Minimal Contribution of Marrow-Derived Endothelial Precursors to Tumor Vasculature[1]

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During embryogenesis, vascular and hemopoietic cells originate from a common precursor, the hemangioblast. Recent evidence suggests the existence of endothelial precursors in adult bone marrow cells, but it is unclear whether those precursors have a role in tumor neovascularization. In this report, we demonstrate that murine bone marrow contains endothelial progenitors, which arise from a cell with self-renewing capacity, and can integrate into tumor microvasculature, albeit at a very low frequency. A transgenic double-reporter strategy allowed us to demonstrate definitively that tumor bone marrow-derived endothelial cells arise by transdifferentiation of marrow progenitors rather than by cell fusion. Single cell transplants showed that a common precursor contributes to both the hemopoietic and endothelial lineages, thus demonstrating the presence of an adult hemangioblast. Furthermore, we demonstrate that increased vascular endothelial growth factor (VEGF)-A secretion by tumor cells, as well as activation of VEGF receptor-2 in bone marrow cells does not alter the mobilization and incorporation of marrow-derived endothelial progenitors into tumor vasculature. Finally, in human umbilical cord blood cells, we show that endothelial precursors make up only ~1 in 10^7 mononuclear cells but are highly enriched in the $CD133^+$ cell population. By ruling out cell fusion, we clearly demonstrate the existence of an adult hemangioblast, but the differentiation of marrow stem cells toward the endothelial lineage is an extremely rare event. Furthermore, we show that VEGF-A stimulation of hemopoietic cells does not significantly alter this process. *The Journal of Immunology,* **2005, 175: 2890–2899.**

Tumors require a vascular supply to grow and metastasize. Until recently, tumor vasculature was thought to arise solely through angiogenesis, the mechanism by which new blood vessels arise from preexisting vessels through endothelial cell migration and proliferation (1). However, recent studies have provided evidence that tumor vasculature can also arise though vasculogenesis, a process by which endothelial progenitors are recruited and differentiate in situ into mature endothelial cells to form new blood vessels (2). Evidence for the existence of such endothelial progenitors has come from studies demonstrating the ability of bone marrow-derived cells to incorporate into tumor vasculature (3). However, the exact nature of such endothelial progenitors remains unclear. A population of endothelial precursors, which express markers such as CD34, CD133, and vascular endothelial (VE)[3] growth factor (VEGF) receptor-2 (VEGFR-2), have been shown to exist among human peripheral blood, bone marrow, and cord blood cells (4, 5). When cultured in the presence of angiogenic factors, these endothelial progenitors become adherent and proliferate to form colonies of mature endothelial cells, which express markers such as von Willebrand factor (vWF), VE-cadherin, and CD31 (PECAM-1) (4, 6). Additional studies have also reported that the $CD34^-$ monocyte/macrophage-containing mononuclear cell population can differentiate into endothelial-like cells in vitro (7).

Because of the lack of a definitive marker for endothelial progenitors, the in vivo contribution of endothelial progenitors to tumor neovascularization remains unclear. Studies have reported that, for some tumor types, ~90% of blood vessels are composed of bone marrow-derived endothelial cells (3). However, other groups have reported that they were unable to observe any contribution of endothelial progenitors to the formation of tumor blood vessels (8). This lack of consensus may be explained in part by different experimental settings, such as type of cells used to transplant animals (whole bone marrow vs purified primitive stem cells), tumor type, time frame of the experiment, method of endothelial cell identification, and propensity of different models to induce cell fusion.

In this study, we show that, in addition to contributing to long-term multilineage hematopoiesis, primitive hemopoietic stem cells can generate endothelial progenitors that integrate into the tumor microvasculature. Bone marrow-derived endothelial cells present in tumor blood vessels arise by differentiation from a precursor and not by cell fusion. VEGF-A does not increase the frequency of bone marrow-derived endothelial cells incorporating into tumor

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[3] Abbreviations used in this paper: VE, vascular endothelial; VEGF, VE growth factor; CBEC, cord blood endothelial cell; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; bFGF, basic fibroblast growth factor; eNOS, endothelial specific NO synthase; hPLAP, human placental alkaline phosphatase; HUVEC, human umbilical vein endothelial cell; Lin^-, lineage depleted; MIG, MSCV-IRES-GFP; vWF, von Willebrand factor; FKBP, FK506-binding protein; NLS, nuclear localization signal.

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blood vessels. The rarity of a common precursor from murine marrow differentiating into an endothelial cell is mimicked in human cord blood, where we estimate that only 1 in 10^7 mononuclear cells differentiates into a mature endothelial cell.

