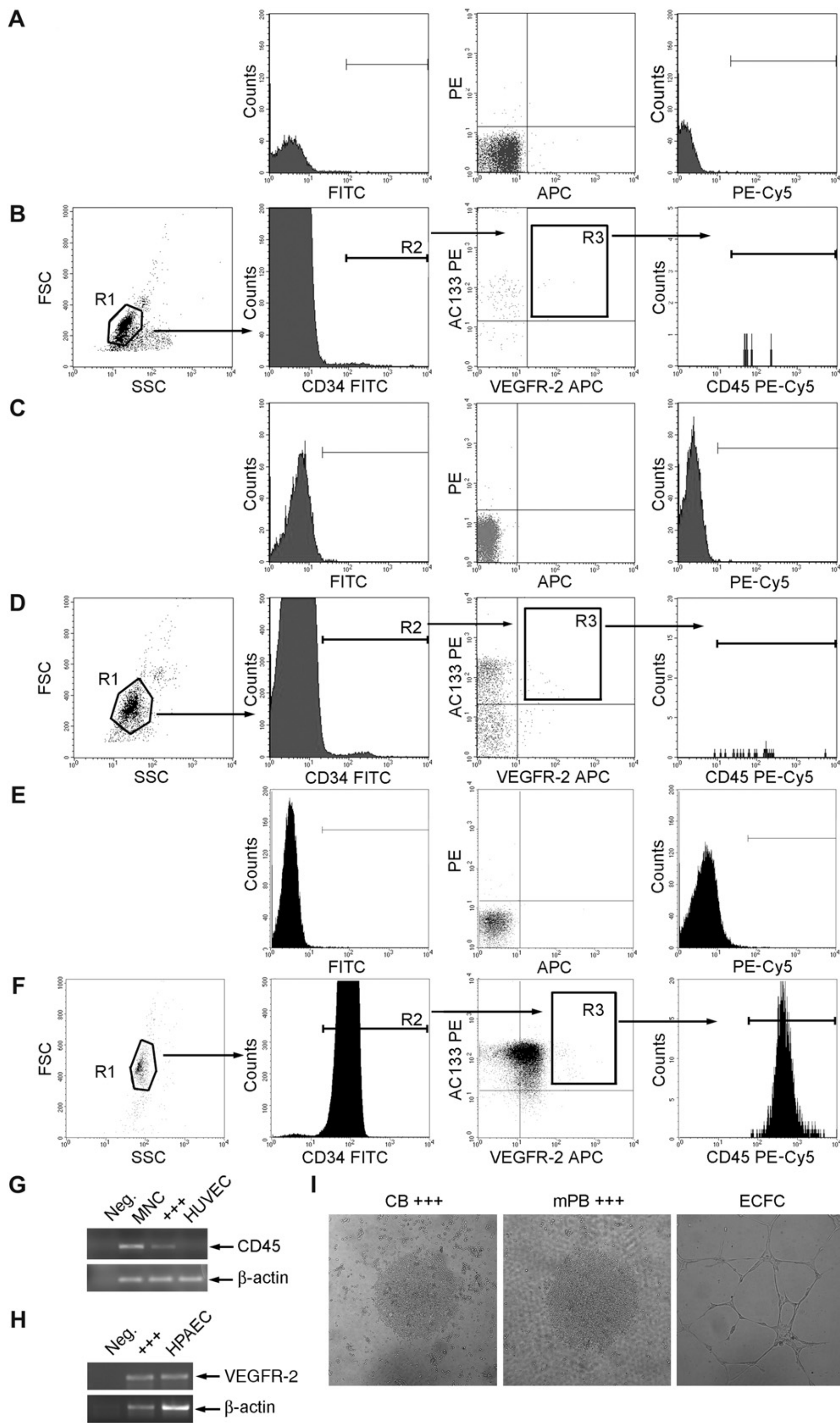
Discussion

New blood vessel formation occurs via angiogenesis, vasculogenesis or arteriogenesis [2,5,36,37]. Since the late 1990s, postnatal vasculogenesis has been purported to be an important mechanism for angiogenesis via BM-derived circulating EPCs [3,4]. Further, experimental and clinical studies suggest that there is a role for EPCs in neoangiogenesis and rejuvenation of the endothelial monolayer of damaged blood vessel walls [1,38,39]. However, despite these observations, identification, isolation, and characterization of EPCs continues to be hampered by not only the absence of specific EC markers to differentiate between EPCs and HPCs, but also functional assays to differentiate between mature ECs and EPCs.

