American Journal of Pathology, Vol. 168, No. 5, May 2006

DOI: 10.2353/ajpath.2006.050556

Vascular Biology, Atherosclerosis and Endothelium Biology

The Origin and *in Vivo* Significance of Murine and Human Culture-Expanded Endothelial Progenitor Cells

Emerson E. Sharpe, III,* Amylynn A. Teleron,* Bin Li,* James Price,*† Mark S. Sands,‡ Kathy Alford,*† and Pampee P. Young*§

From the Departments of Pathology and Internal Medicine,§ Vanderbilt University Medical Center, Nashville, Tennessee; the Veterans Medical Center,† Nashville, Tennessee; and the Department of Internal Medicine,‡ Washington University Medical School, St. Louis, Missouri*

In adults highly purified populations of early hematopoietic progenitors or cells derived from *ex vivo* expanded unmobilized human peripheral blood mononuclear cells contribute to new blood vessel formation. However, the source of these culture-expanded endothelial progenitor cells (CE-EPCs) remains controversial. We demonstrate that *ex vivo* expansion of unmobilized human peripheral blood generated CE-EPCs with similar numbers, kinetics, and antigen expression profile as compared to plating unfractionated $CD34^+/lin^-$-enriched bone marrow mononuclear cells. Both CE-EPC populations uniformly co-expressed myeloid and endothelial markers, suggesting that peripheral blood progenitor enumeration does not correlate with the numbers of early outgrowth CE-EPCs. Using purified myeloid subpopulations obtained from mice harboring the lacZ transgene driven by an endothelial-specific promoter, we showed that the immature myeloid lineage marker $CD31^+$ cells generated CE-EPCs with fourfold greater frequency than mature myeloid populations. Biphenotypic cells co-expressing myeloid/endothelial antigens were not detected in circulating human or murine peripheral blood or bone marrow but were associated with murine tumors. Unlike CE-EPCs, $CD14^+$ leukocytes admixed within tumors did not generate vWF-positive blood vessels during a similarly defined period of tumor growth, but some leukocytes up-regulated the endothelial marker VE-cadherin. Taken together, the data suggest that the local neovascular microenvironment may facilitate vasculogenesis by promoting endothelial differentiation and that CE-EPCs may accelerate such vasculogenesis. *(Am J Pathol 2006, 168:1710–1721; DOI: 10.2353/ajpath.2006.050556)*

There is growing excitement that bone marrow (BM)-derived endothelial progenitor cells (EPCs) can be used to promote revascularization of injured and ischemic tissues or as cellular therapy to treat congenital diseases or cancer growth.[1–4] Several studies support the observation that hematopoietic progenitors can give rise to endothelial cells at sites of neovasculature *in vivo* after intravenous administration.[5,6] Plating highly purified populations of $CD34^+/lin^-$ cells also gives rise to adherent cells that are positive for several endothelial-enriched markers such as KDR, (flk-1), tie-2, and VE-cadherin throughout the course of 3 to 5 weeks.[5,7–9] Concurrently, Kalka and colleagues[10] have reported that after 5 to 7 days of culture *ex vivo* expansion of unmobilized human peripheral blood (hPB) gives rise to adherent cells that express endothelial cell markers. These cells were inferred to be derived from aggressive expansion of the relatively rare numbers of circulating progenitors. Administration of the resultant culture-expanded (CE)-EPCs to immunodeficient, ischemic mice results in their incorporation into new vessels and also promotes overall angiogenesis.[10,11]

Several recent reports have demonstrated that endothelial-like cells can be generated *in vitro* by plating highly purified populations of human $CD14^+$ monocytes.[12–16] Still, the cell type from which CE-EPCs are derived and whether $CD14^+$ cells can generate CE-EPCs that contrib-

Supported by grants from the American Cancer Society, NIH K08HL084020, Pfizer Atorvastatin Research Award, and VA Career Development Award (all to P.P.Y.).

Supplemental material for this article can be found on *http://ajp.amjpathol.org*.

Accepted for publication January 13, 2006.

Address reprint requests to Pampee P. Young, Vanderbilt University School of Medicine, Department of Pathology, 1161 21st Ave. South, U5211 MCN, Nashville, TN 37232. E-mail: pampee.young@vanderbilt.edu.

ute to functional vasculature *in vivo* is unclear.[17,18] Although the phenotype of CE-EPCs has been extensively characterized,[10,13–16] its *in vivo* relevance is unclear. It is not established, for example, if leukocytes that co-express endothelial-specific markers, such as VE-cadherin, are generated in the BM, circulate through the blood, and then home to sites of neovascularization or if they are generated *in situ*. It has also not been resolved if CE-EPCs help accelerate functional blood vessel formation *in vivo* over unmanipulated leukocyte populations.

In this article we provide data that human and murine CE-EPCs are derived from circulating myeloid precursors. Using transgenic mice that express lacZ under the control of an endothelial cell promoter, we further segregate and define the molecular phenotype of the myeloid lineage that can generate adherent cells expressing endothelial markers *in vitro*. Early outgrowth CE-EPCs are bi-phenotypic in that both hematopoietic and endothelial markers are co-expressed. We describe biphenotypic cells associated with murine tumors that are not in the circulation or BM of either human or murine samples. We further show that human CD14-derived CE-EPCs form functional tumor vessels *in vivo*, whereas $CD14^+$ cells and differentiated macrophages cannot. The ease of obtaining CE-EPCs from unmobilized hPB and their ability in multiple models to participate in vasculogenesis has made them an attractive candidate with which to develop cell-based therapy.[19–21] Our data suggest that they accelerate vasculogenesis *in vivo* and support the importance of continued efforts to better characterize and understand their biology.

Materials and Methods

Mice and Cell Lines

NOD/LtSz-scid/β-glucuronidase$^{-/-}$ mice were obtained through collaboration with Dr. M. S. Sands (Washington University, St. Louis, MO).[22] *NOD/SCID/β-glucuronidase*$^{+/-}$ mice were bred in a colony maintained by P.P. Young at the Vanderbilt School of Medicine. Offspring homozygous for the GUSB mutation (*NOD/SCID/β-glucuronidase*$^{-/-}$) were identified by a specific polymerase chain reaction assay.[4,22] β-glucuronidase is a lysosomal enzyme expressed in all cell types, including CE-EPCs and hematopoietic cells. On transplantation into a β-glucuronidase-negative host, cells from normal human and murine donors can be identified by virtue of their β-glucuronidase expression at a level of single cell sensitivity.[23] The *NOD/SCID/β-glucuronidase*$^{-/-}$ tumor line arose spontaneously in a homozygous mouse and was passaged in culture. Tumor cells from passages 10 to 20 were used in these experiments and confirmed to be β-glucuronidase$^{-/-}$ by histochemistry, biochemistry, and polymerase chain reaction assay (B. Li and P.P. Young, unpublished data). Eight- to ten-week-old mice were injected subcutaneously with 2×10^6 β-glucuronidase$^{-/-}$ tumor cells admixed with 100 μl of growth factor-reduced Matrigel (BD Biosciences, San Jose, CA) and experimental cells. After 8 days, the mice were sacrificed and the tumors were removed for histological analysis. Some mice were allowed to generate tumors, injected intravenously with CE-EPCs, and sacrificed 7 days later for histological analysis of tumors. A portion of the tumors were administered to NOD/SCID mice, allowed to generate tumors of ~2 to 4 cm^3, and used to generate suspensions for flow cytometric analysis.

Flk1/LacZ mice (Jackson Laboratory, Bar Harbor, ME) represent mice heterozygous for the flk-1-null mutation in which the target gene was replaced with a promoterless *lacZ* gene from *Escherichia coli*. In the heterozygous mouse, expression of lacZ as well as target receptor protein, flk-1, are subject to regulation by an endothelial cell-specific promoter.[24] Mice heterozygous for the transgene were identified using a specific polymerase chain reaction assay to detect the neomycin cassette, ie, to detect the disrupted allele (sequences available on request). All protocols were approved by the Vanderbilt Institutional Animal Care and Use Committee.

EPC Culture

Normal human PB leukocytes were obtained from human blood donor leukocyte reduction filters (LeukotrapRC; Pall Corporation) from the American Red Cross (Nashville, TN) as previously described[25]; three to four filters were pooled per preparation to reduce donor variability. BM leukocytes were prepared using frozen surplus hBM samples acquired from Vanderbilt Medical Center (Nashville, TN). The bags were thawed at 37°C and then diluted into a thawing medium comprised of Hanks' buffered salt solution (HBSS) with 10% fetal bovine serum (Cambrex, Walkersville, MD), 20 U/ml heparin (Sigma, St. Louis, MO), and 36 U/ml DNase (Sigma), processed with the dead cell removal kit (Miltenyi). MNCs from both PB and BM leukocytes were obtained by centrifugation on Histopaque 1077 (Sigma) gradients according to the manufacturer's instructions. Mononuclear cells (MNCs) were used for cell isolation or resuspended in EGM-2 Bullet kit system (Clonetics, San Diego, CA) consisting of endothelial basal medium, 5% fetal bovine serum, human epidermal growth factor, vascular endothelial growth factor (VEGF), human fibroblast growth factor-B, insulin-like growth factor-1, ascorbic acid, and heparin and directly plated at 1×10^8 cells/cm^2 on 100-mm culture dishes or on 22-mm^2 glass coverslips (placed in six-well plates) coated with human fibronectin (Sigma) diluted 1:50 in HBSS as described.[25] Murine BM cells were obtained as described[26] from flk1/lacZ-positive mice and transgene-negative littermates as controls. Murine MNCs were isolated by density centrifugation over Lympholyte-M (Cedarlane) at $1550 \times g$ for 30 minutes and enumerated and plated or used for cell separation procedures described below. Murine MNCs were plated at 2×10^7 cells/cm^2 on 100-mm culture dishes or on 18-mm diameter circular glass coverslips (placed in 12-well plates) coated with human fibronectin (Sigma) diluted 1:50 in HBSS. Thereafter, the murine CE-EPCs were generated similarly to human samples.