Materials and Methods

Cell culture

NIH 3T3 and GP+E86 (9) cells were cultured in DMEM supplemented with 10% FBS, 2 mM glutamine, and 100 U/ml each of penicillin and streptomycin. B6RV2 lymphoma cells were cultured in RPMI 1640 medium supplemented with 10% FBS and 100 U/ml penicillin and streptomycin. Lewis lung carcinoma (LLC) cells were cultured in DMEM supplemented with 1.5 g/L sodium bicarbonate, 10% FBS, 2 mM glutamine, and 100 U/ml each of penicillin and streptomycin.

Retroviral transduction

The MSCV-IRES-GFP (MIG) and MIG-FK506-binding protein (FKBP)/VEGFR-2 constructs used in this study have been previously described (10). Retroviral transduction of bone marrow cells from C3Pep mice (cross between C3H/HeJ and Pep3b) was performed as previously described (10).

Animals and bone marrow transplants

C3Pep and B6C3 (cross between C3H/HeJ and C57BL/6J) mice were maintained by the Joint Animal Facility of the British Columbia Cancer Research Centre in accordance with regulations approved by the University of British Columbia animal care committee. Flow sorted cells (1×10^6 GFP^+ cells/animal) were injected in the tail vein of lethally irradiated (900 cGy, using a ^{137}Cs source) B6C3 mice. GFP transgenic mice (11) were also used as donors of bone marrow cells for bone marrow transplantation experiments.

To determine the effects of VEGFR-2 activation in bone marrow cells in vivo, B6C3 mice were also transplanted with bone marrow retrovirally transduced with either MIG-FKBP/VEGFR-2 (10) or MIG. Following bone marrow reconstitution, animals were injected i.p. with 10 mg/kg AP-20187 (Ariad Pharmaceuticals) or vehicle (4% ethanol, 10% polyethylene glycol-400, and 1.7% Tween 20 in water) daily for 10 days following tumor implantation.

To determine whether cell fusion contributes to the presence of bone marrow-derived cells in tumor blood vessels, we transplanted Cre deletor mice (Cre recombinase expressed under a ubiquitous promoter) (pCX-nuclear localization signal (NLS)-Cre) (12) with bone marrow harvested from mice transgenic for the *Z/AP* expression vector (13). Single cell transplants were performed as previously described (14). Briefly, bone marrow was harvested from GFP^+CD45.1 C57BL/6 transgenic mice. A c-$Kit^+Lin^-Sca^+$ subset (where Lin^- designates lineage depleted) of the side population was isolated by flow sorting. Individual cells were sorted into 96-well plates. Cells from wells containing one cell were coinjected with stem cell-depleted bone marrow from GFP^- congenic mice into CD45.2 C57BL/6 mice. All transplants were allowed to equilibrate for 4–12 wk. Mice were housed in microisolator units and provided with acidified water (pH 3.0) and 100 mg/l ciprofloxacin (Bayer). GFP engraftment in the peripheral blood was determined by flow cytometry.

Tumor implantation

Tumor cell lines (B6RV2, B6RV2 transduced with VEGF-A_{165} (B6RV2-VEGF), or LLC; 5×10^6 cells/animal) were injected subcutaneously into the dorsa of mice anesthetized with isoflurane. Tumor size was determined by caliper measurements and tumor volume was calculated by a rational ellipse formula ($m_1 \times m_1 \times m_2 \times 0.5236$, where m_1 is the shorter axis and m_2 is the longer axis). Animals were sacrificed 10 days after tumor implantation.

Flow cytometry

Cells were stained as previously described (15). For murine peripheral blood and bone marrow, primary Abs used for analysis were anti-VE-cadherin and anti-VEGFR-2 (BD Pharmingen). Isotype rat IgG (BD Pharmingen) was used as a negative control. Anti-rat IgG-PE (BD Pharmingen) was used as a secondary Ab. For labeling of umbilical cord blood endothelial cells (CBEC), primary anti-VEGFR-2 (Sigma-Aldrich) and secondary goat anti-mouse IgG-FITC (Sigma-Aldrich) Abs were used. Samples were run on an EPICS ELITE-ESP flow cytometer (BD Biosciences), and data were analyzed with FCS Express, version 2 (De Novo software).

Immunofluorescent microscopy

Tumor tissues harvested from transplanted mice were fixed overnight at −20°C in 2% paraformaldheyde-30% glycerol. After washing with PBS, tumors were embedded in OCT compound and sectioned with a cryostat. Sections (8 μm) were stained with Abs against VE-cadherin, CD31, and CD11b (BD Pharmingen). Alexa 594- or Alexa 633-conjugated secondary Abs were used (Molecular Probes). Slides were visualized through a ×40 or ×100 Neofluor objective (numerical aperture 0.75) using a Zeiss Axioplan II Imaging inverted microscope, and images were captured with a 1350EX cooled charge-coupled device digital camera (QImaging) using Northern Eclipse software (Empix Imaging). Alternatively, slides were analyzed on a Bio-Rad Radiance confocal microscope (Bio-Rad Radiance 2000 on a Nikon Eclipse TE300 with a MaiTai Sapphire Laser) using Lasersharp 2000 software for analysis. For the quantitation of bone marrow-derived VE-$cadherin^+$ and $CD31^+$ cells incorporating into tumor blood vessels, between 300 and 500 microvessels per tumor section were counted. Three tumor sections were counted per animal.