The original identification of EPCs was described in 1997 by Asahara et al. [1]. It was reported that plating of freshly isolated CD34 or VEGFR-2–expressing cells from adult PB on fibronectin-coated dishes yielded in vitro clusters of cells that were comprised of round cells centrally and sprouts of spindle-shaped cells at the periphery within 5 days. The CD45 antigen was shown to be expressed on 94.1% of the freshly plated cells and after 7 days, 27.2% of the adherent cells continued to express CD45. An increased expression of CD34, CD31, VEGFR-2, Tie-2, and E selectin was also observed in the adherent cells. Further, injection of the CD34$^+$ MNCs from adult PB (15.7% enriched) into nude mice with induced hind limb ischemia led to engraftment of some human cells in 13.4% of the mouse capillaries. Thus, Asahara et al. [1] concluded that circulating CD34$^+$ MNCs from adult PB contributed to new vessel formation via vasculogenesis in nude mice.

Following Asahara's seminal observations, Peichev et al. [14] extended this work and provided data to demonstrate that the most primitive EPC precursor coexpresses the cell-surface antigens CD34, AC133, and VEGFR-2. In this report, Peichev et al. [14] reported that mature ECs

Figure 2. CD45 expression on CD34$^+$AC133$^+$ vascular endothelial growth factor receptor-2 (VEGFR-2)$^+$ cells. Representative fluorescence-activated cell sorting (FACS) analysis of peripheral blood (PB) mononuclear cells (MNCs) (**A**,**B**), cord blood (CB) MNCs (**C**,**D**) and mobilized peripheral blood (mPB)–derived CD34$^+$ cells (**E**,**F**) stained with isotype controls (**A**,**C**,**E**) or monoclonal antibodies against human CD34, AC133, vascular endothelial growth factor receptor-2 (VEGFR-2) and CD45 (**B**,**D**,**F**). To assess CD45 expression on CD34$^+$AC133$^+$VEGFR-2$^+$ cells, MNCs, or mPB-derived CD34$^+$ cells were gated as described in Figure 1 for the CD34$^+$AC133$^+$VEGFR-2$^+$ subpopulation (R1+R2+R3), and subsequently examined for CD45 coexpression. FACS plots are representative of independent analyses using cells from six different adult donors, four different CB donors, and three different mPB donors. (**G**) Reverse transcriptase polymerase chain reaction (RT-PCR) analysis of CB MNCs (MNC), freshly sorted CB-derived CD34$^+$AC133$^+$VEGFR-2$^+$ cells (+++), or human umbilical vein endothelial cells (HUVECs) for gene expression of CD45 or β-actin. Right lane (Neg) shows the reaction absent of cDNA. (**H**) RT-PCR analysis of freshly sorted CB-derived CD34$^+$AC133$^+$VEGFR-2$^+$ cells (+++), or human pulmonary artery endothelial cells (HPAECs) for gene expression of VEGFR-2 or β-actin. Right lane (Neg) shows the reaction absent of cDNA. RT-PCR results represent three independent experiments using cells from different donors. (**I**) Representative photomicrographs (20× magnification) of freshly sorted CB- or mPB-derived CD34$^+$AC133$^+$VEGFR-2$^+$ cells (CB +++ and mPB +++, respectively) and early passage (P2) CB-derived endothelial colony-forming cells (ECFC) 24 hours after seeding onto Matrigel. CD34$^+$AC133$^+$VEGFR-2$^+$ cells fail to form tube-like structures. Results represent three independent experiments using cells from different donors. APC = allophycocyanin; FITC = fluorescein isothiocyanate; PE = phycoerythrin.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VEGFR-2 APC
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SSC
Counts
CD34 FITC
R2
AC133 PE
VEGFR-2 APC
R3
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CD45 PE-Cy5
G
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MNC
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HUVEC
CD45
β-actin
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β-actin
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mPB +++
ECFC