Antibodies Used for Cell Staining and Flow Cytometry

The following antibodies were obtained and used for analysis: hCD2 (S5.2; BD Biosciences), mCD11b (M1/70; R&D Systems, Minneapolis, MN), hCD14 (TüK4; Caltag, Burlingame, CA), hCD15 (MMA, BD Biosciences), hCD19 (J4.119; IOTest), mCD31 (MEC 13.3, BD Biosciences), hCD34 (QBEnd 10; DAKO, Carpinteria, CA), mCD34 (RAM34, BD Biosciences), hCD45 (HI30, BD Biosciences), hCD45 (5B1, Miltenyi), hCD45 (J33, IOTest), hCXCR4 (44716, R&D Systems), mF4/80 (BM8; eBioscience), polyclonal hFlk-1 (R&D Systems), hFlt-1 (89106, R&D Systems), Ly-6C (RB6–8C5, BD Biosciences), polyclonal htie-1 (Santa Cruz Biotechnology, Santa Cruz, CA), polyclonal mtie-2 (Santa Cruz), hVE-Cadherin (BV9; Cell Sciences), polyclonal VE-Cadherin (BMS158 recognizes human and mouse; Bender Medsystems), human-specific von Willebrand factor (vWF) (VW1–2; Takara), vWF (DAKO), polyclonal goat anti-human β-glucuronidase (provided by M.S. Sands). Appropriate secondary antibodies were purchased and used from Jackson ImmunoResearch (West Grove, PA) and Molecular Probes (Eugene, OR). We also used 293 and human umbilical vein endothelial cells (HUVECs) (American Type Culture Collection, Rockville, MD) as well as human microvascular endothelial cells (kind gift from Dr. T. Takahashi, Vanderbilt University, Nashville, TN).

Histochemistry, Immunofluorescence, and Morphometry

Fluorescent staining of adherent cells on glass coverslips was conducted after 7 days in culture. First, the adherent cells were incubated with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine (DiI)-labeled acetylated low-density lipoprotein (acLDL) (Biomedical Technologies, Stoughton, MA) diluted 1:20 in EBM-2 for 2 hours at 37°C. After fixing these cells with cold acetone, fluorescein isothiocyanate-labeled *Ulex europaeus* agglutinin 1 (UEA-1) lectin (Vector Laboratories, Burlingame, CA), diluted 1:20 in phosphate-buffered saline (PBS), was then applied to the same coverslip for 1 hour. Immunostaining with individual antibodies were performed as previously described.[25] The slides were viewed under an upright fluorescence microscope. The images were visualized using a Zeiss Axioplan 2 microscope (Carl Zeiss MicroImaging, Thornwood, NY). Images were photographed with a CoolSNAP Hq charge-coupled device camera (Photometrics) and acquired with Metamorph version 5.0 software (Universal Imaging Corporation). For β-gal staining, cells on coverslips or cytospins were prepared and stained as described.[27] In parallel, slides were also stained with a polyclonal antibody to β-gal (Novus, Littleton, CO) and analyzed using immunofluorescence. Histochemistry for β-glucuronidase was performed as previously described.[28] CE-EPCs were quantified by counting random high-power fields as previously described.[25] To quantify the number of positive cells, 5 to 10 random fields were digitally captured, and the number of stained cells were compared to the total number of cells as determined by DAPI (Vectashield with 4,6-diaminidino-2-phenylindole; Vector Laboratories)-stained nuclei or total counted cells, respectively. For *in vivo* studies, animals were injected intravenously with biotinylated BS-1 lectin (Vector Laboratories) 30 minutes before sacrifice. Tumors were resected, embedded in OCT, and processed as described before staining.[4] Lectin was detected by rhodamine streptavidin.

Flow Cytometry

Flow cytometric analysis of MNCs and progenitor enumeration were performed as previously described.[25] Analyses of CE-EPCs were performed by detaching cells with ethylenediaminetetraacetic acid, washing with PBS and incubating in PBS/0.5% bovine serum albumin in the presence of various antibodies. All monoclonal antibodies were directly conjugated. Quadrants were established using staining of sample with isotype control antibodies. For intracellular flow analysis (ie, CD68 and vWF), cells were treated with permeabilizing kit (Calbiochem, La Jolla, CA). For UEA-1 lectin, quadrants were established by evaluation of 293 cells (negative control) and HUVECs (positive control). For polyclonal antibodies, quadrants were established by staining cells with irrelevant polyclonal and fluorescent-conjugated secondary antibodies. Tumor-associated cells were obtained for analysis by mincing the tissue to <1 mm^3 and filtering the resulting suspension through with a 70-μm filter. The tumor suspensions were separated on a density gradient, Lympholyte M, for 30 minutes at 1500 $\times$ *g*. The interphase was collected, washed, and analyzed. Nonviable cells, identified by 7-aminoactinomycin D (7-AAD) (Molecular Probes) staining, were excluded. Quantitative analysis and sorting were performed on a FACScan flow cytometer and FACSAria, respectively, and subsequently analyzed using Cellquest software (Becton Dickinson, Mountain View, CA).

Matrigel Tubule Assay

Matrigel (BD Bioscience) was thawed and placed in four-well glass slides at room temperature for 30 minutes to allow solidification. Cells were plated and incubated at 37°C for a half-hour. Media was added to each well thereafter, and tube formation was examined 12 hours later.

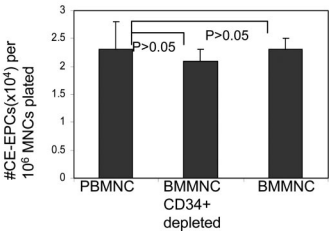
Cell Isolation

Both immunomagnetic and flow sorting techniques were used to obtain highly purified cell populations. Enriched populations of human CD14$^+$, murine CD31$^+$, CD11b$^+$, F4/80$^+$ cells, or CD34 depletion were purified from MNCs by selection with microbeads (Miltenyi Biotec) using a magnetic cell sorter device. Purity was assessed by flow cytometry, and cell viability was determined by trypan blue exclusion and assessed to be $>$95% viable. CD14$^+$ cells were incubated in RPMI with 10% FCS in the pres-

A.

	PB (%)	BM (%)	BM:PB
CD34+/ lin-	0.05±.03	*12.2±4	**240**
CD2	66.1±12	*35.2±15	0.53
CD14	12.5±5	8.3±4	0.66
CD15	3.7±.8	2.2±1.2	0.59
CD19	17.8±4.5	13.9±3.2	0.78

B.



C.

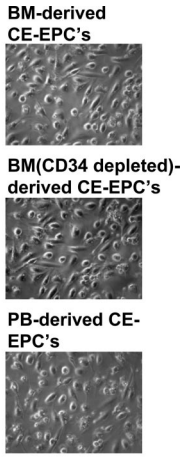


Figure 1. Plating CD34-enriched BM-MNCs generated similar numbers of CE-EPCs as PB-MNCs, which contained significantly fewer progenitors. **A:** Cellular composition of MNCs obtained from PB or thawed BM before culture, as determined by flow cytometry. Data were obtained from four independent experiments. **B:** EPC yield by counting high-power fields from each source per million MNCs plated. **C:** Bright-field images of EPCs generated from MNCs obtained from thawed BM (with and without CD34 depletion) or PB after 7 days in culture. *Statistical significance at $P < 0.05$. Magnifications, ×200.

ence of M-CSF (25 U/ml; Research Diagnostics) to induce macrophage differentiation. $CD31^+$/Ly-$6C^{low\ or\ high}$ populations from bone marrow-derived mononuclear cells (BM-MNCs) from flk-1/lacZ heterozygotes were stained with fluorescent-labeled antibodies and sorted.

Statistical Analysis

Student's *t* test was performed to compare different data sets where appropriate. All data are presented as mean ± SEM; a $P < 0.05$ was interpreted to denote statistical significance.

Results

MNCs from Human BM and PB Generate CE-EPCs within a Time Frame Distinct from Hematopoietic Progenitors

We isolated MNCs from PB and from previously frozen, thawed bone marrow (BM)-derived unfractionated leukocytes, both obtained from normal human donors. Flow cytometric analyses of MNCs were performed to compare the cellular composition (Figure 1A). The numbers of CD2 (T lymphocytes) cells in PB-MNCs was almost twice that found in BM-MNCs. There was no statistical difference found in the numbers of monocytes (CD14), granulocytes (CD15), or B lymphocytes (CD19). As expected, the $CD34^+/lin^-$ progenitor population was dramatically higher (240-fold) in samples obtained from BM ($P < 0.01$). Because culture-expanded endothelial progenitor cells (CE-EPCs) are considered to originate from $CD34^+$ stem cells that lack lineage markers ($CD34^+/lin^-$),[5,29] we expected dramatically higher yields from BM samples on culture. A subset of the BM-MNCs underwent depletion of $CD34^+$ cells (<0.07% final CD34 content; Supplemental Figure 1, see *http://ajp.amjpathol.org*). Culture of equal numbers of viable MNCs derived from each source using conditions that favored CE-EPC growth and adherence resulted in the appearance of endothelial colony forming units by day 3 and near confluent cultures by day 7 (Figure 1C). The cells were analyzed on day 7 after culture (shown) for the ability to uptake DiI-acLDL and stain with UEA-1 lectin and other markers in a manner consistent with endothelial lineage (Supplemental Figure 2, see *http://ajp.amjpathol.org*). The yield of CE-EPCs obtained by day 7 after culture was not statistically different between PB- and BM-MNCs ($P > 0.05$, Figure 1B). Furthermore, depletion of $CD34^+$ progenitors from BM did not impact CE-EPC numbers (Figure 1B). Plating of highly purified (>95%) $CD34^+/lin^-$ cells resulted in adherent cells that endocytosed DiI-acLDL and stained with UEA-1 lectin after 14 days, and a confluent monolayer of spindle-like cells was evident after 3 to 4 weeks of culture (data not shown), a time frame consistent with published reports.[9,30] Taken together, our findings suggested that the origin of CE-EPCs derived from unfractionated human BM or PB (consistently assayed/used by day 7 after culture[21,31]) were not the $CD34^+/lin^-$ cells but likely more abundant committed progenitors.