lacZ *and human placental alkaline phosphatase (hPLAP) staining*

Tumor tissues were fixed in 4% paraformaldehyde for 4 h and cryopreserved by incubating overnight in 15% sucrose in PBS at 4°C. The samples were washed in PBS before being embedded in OCT compound. Sections were fixed in 0.2% glutaraldehyde for 10 min, washed in 100 mM sodium phosphate (pH 7.3) and washing buffer solution (2 mM $MgCl_2$, 0.01% sodium deoxycholate, and 0.02% Nonidet P-40 in 100 mM sodium phosphate, pH 7.3), and stained in fresh X-gal solution (0.5 mg/ml X-gal, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide in washing buffer solution) at 37°C overnight. Following *lacZ* staining, slides were washed three times in PBS for 5 min, and endogenous alkaline phosphatase was inactivated by incubating slides in PBS for 30 min. Slides were rinsed in PBS, washed in alkaline phosphatase buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 10 mM $MgCl_2$), and incubated in a solution containing NBT chloride and 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) (Sigma-Aldrich) for 15–40 min at room temperature in the dark to identify hPLAP. Slides were washed in PBS and either stained for CD31 or dehydrated and mounted in Permount (Fisher Scientific) with coverslips.

ELISA

Detection of VEGF-A in murine serum was assayed by quantitative VEGF ELISA according to the instructions of the manufacturer (R&D Systems).

Endothelial progenitor assays

Endothelial progenitor assays were performed as previously described (4, 5). Mononuclear cells were obtained from human umbilical cord blood. Briefly, cord blood samples from standard or cesarean delivery of full-term infants were harvested into 200-ml plastic bottles containing 40 ml of IMDM that contained 800 U/ml heparin. Ammonium chloride lysis was performed to remove RBC. Mononuclear cells were either selected for the surface marker CD133 using anti-CD133-coupled magnetic microbeads (Miltenyi Biotec) as recommended by the manufacturer or Lin^- using a panel of lineage-specific Abs (CD2, CD3, CD14, CD16, CD19, CD24, CD56, CD66b, glycophorin A; StemCell Technologies) and separated using a magnetic separation device, StemSep (StemCell Technologies). For endothelial progenitor assays, cells were plated onto tissue culture dishes in MCDB 131 medium supplemented with 20% FBS, endothelial cell growth supplement (Sigma-Aldrich), heparin, 5 ng/ml basic fibroblast growth factor (bFGF), and 30 ng/ml VEGF-A. For 3 consecutive days, nonadherent cells were replated into a new tissue culture dish to remove contaminating adherent cells. After 3 days, nonadherent cells were harvested, counted, and resuspended in fresh medium and plated onto tissue culture dishes coated with 0.2% gelatin. Half of the medium was replaced twice weekly. Endothelial colonies were scored after 4 wk, as identified by staining with the P1H12 (CD146) Ab (Chemicon International).

Immunoblotting

Equal amounts of protein (40 μg/lane) from total cellular extracts were separated by SDS-PAGE and assessed by immunoblotting as previously described (10). Anti-endothelial specific NO synthase (eNOS) was from BD Transduction Laboratories, and anti-Tie-1 and anti-Tie-2 were from Santa Cruz Biotechnology.

Endothelial sprouting assay

Endothelial sprouting was assessed by a modification of the method of Nehls and Drenckhahn (16). Briefly, microcarrier beads coated with gelatin

(Cytodex 3; Sigma-Aldrich) were seeded with CBEC or HUVECs. When the cells reached confluence on the beads, equal numbers of beads were embedded in fibrin gels in 96-well plates. For preparation of fibrin gels, bovine fibrinogen was dissolved in MCDB 131 supplemented with 2% FBS at a concentration of 2.5 mg/ml. Aprotinin was added at a concentration of 0.05 mg/ml, and the solution was filtered through a 0.22-μm pore size filter. Fibrinogen solution was supplemented with FGF-2 (15 ng/ml). As a control, fibrinogen solution without angiogenic factor was used. Following transfer of the fibrinogen solution to 96-well plates, cell-coated beads were added at a density of 50 beads/well, and clotting was induced by the addition of thrombin (1.2 U/ml). After clotting was complete, gels were equilibrated with MCDB 131–2% FBS at 37°C. Following 60 min of incubation, the overlying medium was changed for all wells. MCDB 131–2% FBS, either alone or containing FGF-2 (15 ng/ml), was added to the wells. After 3 days of incubation with daily medium changes, the number of capillary-like tubes formed was quantitated by counting the number of tube-like structures per microcarrier bead (sprouts per bead). Only sprouts greater than 150 μm in length and composed of at least three endothelial cells were counted.