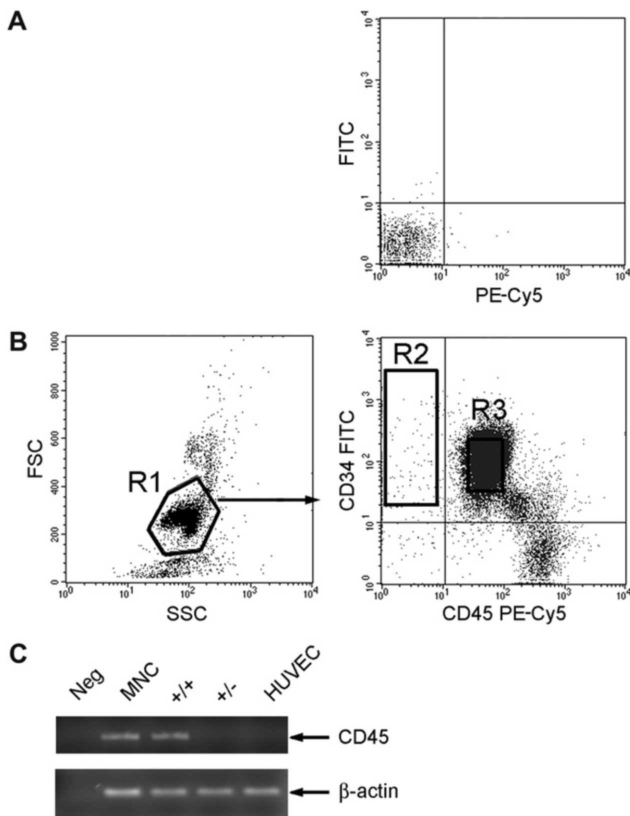

Figure 3. Fluorescence-activated cell sorting (FACS) gating strategy for isolation of CD34$^+$CD45$^-$ and CD34$^+$CD45$^+$ cells. Representative FACS analysis of cord blood (CB) mononuclear cells (MNCs) stained with isotype controls (**A**) or monoclonal antibodies against human CD34 and CD45 (**B**). The lymphocyte population of MNCs (R1) was assessed for expression of CD34 and CD45. CD34$^+$CD45$^-$ cells were isolated by sorting cells, which were contained in gates R1 and R2 and CD34$^+$CD45$^+$ cells were isolated by sorting cells, which were contained in gates R1 and R3. FACS analyses are representative of three independent experiments using cells from different donors. (**C**) Reverse transcriptase polymerase chain reaction (RT-PCR) analysis of CB MNCs (MNC), freshly sorted CB-derived CD34$^+$CD45$^+$ cells (+/+), CD34$^+$CD45$^-$ cells (+/−) or human umbilical vein endothelial cells (HUVECs) for gene expression of CD45 or β-actin. Right lane (Neg) shows the reaction absent of cDNA. Results represent three independent experiments using cells from different donors. FITC = fluorescein isothiocyanate; PE = phycoerythrin.

do not express AC133, but nearly all the CD34$^+$VEGFR-2$^+$ circulating EPCs in fetal liver, CB, or mobilized PB, expressed AC133. These cells also expressed the chemokine receptor CXCR4 and migrated in response to stromal-derived factor-1 or VEGF in vitro. Furthermore, culture of CD34$^+$ cells from human fetal liver with VEGF and fibroblast growth factor-2 resulted in differentiation of these nonadherent cells into adherent AC133$^-$VEGFR-2$^+$acetylated LDL$^+$ cells, which displayed EC morphology. The authors hypothesized that the differentiated adherent EC monolayer was derived from a precursor cell that coexpressed CD34, AC133, and VEGFR-2. However, this observation was not definitively proven, because prospective isolation of CD34$^+$, AC133$^+$, and VEGFR-2$^+$ cells from fetal liver, CB, or mPB and direct plating of the sorted cells into EC media to test for EC colony growth was not performed.