CE-EPCs Co-Expressed Both Endothelial and Monocyte Markers

To further characterize the BM- and PB-derived CE-EPCs, we used both immunofluorescence and flow analysis of day 7 cultured cells. By flow cytometry, the PB-derived CE-EPCs were positive for flt-1 (VEGFR1), flk-1 (VEGFR2 or KDR), tie1, tie2, vWF, CD31, VE-cadherin (CD105), and CD45 (pan-leukocyte), and CD14 (LPS receptor selectively expressed on monocytes) (Figure 2A). The cells were negative for CD2 (T cells), CD19 (B cells), and CD15 (granulocytes) (Figure 2A). The data for BM-MNC-derived CE-EPCs were similar (data not shown). These data were confirmed by antigen expression of each set of CE-EPCs (Supplemental Figure 2, see *http://ajp.amjpathol.org*). Using immunofluorescence analysis of CE-EPCs at day 7, we quantified the percentages of antigen-positive CE-EPCs derived from BM-MNCs (before and after CD34 depletion) and PB-MNCs. The data (Table 1) confirmed that nearly all of the day 7 CE-EPCs derived from PB and BM expressed the pan-hematopoietic marker CD45 and the monocyte marker CD14 (Table 1). The cells co-expressing both CD45 and VE-cadherin from this population were sorted by flow (>95% purity, data not shown) and replated onto coverslips. After an additional 3 weeks in culture, CE-EPCs continued to express endothelial markers (VE-cadherin, flk-1, vWF, and UEA-1 lectin) but down-regulated the hematopoietic markers CD14 and CD45 (Table 1, day 30), consistent with other reports in the literature.[9,14] Importantly, only 2% of day 7 CE-EPCs also stained positive for CD68, a marker for tissue macrophages.[32] This sug-

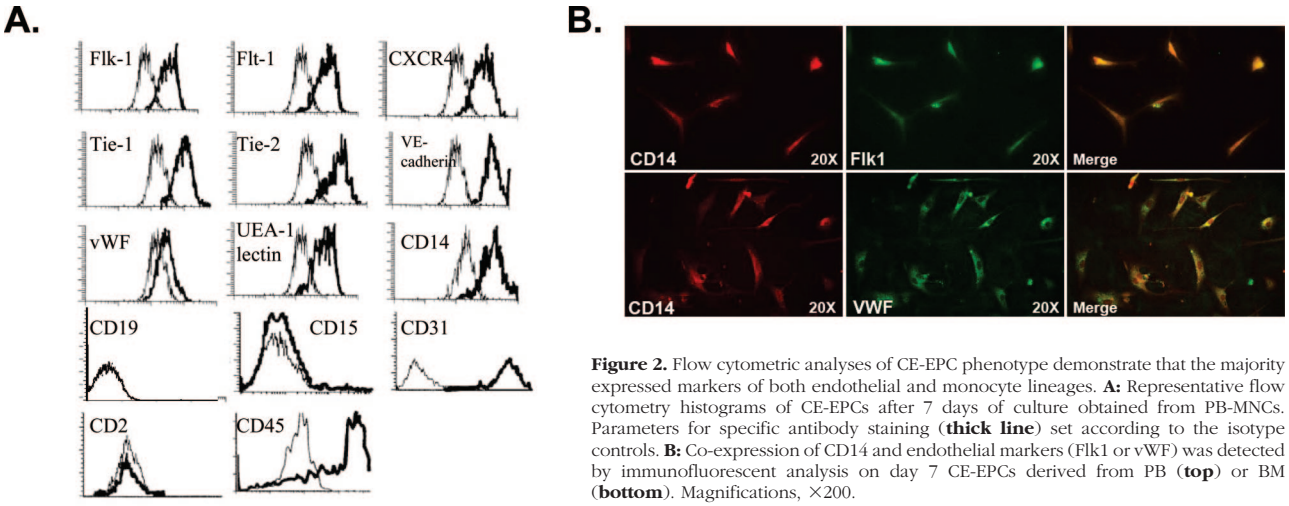


Figure 2. Flow cytometric analyses of CE-EPC phenotype demonstrate that the majority expressed markers of both endothelial and monocyte lineages. **A:** Representative flow cytometry histograms of CE-EPCs after 7 days of culture obtained from PB-MNCs. Parameters for specific antibody staining (**thick line**) set according to the isotype controls. **B:** Co-expression of CD14 and endothelial markers (Flk1 or vWF) was detected by immunofluorescent analysis on day 7 CE-EPCs derived from PB (**top**) or BM (**bottom**). Magnifications, ×200.

gested that human CE-EPCs were likely derived from monocytes but were not mature macrophages (Table 1). To determine whether CE-EPCs co-expressed monocyte and endothelial markers, we demonstrated co-staining by dual immunofluorescence with CD14 and endothelial-specific flk-1 and vWF antibodies. Greater than 90% of the cells that were vWF- or flk-1-positive were also positive for CD14 (Figure 2B).

Culturing of Human CD14-Positive, but Not Negative, Fraction Yielded CE-EPCs ex Vivo

To investigate whether CE-EPCs were derived from monocytic cells, we purified $CD14^+$ and $CD14^-$ cells from PB-MNCs. The purity of the $CD14^+$ population for all experiments was greater than 95%, and the $CD14^-$ population contained less than 2.0% $CD14^+$ cells (Figure 3A). We further determined that the $CD34^+$ fraction segregated, as expected, with the $CD14^-$ cells (Supplemental Figure 3, see *http://ajp.amjpathol.org*). By dual-color flow cytometric analysis we demonstrated that the $CD14^+$ cell-derived CE-EPCs co-expressed VE-cadherin and CD14. HUVECs were used as positive controls because they constitutively expressed VE-cadherin and were $CD14^-$ (Figure 3B) and $CD45^-$ (data not shown). Absence of VE-cadherin expression on $CD14^+$ cells before culture was confirmed (Figure 3B). Also, a subset of $CD14^+$ monocytes were plated in culture conditions designed to promote differentiation into macrophages. The majority of the cells under this culture condition were $CD68^+$ and did not co-express VE-cadherin (Figure 3B). The $CD14^-$ subset was cultured in parallel but failed to yield adherent cells with spindle-shaped morphology by day 7 (data not shown). In a Matrigel tube-formation assay $CD14^+$ cells before culture failed to generate tubes under culture conditions that demonstrated extensive network formation using control human microvascular endothelial cells (Figure 3C). By contrast, CE-EPCs generated from $CD14^+$ cells formed tube networks on Matrigel, albeit less extensively than human microvascular endothelial cells (Figure 3C).

CD14-Derived CE-EPCs Give Rise to Blood Vessels in Vivo

To study the fate of human CE-EPCs *in vivo* we used the β-glucuronidase-deficient NOD/SCID (immunodefi-

Table 1. Phenotype of CE-EPCs Generated from Different Sources of MNCs at Day 7 and Day 30 of Culture

Marker	7 Days BM (%)	7 Days PB (%)	7 Days BM (CD34 depleted) (%)	30 Days BM (%)	30 Days PB (%)
Flk-1	60 + 12	54 + 22	66 ± 18	79 + 10	88 + 9
Flt-1	57 + 19	55 + 16	78 ± 32	ND	ND
CXCR4	47 + 24	60 + 17	ND	ND	ND
Tie-1	72 + 15	58 + 12	ND	ND	ND
Tie-2	55 + 20	64 + 14	68 ± 14	ND	ND
VE-cadherin	88 + 8	93 + 12	78 ± 22	94 + 7	88 + 7
vWF	12 + 7	16 + 5	10 ± 12	22 + 14	38 + 15
UEA-1 lectin	78 + 16	68 + 12	75 ± 21	94 + 5	92 + 6
CD14	92 + 5	93 + 5	96 ± 14	3 + 1	3 + 4
CD45	90 + 9	95 + 2	99 ± 5	8 + 2	3 + 2
CD68	2 + 5	4 + 6	ND	ND	ND

Indirect immunofluorescent staining of CE-EPCs was utilized to determine the percentage of antigen-positive cells in five random fields relative to total number of cells as determined by concomitant staining with DAPI. ND, not done. The data represents more than 3 independent experiments. *Denotes statistical significance with $P < 0.05$.