Statistical analysis

Results were analyzed by ANOVA to ascertain differences between groups, followed by a Tukey test to correct for multiple comparisons.

Results

Determination of the existence of bone marrow-derived endothelial cells

To quantify endothelial progenitor activity in vivo, chimeric mice reconstituted with GFP^+ bone marrow were generated. Typically, mice used in these experiments had between 25% and 65% GFP^+ cells in the peripheral blood and bone marrow. Flow cytometry showed reconstitution of lymphoid and myeloid lineages in transplanted mice 6 wk posttransplant (Fig. 1*A*). VEGFR-2, which is a marker of primitive circulating endothelial progenitors, was also detected in a small fraction of bone marrow and peripheral blood GFP^+ cells (3). To identify marrow-derived endothelial cells in tumor microvasculature, GFP-transplanted mice were implanted s.c. with B6RV2 tumor cells. Ten days postimplantation, mice were sacrificed, and blood vessels were analyzed in tumor tissue sections by fluorescence microscopy. Most studies that examined the contribution of bone marrow-derived cells have used either CD31 or vWF. However, although both are strongly expressed in endothelial cells, they are also expressed on subsets of hemopoietic cells such as monocytes/macrophages (CD31), granulocytes (CD31), and platelets (vWF, CD31) (17, 18). Because leukocytes and platelets can be found in close association with the vascular wall, it may be difficult to differentiate vWF^+ or $CD31^+$ bone marrow-derived leukocytes from tumor endothelial cells, thereby leading to incorrect identification of marrow-derived endothelial cells. To avoid misidentification of endothelial cells, we used Abs against CD31 as well as VE-cadherin, which is expressed specifically on vascular endothelial cells (19). To account for different levels of GFP^+ cell engraftment in transplanted animals, results were normalized for the proportion of GFP^+ cells in the peripheral blood of the mice at the time of sacrifice.

Immunofluorescence staining and confocal microscopy of B6RV2 tumors grown in B6C3 mice showed extensive infiltration of GFP^+ cells throughout the tumor. GFP^+ bone marrow-derived cells were detected in tumor blood vessels at a very low frequency using either epifluorescence or confocal microscopy. Fig. 1*B* displays representative images of $CD31^+$ and VE-$cadherin^+$ blood vessels containing at least one bone marrow-derived cell. Because macrophages may be closely apposed to the endothelium and could therefore be misinterpreted as endothelial cells, sections were also costained with VE-cadherin and the monocyte/macrophage marker CD11b to confirm that the GFP^+ cells were not hemopoietic (Fig. 1). We did observe large numbers of $CD11b^+$VE-$cadherin^-GFP^+$ cells in tumors, which represent a population of macrophages. However, double staining for VE-cadherin and CD11b was not observed in the GFP^+ marrow-derived cells in the vessel walls, ruling out the possibility of macrophages being misinterpreted as endothelial cells and confirming the presence of rare marrow-derived endothelial cells in B6RV2 tumors. These marrow-derived cells arise from a cell that has the ability to self-renew, as we also detected $CD31^+$ and VE-$cadherin^+$ bone marrow-derived cells in the tumor vasculature of serially transplanted animals (data not shown).

We then quantified the relative contribution of marrow cells to the formation of microvessels in B6RV2 tumors. As a source of GFP^+ bone marrow cells in transplanted animals, we used either cells harvested from GFP^+ transgenic mice (11) that were immediately injected into lethally irradiated recipient mice (Fig. 1*D*) or bone marrow cells harvested from mice that were expanded ex vivo (2 days) before transduction with a retroviral vector encoding GFP (MIG) (Fig. 1*E*). This approach allowed us to determine whether ex vivo culture of bone marrow cells had deleterious effects on the endothelial progenitor pool present in the marrow. In both approaches, $<1\%$ of B6RV2 blood vessels were found to have incorporated GFP^+ marrow-derived endothelial cells. Ex vivo transduction of marrow cells before transplantation did not result in significant differences in the proportion of marrow-derived endothelial cells in the tumor microvasculature compared with mice transplanted with unmanipulated marrow, indicating that in vitro culture of marrow cells does not result in significant loss of endothelial progenitor activity within the time frame of this experiment.