In addition, in a human in vivo model, Peichev et al. [14] also provided data to demonstrate that the surface of implanted human left ventricular assist devices was colonized with AC133$^+$VEGFR-2$^+$ cells, suggesting that the circulating CD34$^+$ cells expressing AC133 and VEGFR-2 is a functionally distinct population of EPCs that may play a role in neoangiogenesis. Subsequently, Reyes et al. [13] also identified a human postnatal CD34$^-$, vascular endothelial cadherin$^-$ (VE-cadherin$^-$), AC133$^+$, VEGFR-2$^+$ (CD34$^-$VE-cadherin$^-$AC133$^+$VEGFR-2$^+$) multipotent adult progenitor BM cell that gives rise to cells that express EC surface proteins, functions in vitro as mature ECs and contributes to neoangiogenesis during in vivo tumor angiogenesis and wound healing.

Largely based on these important studies, a widely accepted definition for the most primitive EPC precursor is a cell which coexpresses CD34, AC133, and VEGFR-2 [1,5,12–16]. In fact, this EPC definition has formed the basis of many clinical studies designed to determine the role of EPCs in a variety of vascular disorders. Specifically, numerous articles have correlated the concentration of

Table 3. CFU-EC and ECFC colony frequency in CB-derived MNCs, CD34$^+$CD45$^-$ and CD34$^+$CD45$^+$ cells

	MNCs		CD34$^+$CD45$^-$		CD34$^+$CD45$^+$	
Experiment no.	CFU-EC	ECFC	CFU-EC	ECFC	CFU-EC	ECFC
1	1.8*	2.0*	0*	666*	0*	0*
2	2.0	0.3	0	400	0	0
3	0.4	1.0	0	150	0	0
Average	1.4	1.1	0	405	0	0
SEM	0.5	0.5	0	149	0	0

CB = cord blood; CFU-EC = colony-forming unit endothelial cells; ECFC = endothelial colony-forming cells; MNC = mononuclear cells; SEM = standard error of mean.
*Frequency of CFU-EC or ECFC colony formation per 10^6 cells plated.

Table 4. LPP- and HPP-CFC colony frequency in CB-derived MNCs, CD34$^+$CD45$^-$, and CD34$^+$CD45$^+$ cells

	MNCs		CD34$^+$CD45$^-$		CD34$^+$CD45$^+$	
Exp no.	LPP-CFC	HPP-CFC	LPP-CFC	HPP-CFC	LPP-CFC	HPP-CFC
1	0.67*	0.33*	0*	0*	160*	340*
2	4.0	7.5	0	0	190	320
3	1.5	2.33	0	0	6.33	25.3
Average	2.06	3.39	0	0	119	228
SEM	1.00	2.14	0	0	56.9	102

CB = cord blood; CFC = colony-forming cells; HPP = high proliferative potential; LPP = low proliferative potential; MNC = mononuclear cells; SEM = standard error of mean.
*Frequency of LPP- or HPP-CFC colony formation per 10^4 cells plated.

$CD34^+AC133^+VEGFR\text{-}2^+$, $CD34^+VEGFR\text{-}2^+$, or $CD34^+$ cells with the risk of development of adverse cardiovascular outcomes and in general an inverse correlation with each of these subsets and the highest-risk category exists [5,7,17,40,41]. Thus, enumeration of $CD34^+AC133^+VEGFR\text{-}2^+$ cells in adult PB appears to serve as a predictor for patient cardiovascular risk, though the biologic mechanism for this observation remains unclear, especially given limitations on the precise functional phenotype of circulating $CD34^+AC133^+VEGFR\text{-}2^+$ cells.