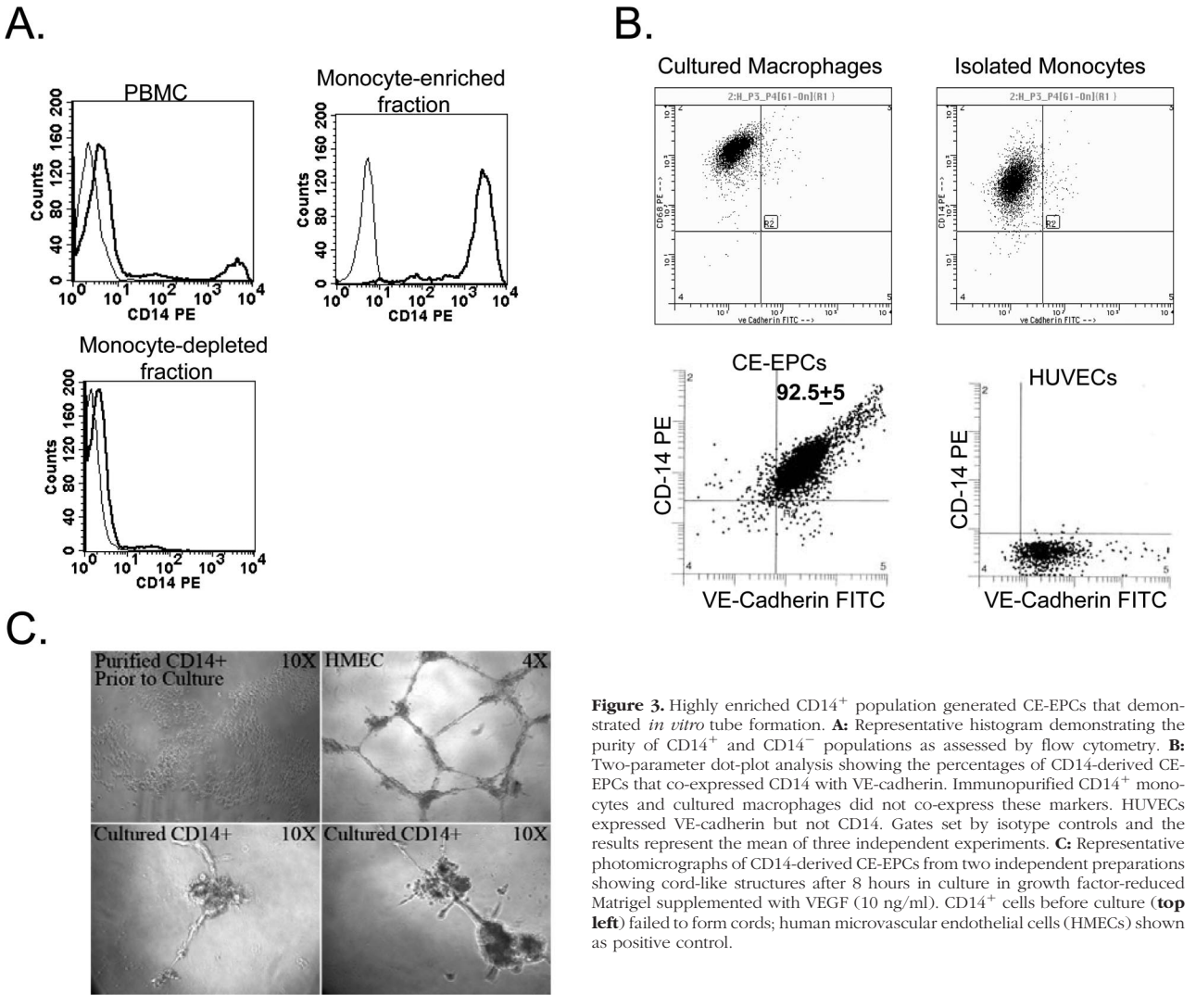


Figure 3. Highly enriched $CD14^+$ population generated CE-EPCs that demonstrated *in vitro* tube formation. **A:** Representative histogram demonstrating the purity of $CD14^+$ and $CD14^-$ populations as assessed by flow cytometry. **B:** Two-parameter dot-plot analysis showing the percentages of CD14-derived CE-EPCs that co-expressed CD14 with VE-cadherin. Immunopurified $CD14^+$ monocytes and cultured macrophages did not co-express these markers. HUVECs expressed VE-cadherin but not CD14. Gates set by isotype controls and the results represent the mean of three independent experiments. **C:** Representative photomicrographs of CD14-derived CE-EPCs from two independent preparations showing cord-like structures after 8 hours in culture in growth factor-reduced Matrigel supplemented with VEGF (10 ng/ml). $CD14^+$ cells before culture (**top left**) failed to form cords; human microvascular endothelial cells (HMECs) shown as positive control.

cient) mouse strain (*NOD/SCID/β-glucuronidase*$^{-/-}$)[22] and β-glucuronidase-negative tumor cells. Because the tumor itself is β-glucuronidase-negative, the presence of any unmodified human cell can be detected by histochemical staining to detect β-glucuronidase enzyme activity. To test whether CD14-derived CE-EPCs can participate in tumor vasculogenesis, we administered intravenously 6×10^6 CD14-derived CE-EPCs into β-glucuronidase$^{-/-}$ mice harboring ~0.5-cm^3 tumor mass ($n = 4$). Mice were sacrificed 1 week later, and the tumor cryosections were analyzed for contribution of CE-EPCs to functional vessels by fluorescence co-localization using human-specific vWF and BS-1 lectin. We identified some CE-EPC-derived vessels in three of four mice, demonstrating that CD14-derived CE-EPCs can home to site of tumor growth and contribute to functional vessels *in vivo* (Figure 4, A–C). To compare the potential of the CE-EPCs versus myeloid cells to generate functional vessels *in situ*, we admixed 1×10^5 cells with 2×10^6 β-glucuronidase$^{-/-}$ tumor cells and injected them subcutaneously into the flank of *NOD/SCID/β-glucuronidase*$^{-/-}$ mice. Animals were sacrificed 8 days later, and tumor cryosections were analyzed for β-glucuronidase immunohistochemistry to detect any administered cell (Figure 4D). Functional vessels were detected by co-staining for human-specific vWF and BS-1 lectin. Transplanted CE-EPCs were detected in abundance (Figure 4, D and E) and had organized to form vascular structures that were positive for human-specific vWF (Figure 4, G and J). Many of the vWF-positive vessels co-localized with BS-1 lectin (which marks murine vessels) (Figure 4, H and K), suggesting that a portion of the vascular structures constituted functional vessels. By contrast, tumors admixed with freshly isolated $CD14^+$ cells (Figure 4, M and N), $CD68^+$ macrophages (data not shown), or $CD14^-$ (data not shown) cells failed to generate any vascular structures despite detection of abundant β-glucuronidase$^+$ (donor) cells by histochemistry (Figure 4F). Although the $CD14^+$ and $CD68^+$ leukocytes did not express vWF, we examined if an earlier vascular marker, such as VE-cadherin,[33] could be identified. We were surprised to identify a number of cells co-expressing β-glucuronidase and VE-cadherin (using a human-specific antibody) within the tumor containing admixed $CD14^+$ leukocytes (Figure 4, O–Q), suggest-

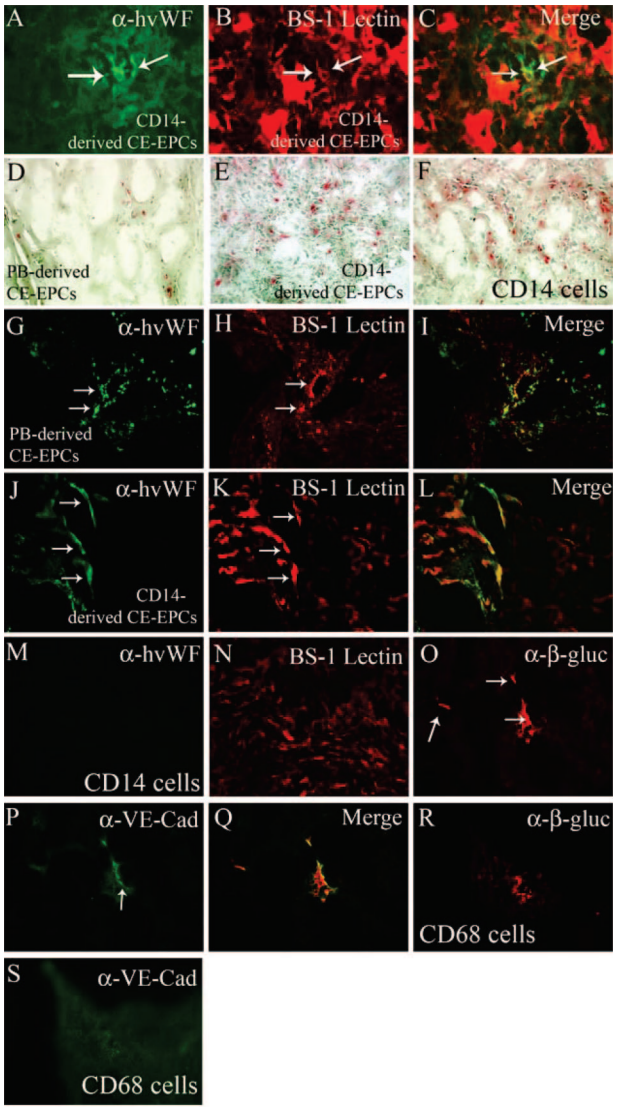


Figure 4. CE-EPCs contributed to functional vessels *in vivo* when transplanted into heterotopic tumors. Ten-μm tumor cryosections from NOD/SCID/β-glucuronidase$^{-/-}$ mouse transplanted with CE-EPCs. For **A–C**, mice harboring 0.5-cm^3 subcutaneous tumors were injected intravenously with CD14-derived CE-EPCs; tumors were analyzed 1 week later for EPC-derived vessels by staining with human-specific vWF (α-hvWF, **A**), BS-1 lectin (**B**), and merge (**C**). The remaining panels represent tumors resulting from admixing tumor cells with various cell types (as designated on panels). Histochemistry for β-glucuronidase enzyme activity (red) (**D–F**) shows abundant numbers of administered cells within tumors 8 days after administration of all cell populations. Sections serial to these were analyzed by co-immunofluorescence using an anti-human-vWF (**G**, **J**, **M**) and BS-1 lectin (**H**, **K**, **N**) to demonstrate evidence of functional vessels from both PB-MNC (**G–I**) and CD14$^+$ (**J–L**) cell-derived CE-EPCs. Human-derived vessels were not detected in murine tumors admixed with human CD14$^-$ cells (**M**). Some of the CD14$^+$ admixed cells up-regulated the endothelial cell marker VE-cadherin as demonstrated by co-localization of the human marker anti-β-glucuronidase (α-β-gluc) (**O**) and anti-human VE-cadherin (**P** and **Q** represent the merge). CD68$^+$ macrophages expressed β-glucuronidase (**R**) but were not found to express VE-cadherin (**S**).

ing that CD14$^+$ leukocytes can up-regulate an endothelial-specific marker *in vivo* in a tumor microenvironment. VE-cadherin expression was not detected within CD68$^+$ macrophage-containing tumor cryosections (Figure 4, R and S).