Determination of the existence of an adult hemangioblast

Recent evidence demonstrating the ability of marrow cells to fuse with parenchymal cells of the liver and other organs has cast doubt on the contribution of the bone marrow to the regeneration of various tissues (20, 21). Given that marrow-derived endothelial cells integrating into angiogenic microvasculature appears to be a rare event, we attempted to determine whether the rare GFP^+ endothelial cell was a result of cell fusion. Others have used DNA content analysis to determine the presence or absence of cell fusion, but this analysis can be misleading as fused cells have been shown to lose cellular DNA, which could potentially lead to inaccurate interpretation of results (22–25). We thus used a transgenic double-reporter strategy to determine whether bone marrow-derived endothelial cells observed in B6RV2 tumors arise by differentiation or fusion. Marrow cells were harvested from *Z/AP* double reporter transgenic mice, which express a transgene consisting of the *lacZ* gene flanked by two *loxP* sites, and followed by the hPLAP reporter (13). Thus bone marrow cells from these animals express the *lacZ* reporter gene. With Cre-mediated excision, however, removal of the *lacZ* gene permits expression of the second reporter, hPLAP. Mice that constitutively express Cre recombinase (pCX-NLS-Cre) were transplanted with marrow harvested from *Z/AP* mice (12). If putative marrow-derived endothelial cells result from differentiation of marrow stem cells, they will express the *lacZ* gene and stain blue in the presence of X-gal reagent. However, if marrow cells fuse with endothelial cells from the recipient mice (pCX-NLS-Cre), Cre recombinase will excise the *lacZ* gene, permitting expression of the hPLAP reporter. Fused cells will therefore appear purple when stained for alkaline phosphatase with the NBT/BCIP reagent. B6RV2 tumors harvested from pCX-NLS-Cre mice transplanted with bone marrow harvested from Z/AP mice were cryosectioned and stained with X-gal (*lacZ*) and NBT/BCIP (hPLAP), followed by CD31 and diaminobenzidine staining (Fig. 2). Because diaminobenzidine produces a