While the proteins CD34, AC133, and VEGFR-2 are thought to be present on EPCs, it is important to note that these antigens are also expressed on subpopulations of HPCs, including LPP- and HPP-CFCs. Further, CD34 and AC133 are also expressed on human HSCs. Remarkably, despite this observation and lack of definitive data to demonstrate that $CD34^+AC133^+VEGFR\text{-}2^+$ cells directly yield EC colonies in a clonogenic assay, $CD34^+AC133^+VEGFR\text{-}2^+$ cells have never been isolated and simultaneously plated in hematopoietic or EC clonogenic assays to determine the type of colony-forming potential harbored by these rare circulating cells.

In this report, utilizing CB and mPB, we isolated and purified $CD34^+AC133^+VEGFR\text{-}2^+$ cells by FACS and assayed for the presence of clonogenic CFU-ECs, ECFCs, and hematopoietic LPP- and HPP-CFCs. For each assay, we plated either unfractionated CB MNCs or mPB $CD34^+$ cells to calculate the frequency of each CFC type in CB and mPB. We then compared the frequency of the different CFCs in the assays inoculated with the sorted $CD34^+AC133^+VEGFR\text{-}2^+$ cells to determine if these cells were enriched for a particular type of CFC activity. Surprisingly, we found that freshly isolated CB- and mPB-derived $CD34^+AC133^+VEGFR\text{-}2^+$ cells are highly enriched for hematopoietic LPP- and HPP-CFCs, yet were devoid of any CFU-EC or ECFC activity. Consistent with this clonogenic phenotype, we also provide novel data to demonstrate that in fact $CD34^+AC133^+VEGFR\text{-}2^+$ cells isolated from adult PB, CB, or mPB universally express the cell-surface antigen, CD45, confirming their hematopoietic cell identity. Finally, in contrast to ECFCs, we also show that CB- and mPB-derived $CD34^+AC133^+VEGFR\text{-}2^+$ cells do not form capillary-like structures in Matrigel assays.

As the $CD34^+AC133^+VEGFR\text{-}2^+$ cells are a rare cell subpopulation of the $CD34^+$ pool of cells, we also performed additional studies to determine whether it was feasible to isolate each of the endothelial and hematopoietic CFCs from $CD34^+$ CB cells. This was a particularly important experiment because freshly isolated $CD34^+$ cells from BM or CB are currently being used for angiogenic therapies in treating different cardiovascular disorders. We costained the CB cells with a monoclonal antibody to common leukocyte antigen, CD45, to assess whether cellular expression of this antigen would facilitate dissection of endothelial from hematopoietic CFC activity. Most CB CD34–expressing cells coexpress CD45. However, there are rare cells that are $CD34^+CD45^-$. We demonstrated that ECFC activity is greatly enriched in the $CD34^+CD45^-$ cell subpopulation, but this fraction is devoid of hematopoietic activity. We also showed that $CD34^+CD45^-$ cells did not yield CFU-ECs. This is not surprising given that we recently generated human clonal data to demonstrate that CFU-ECs and their progeny are hematopoietic-restricted cells, which express macrophage-specific cellular proteins and function as macrophages to ingest bacteria [34]. In contrast, $CD34^+CD45^+$ cells are greatly enriched in hematopoietic LPP- and HPP-CFC activity, but not CFU-EC or ECFC activity. Thus, CB EPCs are $CD34^+CD45^-$ restricted.

In conclusion, our data provides novel evidence that circulating $CD34^+AC133^+VEGFR\text{-}2^+$ and $CD34^+CD45^+$ cells are distinct, primitive HPC subpopulations and not EPCs. Therefore, the biologic mechanism for the correlation of $CD34^+AC133^+VEGFR\text{-}2^+$ with cardiovascular disease needs to be reexamined. Furthermore, it is interesting to note that many cell-based therapies, which currently infuse unfractionated MNCs or sorted $CD34^+$ cells to treat a variety of cardiovascular diseases, have demonstrated limited efficacy in improving long-term clinical outcomes. Based on our data, it is likely that most of the infused cells are hematopoietic and have little capacity to form new blood vessels, which emphasizes the importance of determining the precise biologic potential of cells when considering cell-based therapies for vessel regeneration. Future studies directed at sorting different cell populations, which harbor EC colony- and vessel-forming capacity, could potentially uncover new cell-based therapeutic avenues for treating cardiovascular disease.

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