Myeloid Precursor and Monocyte Fractions Generate CE-EPCs More Efficiently than Fractions Enriched with Markers of Differentiated Macrophage Lineage

Immunomagnetically purified murine CD31$^+$ monocytes and CD31$^-$ cells were obtained from BM-MNCs and plated on fibronectin-coated dishes to generate CE-EPCs. Contamination of immunopurified CD31 cells with mature endothelial cells, which can also express CD31, were excluded by flow analysis with anti-VE-cadherin, anti-vWF, and anti-PIHI2 (Supplemental Figure 4, see *http://ajp.amjpathol.org*). Confluent cultures of spindle-shaped cells were evident by 4 days; this faster time course for generating murine CE-EPCs from cultured cells has been previously reported.[6] Unfractionated MNCs (data not shown) and CD31$^+$ cells generated adherent cells that endocytosed DiI-acLDL and stained with BS-1 lectin (marker for murine endothelial cells), flt-1, and flk-1 (Figure 5A, top). By contrast, CD31$^-$ cells generated an adherent monolayer that was morphologically indistinguishable but did not stain with BS-1 lectin or uptake DiI-acLDL (Figure 5A, bottom).

Recently, a series of monoclonal antibodies have been used to characterize the development of mature macrophages from early myeloid precursors in mouse BM.[34–36] These include ER-MP12 (CD31 or PECAM-1) and ER-MP20 (Ly-6C, a monocyte differentiation antigen). Long-term hematopoietic stem cell activity resides in the CD31$^{hi\text{-}med}$/Ly-6C$^{-/low}$ population. The down-regulation of CD31 and Ly-6C occurs as more expression increases for mature macrophage markers such as CD11b (Mac-1) and F4/80.[34–36] Figure 5B shows a diagrammatic representation of the expression of these antigens during monocyte and macrophage differentiation.

To better define the myeloid population in unfractionated MNCs that give rise to CE-EPCs, we used a combination of immunomagnetic and fluorescence-activated cell sorting (FACS) strategies using BM-MNCs isolated from mice that expressed LacZ under an endothelial-specific promoter, flk-1 (VEGFR2). Various fractions (Table 2) were isolated, and the purity of each population was confirmed by flow cytometry and found to be >95% pure (data not shown). These markers enabled the sep-

Table 2. Myeloid Subpopulations and CE-EPC Generation

Fraction	Phenotype	LacZ-positive (%)
1	CD31$^+$	87 ± 12
2	CD31$^+$/CD11b$^-$	89 ± 11
3	CD31$^+$/LY-6C^{hi}	95 ± 16
4	CD31$^+$/Ly-6C^{low}	88 ± 17
5	CD31$^-$/CD11b$^+$	*21 ± 8
7	CD31$^-$/F480$^+$	*20 ± 11
8	CD31$^-$	*5.3 ± 5

Summary of percentages of lacZ-positive adherent cells out of total numbers of adherent cells obtained upon culturing designated myeloid populations.
* Denotes statistical significance with $P < 0.05$.

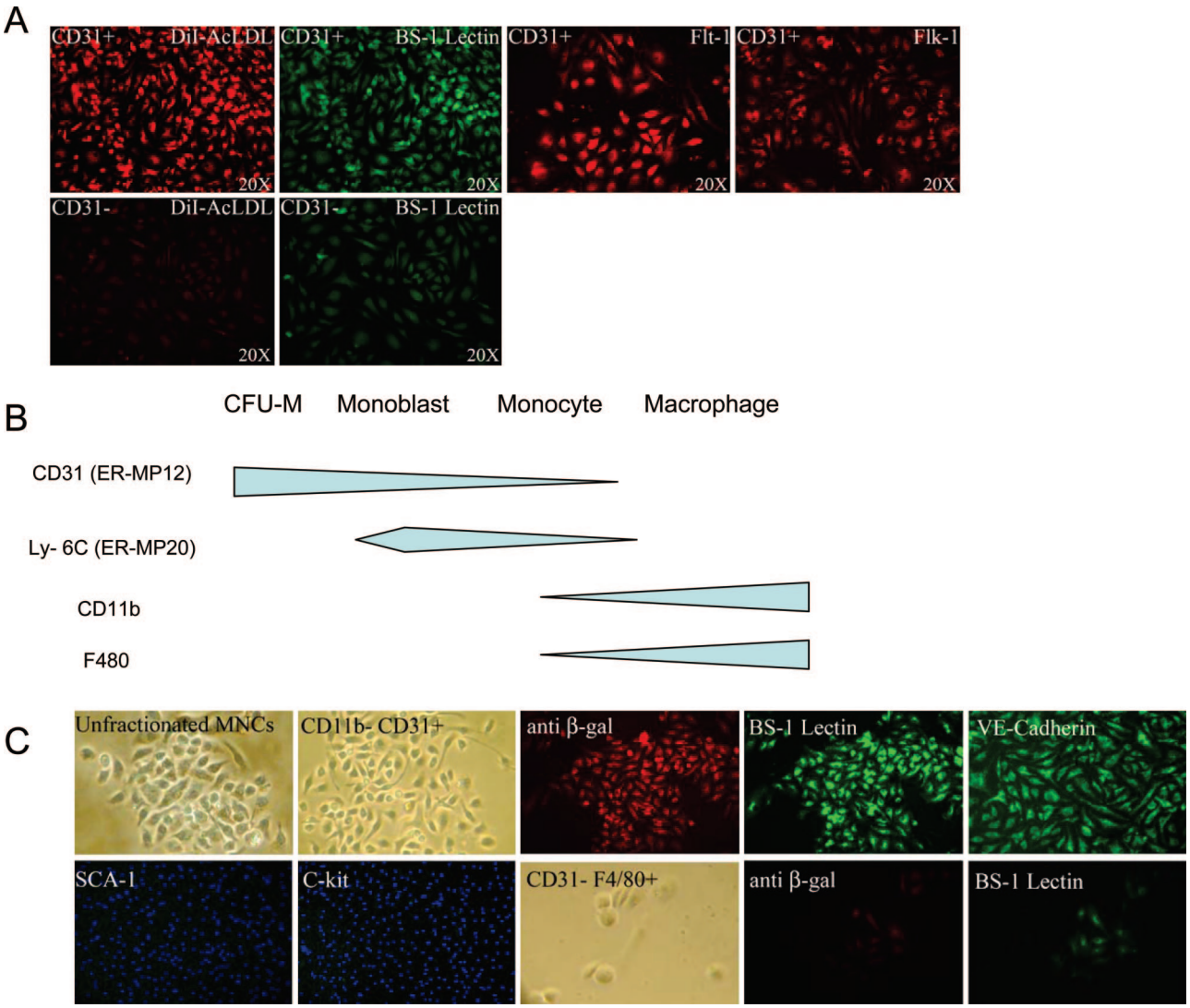


Figure 5. Immature myeloid subpopulations generate CE-EPC with greater efficiency. **A:** Fluorescence microscopy illustrated that adherent cells generated from $CD31^+$ population obtained from normal mice were positive for uptake of DiI-acLDL, binding of fluorescein isothiocyanate-labeled BS-1 lectin (demonstrated in merge), as well as flt-1 and flk-1, detected by indirect immunofluorescence. Culture of $CD31^-$ cells generated adherent cells that failed to endocytose acLDL and were negative for BS-1 lectin staining **B:** Diagram of antigen expression during murine myeloid differentiation, adopted in part from de Braijn and colleagues.[35] **C:** Representative photomicrographs from cultured cells isolated from Flk1/lacZ transgenic mouse BM-MNCs. Myeloid populations designated in Table 2 were immunomagnetically or flow sorted to purity of >95%, as assessed by flow cytometry, and cultured. Cells were stained after 7 days in culture for β-gal expression to quantify numbers of positive cells and analyzed by immunofluorescence to confirm expression of β-gal transgene and murine endothelial marker, BS-1 lectin. Cells derived from unfractionated MNCs (**first panel**), $CD11b^-/CD31^+$ (**panels 2 to 7**) and $CD31^-/F480^+$ (**last 3 panels**) cells that consistently generated high or low percentages of $lacZ^+$/BS-1 $lectin^+$ CE-EPCs, respectively, as summarized in Table 2.

aration of myeloid cells into fractions that comprised precursor populations ($CD31^+/Ly\text{-}6C^{low}$), immature monocytes ($CD31^+$, $Ly\text{-}6C^{high}$, or $CD31^+/CD11b^-$), mature monocytes ($CD31^+/CD11b^+$), and macrophages ($CD31^-/CD11b^+$ or $CD31^-/F480^+$). Equal numbers of viable cells, as determined by trypan blue exclusion, were plated on fibronectin-coated dishes and maintained in culture that promoted CE-EPC outgrowth. The cells were stained at day 7 to evaluate the percentage of cells expressing lacZ (as a marker of flk-1 expression) and BS-1 lectin (Figure 5C, Table 2). Flk1 is known also to be expressed by a subset of very early progenitors, however, our culture-expanded population failed to express the early progenitor markers, Sca-1 and cKit (Figure 5C). These data suggested that murine CE-EPCs were generated primarily from $CD31^+$ monocytes. Highly differentiated macrophages, especially those that have downregulated CD31, generated significantly fewer CE-EPCs as demonstrated by a more than fourfold decrease in percentages of cells expressing lacZ and BS-1 lectin.