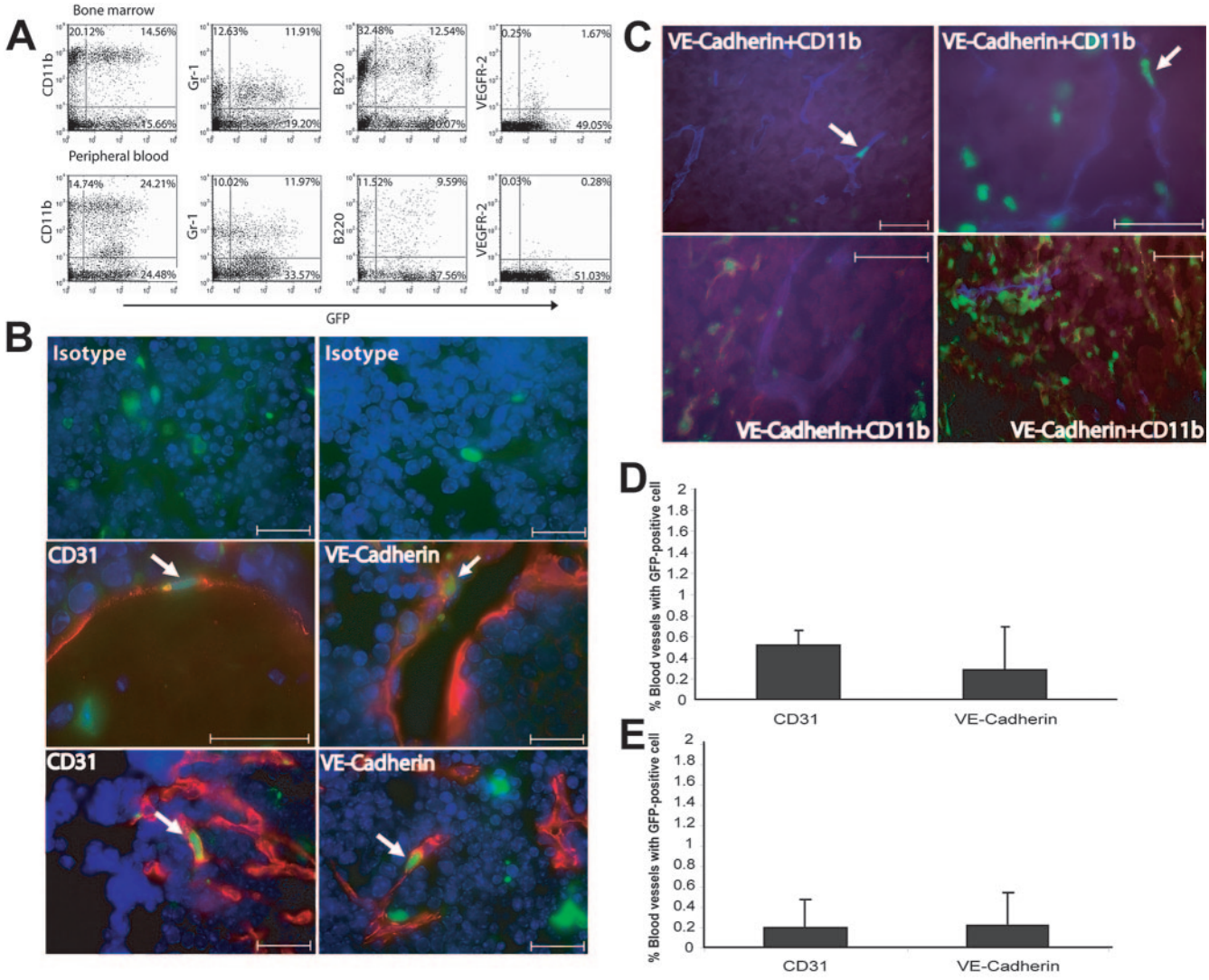

FIGURE 1. Bone marrow-derived endothelial cells incorporate into tumor vasculature at low frequency. *A*, Flow cytometry showing reconstitution of the myeloid (CD11b, Gr-1), lymphoid (B220), and endothelial progenitor (VEGFR-2) populations in MIG-transplanted mice 6 wk posttransplant. *B*, Four to 6 wk following marrow transplantation with GFP^+ mononuclear cells, mice were implanted s.c. with B6RV2 tumor cells. Ten days following implantation, tumors were sectioned and immunostained with either anti-CD31 or anti-VE-cadherin Abs (red) and examined for the presence of marrow-derived cells (GFP^+, green) coexpressed with endothelial markers. 4′,6′-Diamidino-2-phenylindole was used to counterstain nuclei (blue). Isotype Ig-stained sections were used as control. Original magnification, ×1000. Scale bars represent 25 μm. *C*, To confirm the phenotype of bone marrow-derived endothelial cells, B6RV2 tumor sections were immunostained with anti-CD11b Ab labeled with PE (red) and anti-VE-cadherin-Alexa 350 Ab (blue). Original magnification, ×400, except *upper right*, ×1000. Scale bars represent 50 μm. *D* and *E*, Proportion of blood vessels that incorporated GFP^+ bone marrow-derived endothelial cells in B6RV2 tumors from mice previously transplanted with GFP^+ bone marrow from GFP transgenic donors (*D*) or with bone marrow transduced ex vivo with a GFP-encoding vector (MIG) (*E*). Data represent means ± SEM of GFP^+ cells per vessel. At least three sections per tumor and four tumors per group were analyzed.

brown color that could potentially mask the purple color generated by NBT/BCIP, tumor sections were carefully examined after NBT/BCIP staining and before CD31 staining. In tumor sections obtained from five different animals, we were able to observe a rare proportion of blood vessels containing $lacZ^+$ cells (Fig. 2*A*), indicating that marrow-derived endothelial precursors in the tumor microvasculature arise by differentiation. In contrast, $hPLAP^+$ endothelial cells were not observed, ruling out cell fusion as a mechanism in the generation of marrow-derived endothelial cells. The functionality of the system was confirmed in spleen sections demonstrating hPLAP activity, thereby demonstrating fusion of transplanted marrow cells with host cells in this organ (Fig. 2*B*).

In the embryo, a single cell, the hemangioblast, can give rise to cells of both hemopoietic and endothelial lineages. In adult animals, however, the existence of hemangioblasts is still unclear. To determine whether adult marrow contains a single precursor for endothelial and hemopoietic cells, mice were transplanted with a single GFP^+ hemopoietic stem cell, implanted with a s.c. tumor and examined for GFP^+ tumor endothelial cells. To confirm that the single cells injected were hemopoietic stem cells, their ability to reconstitute all blood lineages was tested by staining peripheral blood with Abs against myeloid and lymphoid cells as previously described (14). Only animals with >15% GFP^+ blood leukocytes and showing contribution of GFP^+ cells to all blood lineages were chosen for further analyses. Extensive analyses of both B6RV2 and LLC tumors from single cell-transplanted mice demonstrated a low number of blood vessels (~1%) that incorporated bone marrow-derived cells that stained positive for endothelial markers (Fig. 3). The presence of bone marrow-derived endothelial progenitors that incorporated into the vasculature of animals transplanted with a single