Cells Expressing Both Endothelial and Hematopoietic Markers Were Not Detected in Murine or Human BM or in PB but Were Detected in Tumor Tissue

CE-EPCs derived from both human and murine hematopoietic cells co-expressed in culture both endothelial (ie,

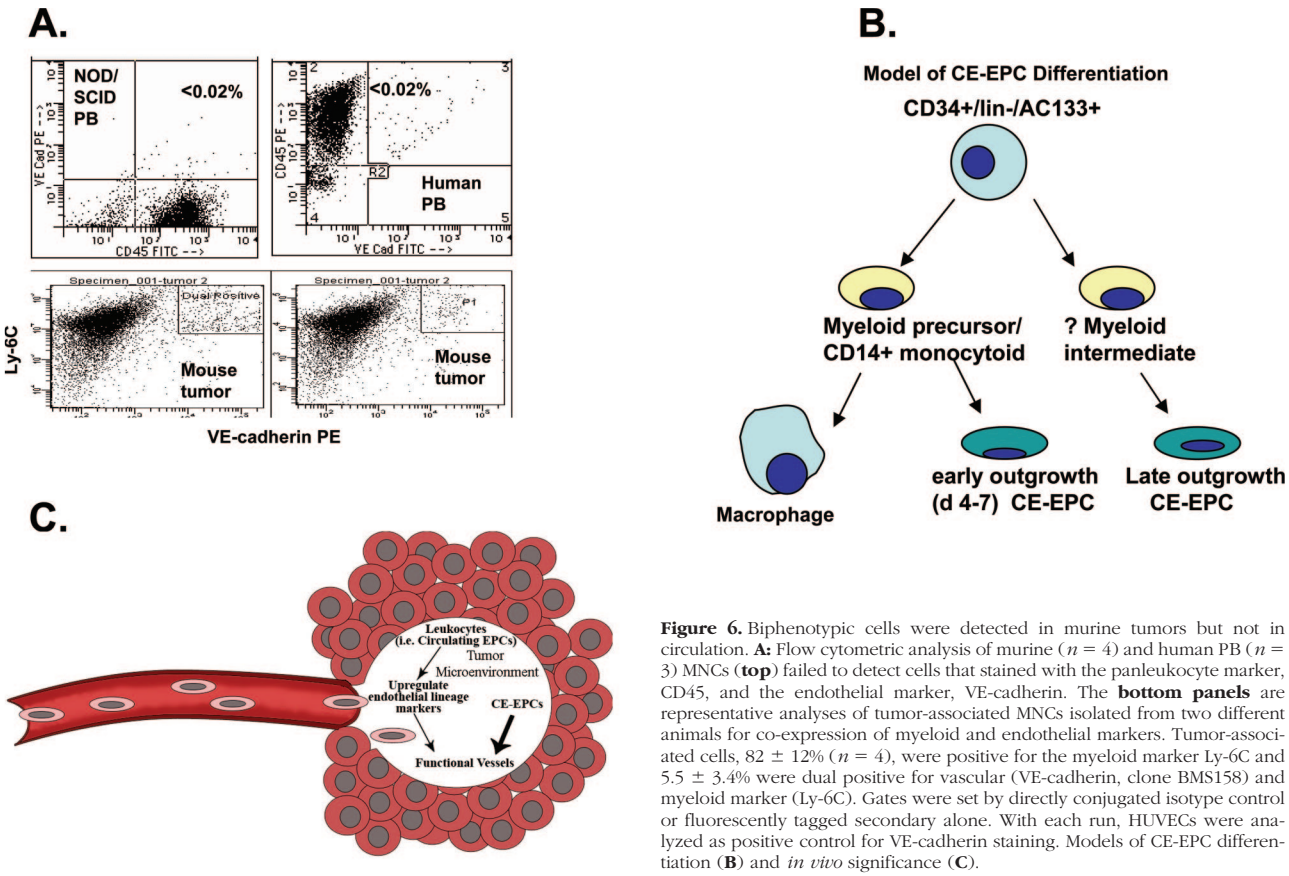


Figure 6. Biphenotypic cells were detected in murine tumors but not in circulation. **A:** Flow cytometric analysis of murine ($n = 4$) and human PB ($n = 3$) MNCs (**top**) failed to detect cells that stained with the panleukocyte marker, CD45, and the endothelial marker, VE-cadherin. The **bottom panels** are representative analyses of tumor-associated MNCs isolated from two different animals for co-expression of myeloid and endothelial markers. Tumor-associated cells, 82 ± 12% ($n = 4$), were positive for the myeloid marker Ly-6C and 5.5 ± 3.4% were dual positive for vascular (VE-cadherin, clone BMS158) and myeloid marker (Ly-6C). Gates were set by directly conjugated isotype control or fluorescently tagged secondary alone. With each run, HUVECs were analyzed as positive control for VE-cadherin staining. Models of CE-EPC differentiation (**B**) and *in vivo* significance (**C**).

VE-cadherin, flk-1, tie-2) and hematopoietic markers (ie, CD45 and CD14). It is not established if biphenotypic cells, resembling the CE-EPCs, circulate in PB or reside in the BM. We analyzed murine (NOD/SCID and C57BL/6) and human PB (Figure 6A) and BM (data not shown) to determine whether cells of mixed phenotype could be detected *in vivo*. We failed to detect any $CD45^+$/VE-$cadherin^+$ cells in either murine or human PB-MNC samples. VE-cadherin is recognized to be a very early marker denoting endothelial lineage in both mice and human vascular development.[37,38] We next examined if biphenotypic cells, specifically those co-expressing myeloid and endothelial markers, could be isolated from sites of tumor vasculogenesis. Using the NOD/SCID tumor model, we analyzed tumors from multiple mice ($n = 4$) that resulted after subcutaneous administration of 1×10^6 β-glucuronidase$^{-/-}$ sarcoma cells. A small population (5.5 ± 3.4%) of the MNCs obtained from the minced tumor suspension demonstrated co-expression of VE-cadherin and the myeloid marker Ly-6C by dual-color flow cytometry (Figure 6A, bottom).

Discussion

Cell therapy with EPCs to promote vasculogenesis became more tenable when it was demonstrated that culture expansion of MNCs derived from diverse sources, ie, BM, cord blood, GCSF-mobilized PB, and more recently, unmobilized PB, by 1 week led to the formation of adherent cells that expressed many endothelial markers.[5,10,39–41] Unlike differentiated endothelial cells, these could home to sites of new blood vessels and contribute to functional vasculature.[10,11,42]

It remains widely accepted that the origin of early outgrowth CE-EPCs generated from unfractionated MNCs is the hematopoietic progenitor cell, regardless of whether they are derived from a stem cell-enriched (ie, BM or cord blood) or -depleted (ie, unmobilized PB) source.[10,18,43–45] Consequently, many studies quantifying circulating EPCs in physiological and pathological states have correlated the numbers of CE-EPCs derived from unmobilized PB to hematopoietic progenitor enumeration.[18,43,44,46] However, it is estimated that ~1.6–3×10^5 CE-EPCs can be obtained from 1 ml of hPB (containing ~4×10^6 MNCs; $CD34^+/lin^-$ content of ~2×10^3 cells).[10,25] This would require a greater than 80- to 150-fold enrichment throughout 7 days in culture, assuming that nearly 100% of $CD34^+$ cells were successfully expanded.[10,25] These observations have suggested that CE-EPCs generated from unfractionated hPB-MNCs were derived from another more abundant source.

Our study showed that the kinetics of differentiation and the EPC yield were similar between human BM- and PB-MNCs, although the BM contains >200-fold higher

numbers of early progenitors. We show that CE-EPCs derived from both sources were biphenotypic and co-expressed hematopoietic (panleukocyte, CD45, and monocyte, CD14) and endothelial markers. We also showed that highly purified populations of $CD14^+$, but not $CD14^-$, cells from human PB generated adherent cells in culture that had the same surface phenotype and within the same time frame as CE-EPCs generated from PB- or BM-MNCs. Furthermore, CD14-derived CE-EPCs also homed to tumor sites and contributed to functional blood vessels *in vivo*. Additionally, murine BM $CD31^+$ cells, a marker on immature myeloid progenitors and circulating monocytes,[34–36,47] selectively enriched for CE-EPC-generating cells, supporting the hypothesis that myeloid cells also generate murine CE-EPCs. We further segregated myeloid subpopulations to demonstrate that endothelial-like cells can be generated from both immature ($CD31^+$/Ly-$6C^{low}$) and more mature ($CD31^+$/$CD11b^+$) myeloid populations. Recently murine $CD31^+$/$CD11c^+$ leukocytes have been demonstrated to play a role in tumor vasculogenesis.[48]

There are three lines of evidence that suggest that mature macrophages do not generate CE-EPCs efficiently. Human CE-EPCs did not express the macrophage marker CD68. *In vitro*-differentiated CD68-positive macrophages failed to form vascular structures or up-regulate VE-cadherin when admixed with tumor cells *in vivo*. Using the flk1/lacZ transgenic model that allowed for genetic marking of endothelial cells, we demonstrated that mature macrophage fractions ($CD31^-$/$CD11b^+$ or $CD31^-$/$F480^+$) isolated from transgenic mice that contained lacZ driven by an endothelial promoter generated lacZ- and BS-1 lectin-positive CE-EPCs with more than fourfold less efficiency than immature monocyte populations.

Although several reports have demonstrated that only the $CD14^+$ population generated endothelial-like cells in culture,[13,15,16,49,50] one report successfully cultivated EPC-like cells from both $CD14^+$ and $CD14^-$ populations.[17] One difference was that the latter study used CE-EPCs after 4 days in culture; however, this cannot explain the discrepant results and requires further study. The media and culture conditions appear to be similar among these studies, including ours. Unlike the previous studies, however, we have also examined the role of myeloid transdifferentiation using murine cells, supporting the experimental data that suggest that CE-EPCs are primarily derived from the circulating myeloid populations.

We confirmed previous reports that culture of highly purified $CD34^+$/lin^- generated CE-EPCs after longer periods of culture (3 to 4 weeks).[9,30,51] Several groups have reported on two distinct CE-EPC populations, involving early (7 to 10 days) and late (3 to 4 weeks) outgrowth.[16,52,53] It was shown that the late outgrowth CE-EPCs from hPB were derived from $CD14^-$ population, whereas the early outgrowth from $CD14^+$.[16] Importantly, human PB CE-EPCs used in *in vivo* assays, for human therapy or to quantify colony-forming units of EPCs, were harvested/quantified after no later than 7 days of culture in all published studies (for compilation of many studies, see reviews[21,31]). During that time frame, our studies suggest that the origin of the CE-EPCs, regardless of the source, is derived primarily from circulating monocytes and, hence, does not likely reflect the role/contribution of circulating $CD34^+$ progenitors in their generation.

Our finding that the $CD34^+$/lin^- progenitor population segregated with the $CD14^-$ population is consistent with these published observations Taken together, this would support the hypothesis that the early outgrowth CE-EPCs are derived from committed myeloid cells, whereas the late outgrowth may be derived from the immature progenitor population that segregated with the $CD14^-$ fraction (Figure 6B). Interestingly, evaluation of cultured $CD34^+$/$AC133^+$/lin^- progenitors generated many adherent cells that transiently expressed CD14 and CD45.[9] This would support a model of differentiation in which the generation of CE-EPCs from immature progenitors occurred via a myeloid intermediate (Figure 6B).

The CE-EPCs demonstrated both leukocyte and endothelial phenotypes. The relationship of CE-EPCs to *in vivo* EPC populations is not well understood. Furthermore, the differentiation cascade that leads to the formation of a functional endothelial cell from circulating leukocytes, either *in vivo* or *in vitro*, is not known. We failed to detect cells that co-expressed hematopoietic and endothelial markers in both murine and human PB or BM but did detect them associated with mouse tumors. This suggests that this biphenotypic cell may not be part of the normal differentiation cascade of the hematopoietic progenitor but may be generated from vascular leukocytes present at the tumor site by specific local cues (Figure 6C). An alternative explanation may be that these biphenotypic cells are generated during toxic or ischemic insult and do not exist in the circulation of healthy animals, and hence, were not detected in our sampling of PB or BM from healthy mice or humans. Vasculogenesis has been well described in ischemia/wounds as well as tumors.[1,3] Various cytokines, such as VEGF and erythropoietin for example, are up-regulated in the circulation after ischemic injury and may serve as *in vivo* signals for generation of biphenotypic cells in the circulation.[6,18] We hope to examine if biphenotypic cells are restricted to sites of vasculogenesis or circulate under certain pathological states using mouse models of ischemic injury and recombinant proteins in future studies.

Our work also suggests that the circulating population(s) of EPCs *in vivo* is distinct from CE-EPCs; currently both populations are designated as "EPCs" in the literature. There is a large discrepancy concerning the physiological relevance of tumor vasculogenesis among different reports, ranging from 0 to >90%.[54,55] These observations may be explained by a model in which contribution of vasculogenesis is determined by the local cytokine and cellular milieu (Figure 6C). Tumor-associated biphenotypic cells expressing myeloid/endothelial markers have recently been described by others in murine and human cancer.[56,57]

Unlike CE-EPCs, $CD14^+$ cells admixed with tumors did not generate functional vessels within that observed period, but a portion was found to up-regulate VE-cadherin, supporting the idea that the neovascular microenviron-

ment may induce the generation of an endothelial phenotype in leukocytes. A longer time course of analysis may be necessary to evaluate their function. Vascular plasticity of myeloid cells has been described during ischemic injury in murine hearts overexpressing the chemokine MCP-1.[58] Studies are underway to delineate factors that promote vasculogenesis. Our finding that CE-EPCs successfully generated functional vessels in a tumor model adds additional support toward using them to promote tissue vasculogenesis.

Acknowledgments

We thank Dr. Jeffrey Davidson for help in establishing the flk/lacZ colony and Dr. Samuel Santoro for critical reading of this manuscript.

References

1. Crosby JR, Kaminski WE, Schatteman G, Martin PJ, Raines EW, Siefert RA, Bowen-Pope DF: Endothelial cells of hematopoietic origin make a significant contribution to adult blood vessel formation. Circ Res 2000, 87:728–735
2. Kaushal S, Amiel GE, Guleserian KJ, Shapira OM, Perry T, Sutherland FW, Rabkin E, Moran AM, Schoen FJ, Atala A, Soker S, Bischoff J, Mayer JEJ: Functional small-diameter neovessels created using endothelial progenitor cells expanded ex vivo. Nat Med 2001, 7:1035–1040
3. Raffi S, Lyden D: Therapeutic stem and progenitor cell transplantation for organ vascularization and regeneration. Nat Med 2003, 9:702–712
4. Young PP, Hofling AA, Sands MS: VEGF increases engraftment of bone marrow-derived endothelial progenitor cells (EPCs) into vasculature of newborn murine recipients. Proc Natl Acad Sci USA 2002, 99:11951–11956
5. Asahara T, Murohara T, Sullivan A, Silver M, Van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM: Isolation of putative progenitor endothelial cells for angiogenesis. Science 1997, 275:964–967
6. Takahashi T, Kalka C, Haruchika M, Chen D, Silver M, Klearny M, Magner M, Isner JM, Asahara T: Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. Nat Med 1999, 5:434–438
7. Peichev M, Naiyer AJ, Pereira D, Zhu Z, Lane WJ, Williams M, Oz MC, Hicklin DJ, Witte L, Moore MAS, Rafii S: Expression of VEGFR-2 and AC133 by circulating human CD34+ cells identifies a population of functional endothelial precursors. Blood 2000, 95:952–958
8. Gehling UM, Ergun S, Schumacher U, Wagener C, Pantel K, Otte M, Schuch G, Schafhausen P, Mende T, Kilic N, Kluge K, Schafer B, Hossfeld DK, Fiedler W: In vitro differentiation of endothelial cells from AC133-positive progenitor cells. Blood 2000, 95:3106–3112
9. Quirici N, Soligo D, Servida F, Bossolasco P, Deliliers GL: Endothelial cells derived from human bone marrow CD133+ cells. Br J Hematol 2001, 115:186–194
10. Kalka C, Masuda H, Tomono T, Kalka-Moll WM, Silver M, Kearney M, Li T, Isner JM, Asahara T: Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization. Proc Natl Acad Sci USA 1999, 97:3422–3427
11. Schatteman G, Hanlon HD, Jiao C, Dodds SG, Christy BA: Blood-derived angioblasts accelerate blood-flow restoration in diabetic mice. J Clin Invest 2000, 106:571–578
12. Zhao Y, Glesne D, Humerman E: A human peripheral blood monocyte-derived subset acts as pluripotent stem cells. Proc Natl Acad Sci USA 2003, 100:2426–2431
13. Pujol BF, Lucibello FC, Gehling UM, Lindemann K, Weidner N, Zuzarte M, Adamkiewicz J, Elsasser H, Muller R, Havemann K: Endothelial-like cells derived from human CD14 positive monocytes. Differentiation 2000, 65:287–300
14. Schmeisser A, Garlichs CD, Zhang H, Eskafi S, Graffy C, Ludwig J, Strasser RH, Daniel WG: Monocytes coexpress endothelial and macrophagocytic lineage markers and form cord-like structures in Matrigel under angiogenic conditions. Cardiovasc Res 2001, 49:671–680
15. Rehman J, Jingling L, Orschell CM, March KL: Peripheral blood "endothelial progenitor cells" are derived from monocyte/macrophages and secrete angiogenic growth factors. Circulation 2003, 107:1164–1169
16. Gulati R, Jevremovic D, Peterson TE, Chatterjee S, Shah V, Vile RG, Simari RD: Diverse origin and function of cells with endothelial phenotype obtained from adult human blood. Circ Res 2003, 93:1023–1025
17. Urbich C, Heeschen C, Aicher A, Dernbach E, Zeiher AM, Dimmeler S: Relevance of monocytic features for neovascularization capacity of circulating endothelial progenitor cells. Circulation 2003, 108: 2511–2516
18. Bahlmann FH, Groot K, Spandau J, Landry AL, Hertel B, Duckert T, Boehm SM, Menne J, Haller H, Fliser D: Erythropoietin regulates endothelial progenitor cells. Blood 2004, 103:921–926
19. Assmus B, Schachinger V, Teupe C, Britten M, Lehmann R, Dobert N, Grunwald F, Aicher A, Urbich C, Martin H, Hoelzer D, Dimmeler S, Zeiher AM: Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction (TOPCARE-AMI). Circulation 2002, 106:3009–3017
20. Moore XL, Lu J, Sun L, Zhu CJ, Tan I, Wong MC: Endothelial progenitor cells' "homing" specificity to brain tumors. Gene Ther 2004, 11:811–818
21. Hristov M, Weber C: Endothelial progenitor cells: characterization, pathophysiology, and possible clinical relevance. J Cell Mol Med 2004, 8:498–508
22. Hofling AA, Vogler C, Creer MH, Sands MS: Engraftment of human CD 34+ cells leads to widespread distribution of donor derived cells and correction of tissue pathology in a novel murine xenotransplantation model of lysosomal storage disease. Blood 2002, 101:2054–2063
23. Freeman BJ, Roberts MS, Vogler C, Nicholes A, Hofling AA, Sands MS: Behavior and therapeutic efficacy of beta-glucuronidase-positive mononuclear phagocytes in a murine model of mucopolysaccharidosis type VII. Blood 1999, 94:2142–2150
24. Shalaby F, Rossant J, Yamaguchi TP, Gertenstein M, Wu XF, Breitman M, Schuh AC: Failure of blood island formation and vasculogenesis in Flk-1 deficient mice. Nature 1995, 376:62–66
25. Teleron AA, Carlson B, Young PP: Blood donor WBC reduction filters as a source of human peripheral blood-derived endothelial progenitor cells (EPCs). Transfusion 2004, 45:21–25
26. Young PP, Fantz CR, Sands MS: VEGF disrupts the neonatal blood-brain barrier and increases life span after non-ablative BMT in a murine model of congenital neurodegeneration caused by a lysosomal enzyme deficiency. Exp Neurol 2004, 188:104–114
27. Hofling AA, Devine S, Vogler C, Sands MS: Human CD34+ hematopoietic progenitor cell-directed lentiviral-mediated gene therapy in a xenotransplantation model of lysosomal storage disease. Mol Ther 2004, 9:856–865
28. Young PP, Vogler C, Hofling AA, Sands MS: Biodistribution and efficacy of donor lymphocytes in a murine model of lysosomal storage disease. Mol Ther 2003, 7:52–61
29. Rafii S: Circulating endothelial precursors: mystery, reality and promise. J Clin Invest 2000, 105:17–19
30. Hildbrand P, Cirulli V, Prinsen RC, Smith KA, Torbett BE, Salomon DR, Crisa L: The role of angiopoietins in the development of endothelial cells from cord blood CD34+ progenitors. Blood 2004, 104:2010–2019
31. Urbich C, Dimmeler S: Endothelial progenitor cells characterization and role in vascular biology. Circ Res 2004, 95:343–353
32. Micklem K, Rigney E, Cordell J, Simmons D, Stross P, Turley H, Seed B, Mason D: A human macrophage-associated antigen (CD68) detected by six different monoclonal antibodies. Br J Hematol 1989, 73:6–11
33. Wang L, Li L, Shojaei F, Levac K, Cerdan C, Menendez P, Martin T, Rouleau A, Bhatia M: Endothelial and hematopoietic cell fate of human embryonic stem cells originates from primitive endothelium with hemangioblastic properties. Immunity 2004, 21:31–41
34. Kennedy DW, Abkowitz JL: Mature monocytic cells enter tissues and engraft. Proc Natl Acad Sci USA 1998, 95:14944–14949
35. de Braijn MF, Ploemacher RE, Mayen AE, Voerman JS, Slieker WA,