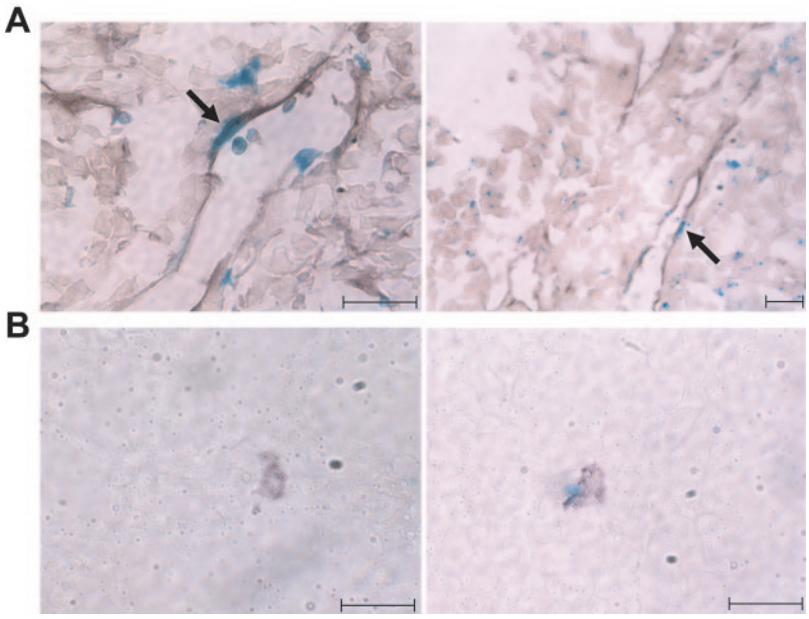

FIGURE 2. Marrow-derived endothelial cells arise by differentiation and not by cell fusion. B6RV2 tumors (*A*) and spleens (*B*) were harvested from PCX-NLS-Cre mice transplanted with Z/AP bone marrow. Sections were stained for *lacZ* (blue) and alkaline phosphatase activity and with anti-CD31 Ab (brown). Arrows indicate marrow-derived endothelial cells. Original magnification, ×1000 (*left*) and ×400 (*right*). Scale bars represent 25 μm.

hemopoietic stem cell indicates that primitive hemopoietic stem cells have the potential to give rise to endothelial precursors and may therefore represent a population of adult hemangioblasts.

Role of VEGF and VEGFR-2 in the mobilization and differentiation of bone marrow-derived endothelial cells

VEGF-A is a potent inducer of both angiogenesis and vasculogenesis and has been reported to mobilize VEGFR-2$^+$ endothelial progenitors from the bone marrow (26). To determine whether increased VEGF-A secretion by tumor cells would lead to an increase in the contribution of bone marrow-derived endothelial progenitors to the tumor vasculature, B6RV2 cells were transduced with a retroviral vector encoding the human VEGF-A_{165} cDNA (MSCVneo-VEGF-A_{165}). Human VEGF-A has previously been shown to be biologically active in mice (27, 28). Tumor cells were implanted s.c. in mice transplanted with GFP$^+$ marrow; after 10 days, they were harvested, cryosectioned, and stained with CD31 and VE-cadherin, and GFP$^+$ endothelial cells incorporating into tumor vasculature were quantified. B6RV2-VEGF tumors had an increased growth rate and displayed increased vascularity compared with B6RV2 wild-type tumors (Fig. 4, *C* and *D*). Tumor size averaged 134.4 ± 18.5 mm^3 for B6RV2 and 515.9 ± 186.9 mm^3 for B6RV2-VEGF after 10 days. However, there was no increase in the integration of marrow cells into the neovasculature of B6RV2-VEGF tumors compared with wild-type B6RV2 tumors (Fig. 4, *A* and *B*). To ensure that the VEGF-A secreted by tumor cells was able to perfuse the bone marrow, we quantitated VEGF-A concentrations in the sera of mice implanted with B6RV2-VEGF and wild-type B6RV2 tumors by ELISA. Human VEGF-A was detected in the serum of mice implanted with B6RV2-VEGF tumors (28.0 ± 4.7 pg/ml). In comparison, levels of VEGF-A in the serum of mice implanted with wild-type B6RV2 tumors were below the limit of detection of this assay. Because increased VEGF-A serum levels did not result in a greater proportion of bone marrow-derived endothelial cells lining tumor capillaries, we asked whether this reflected the inability of endothelial progenitors to efficiently home to tumors. Mice were implanted with either B6RV2 or B6RV2-VEGF tumor cells or were injected with medium only (sham injection) to determine whether increased VEGF-A serum levels could increase the proportion of circulating VEGFR-2$^+$ cells. Ten days following tumor implantation, peripheral blood was harvested and stained for VEGFR-2. Mice implanted with either B6RV2 or B6RV2-VEGF tumors did not display any increase in the proportion of circulating VEGFR-2$^+$ cells compared with sham-injected mice (Fig. 4*E*). These results indicate that increased VEGF-A secretion by tumor cells does not result