van Ewijk W, Leenen PJ: High level expression of the ERMP58 antigen on mouse bone marrow hematopoietic progenitor cells marks commitment to the myeloid lineage. Eur J Immunol 1996, 26:2850–2858
36. Nikolic T, de Braijn MF, Lutz MB, Leenen PJ: Developmental stages of myeloid dendritic cells in the mouse bone marrow. Int Immunol 2003, 15:515–524
37. Breier G, Breviario F, Caveda L, Berthier R, Schnurch H, Gotsch U, Vestweber D, Risau W, Dejana E: Molecular cloning and expression of murine vascular endothelial cadherin in early stage development of cardiovascular system. Blood 1996, 87:630–641
38. Lampugnani MG, Resnati M, Raiteri M, Pigott R, Piscane A, Houen G, Ruco LP, Dejana E: A novel endothelial specific membrane protein is a marker of cell-cell contacts. J Cell Biol 1992, 118:1511–1522
39. Hatzopoulos AK: Isolation and characterization of endothelial progenitor cells from mouse embryos. Development 1998, 125:1457–1468
40. Asahara T, Masuda H, Takahashi T, Kalka C, Pastore C, Silver M, Kearne M, Magner M, Isner JM: Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. Circ Res 1999, 85:221–228
41. Ricousse-Roussanne S, Barateau V, Contreres J, Boval B, Kraus-Berthier L, Tobelem G: Ex vivo differentiated endothelial and smooth muscle cells from human cord blood progenitors home to the angiogenic tumor vasculature. Cardiovasc Res 2004, 62:176–184
42. Urbich C, Heeschen C, Aicher A, Sasaki K, Bruhl T, Farhadi MR, Vajkoczy P, Hofmann WK, Peters C, Pennacchio LA, Abolmaali ND, Chavakis E, Reinheckel T, Zeiher AM, Dimmeler S: Cathepsin L is required for endothelial progenitor cell-induced neovascularization. Nat Med 2005, 11:206–213
43. Vasa M, Fichtlscherer S, Aicher A, Adler K, Urbich C, Martin H, Zeiher AM, Dimmeler S: Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. Circ Res 2001, 89:e1–e7
44. Valgimigli M, Rigolin GM, Fucili A, Della Porta M, Soukhomovskaia O, Malagutti P, Bugli AM, Bragotti LZ, Francolini G, Mauro E, Castoldi G, Ferrari R: CD34+ and endothelial progenitor cells in patients with various degrees of congestive heart failure. Circulation 2004, 110:1209–1212
45. Hill JM, Zalos G, Halcox JPJ, Schenke WH, Waclawiw MA, Quyyumi AA, Finkel T: Circulating endothelial progenitor cells vascular function, and cardiovascular risk. N Engl J Med 2003, 248:593–600
46. Werner N, Kosiol S, Schiegl T, Ahlers P, Walenta K, Link A, Bohm M, Nickenig G: Circulating endothelial progenitor cells and cardiovascular outcomes. N Engl J Med 2005, 353:999–1007
47. de Braijn MF, Slieker WA, van der Loo JC, Voerman JS, van Ewijk W, Leenen PJ: Distinct mouse bone marrow macrophage precursors identified by differential expression of ER-MP12 and ER-MP20 antigens. Eur J Immunol 1994, 24:2279–2284
48. Conejo-Garcia JR, Benencia F, Courreges M, Kang E, Mohamed-Hadley A, Buckanovich RJ, Holtz DO, Jenkins A, Na H, Zhang L, Wagner DS, Katsaros D, Caroll R, Coukos G: Tumor-infiltrating dendritic cell precursors recruited by a b-defensin contribute to vasculogenesis under the influence of VEGF-A. Nat Med 2004, 10:950–958
49. Romagnani P, Annunziato F, Liotta F, Lazzeri E, Manzzinghi B, Frosali F, Cosmi L, Maggi L, Lasagni L, Scheffold A, Kruger M, Dimmeler S, Marra F, Gensini G, Maggi E, Romagnani S: CD14+CD34low cells with stem cell phenotype and functional features are the major source of circulating endothelial progenitors. Circ Res 2005, 97:314–322
50. Elsheikh E, Uzunel M, He Z, Holgersson J, Nowak G, Sumitran-Holgersson S: Only a specific subset of human peripheral-blood monocytes has endothelial-like functional capacity. Blood 2005, 106:2347–2355
51. Shi Q, Rafii S, Wu M, Wijelath ES, Yu C, Ishida A, Fujita Y, Kothari S, Mohle R, Sauvage LR, Moore MAS, Storb RF, Hammond WP: Evidence for circulating bone marrow-derived endothelial cells. Blood 1998, 92:362–367
52. Hur J, Yoon CH, Kim HS, Choi JH, Kang HJ, Hwang KK, Oh BH, Lee MM, Park YB: Characterization of two types of endothelial progenitor cells and their different contributions to neovasculogenesis. Hypertension 2004, 24:1–6
53. Lin Y, Weisdorf DJ, Solovey A, Hebbel RP: Origins of circulating endothelial cells and endothelial outgrowth from blood. J Clin Invest 2000, 105:71–77
54. Lyden D, Hattori K, Dias S, Costa C, Blaikie P, Butros L, Chadburn A, Heissig B, Marks W, Witte L, Wu Y, Hicklin DJ, Zhu Z, Hackett NR, Crystal RG, Moore MAS, Hajjar KA, Manova K, Benezra R, Rafii S: Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. Nat Med 2001, 7:1194–1201
55. Shinde Patil VR, Friedrich EB, Wolley AE, Gerszten RE, Allport JR, Weissleder R: Bone marrow-derived lin(−)c-kit(+)sca-1+ stem cells do not contribute to vasculogenesis in Lewis lung carcinoma. Neoplasia 2005, 7:234–240
56. Conejo-Garcia JR, Buckanovich RJ, Benencia F, Courreges M, Rubin SC, Carroll RG, Coukos G: Vascular leukocytes contribute to tumor vascularization. Blood 2005, 105:679–681
57. Yang L, DeBusk LM, Fukuda K, Fingleton B, Green-Jarvis B, Shyr Y, Matrisian LM, Carbone DP, Lin PC: Expansion of myeloid immune suppressor Gr+CD11b+ cells in tumor bearing host directly promotes tumor angiogenesis. Cancer Cell 2004, 6:409–421
58. Moldovan NI, Goldschmidt-Clermont PJ, Parker-Thornburg J, Shapiro SD, Kolattukudy PE: Contribution of monocytes/macrophages to compensatory neovascularization: the drilling of metalloelastase-positive tunnels in ischemic myocardium. Circ Res 2000, 87:341–343