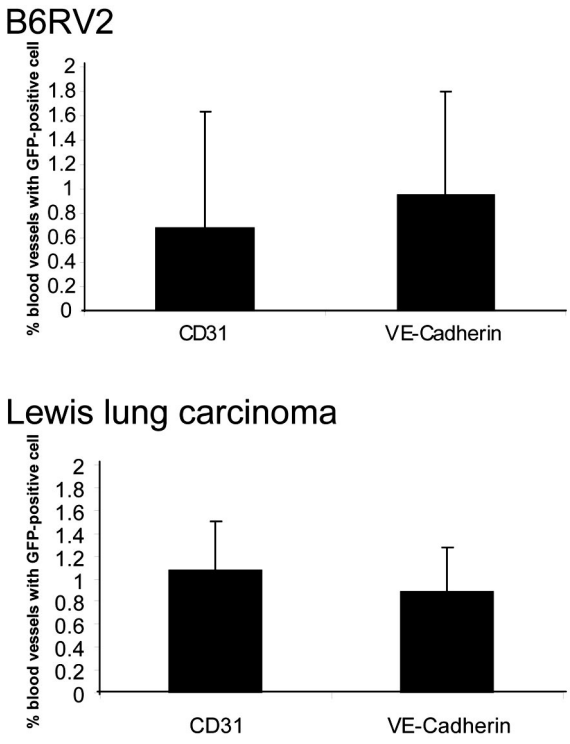

FIGURE 3. A single hemopoietic stem cell can give rise to endothelial progenitor cells that incorporate into tumor blood vessels. B6RV2 and Lewis lung carcinoma tumors were implanted into the dorsa of mice transplanted with a single GFP$^+$ hemopoietic stem cell. Tumor sections were quantified for the presence of GFP$^+$ marrow-derived cells present in blood vessels stained with either CD31 or VE-cadherin. Data represent means ± SEM of GFP$^+$ cells per vessel. At least three sections per tumor and three tumors per group were analyzed.

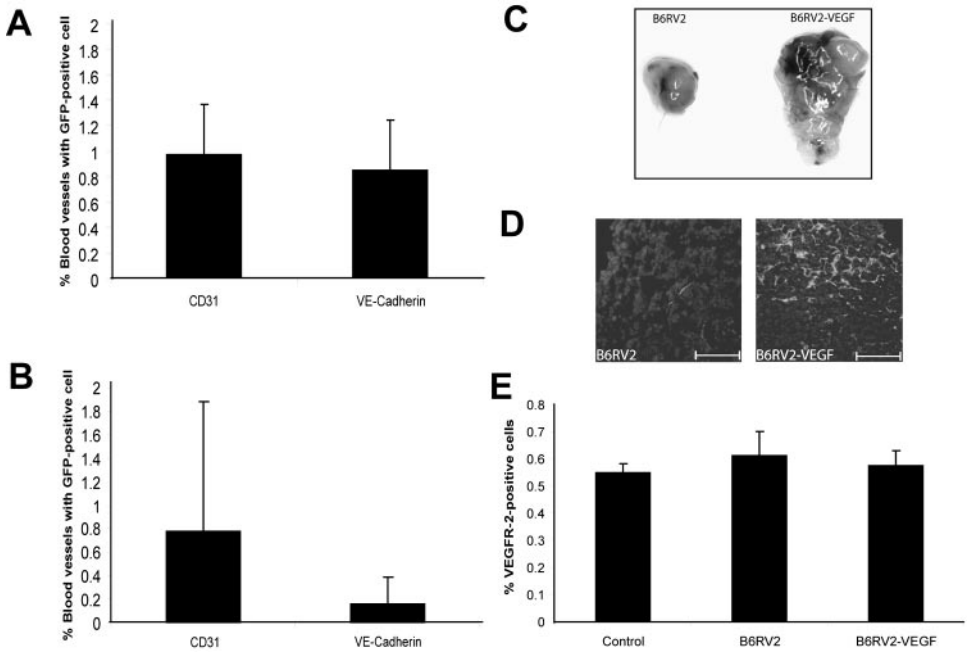

FIGURE 4. VEGF-A secretion by tumor cells does not increase the contribution of marrow-derived cells in tumor vasculature. B6RV2-VEGF tumors were implanted s.c. in mice previously transplanted with GFP^+ bone marrow from GFP transgenic donors (*A*) or with marrow transduced ex vivo with a GFP-encoding vector (MIG) (*B*). Data represent means ± SEM of GFP^+ cells per vessel. At least three sections per tumor and four tumors per group were analyzed. *C*, Image displaying the increased tumor mass of B6RV2-VEGF tumors (*left*) compared with B6RV2 tumors (*right*) 10 days after implantation. *D*, CD31 staining of B6RV2 and B6RV2-VEGF tumors demonstrating increased vascular density in B6RV2-VEGF tumors. At least three sections per tumor and four tumors per group were analyzed. *E*, B6RV2 and B6RV2-VEGF tumors were implanted s.c. in the dorsa of B6C3 mice. Tumor-free mice (sham-injected) were used as control. Ten days postimplantation, peripheral blood was harvested and stained for VEGFR-2. The proportion of nucleated cells positive for VEGFR-2 was determined by flow cytometry. Original magnification, ×400. Scale bars represent 50 μm.

in an increased proportion of marrow-derived cells incorporating into tumor blood vessels, suggesting a lack of mobilization of VEGFR-2^+ cells by the concentrations of VEGF-A secreted by these tumors into serum.

Because serum VEGF-A levels produced by B6RV2-VEGF tumors may not have been sufficient to mobilize bone marrow endothelial progenitors, we used a previously described strategy to activate VEGFR-2 in marrow cells with a chemical inducer of dimerization (AP-20187) and examined whether activation of this receptor was sufficient to mobilize endothelial progenitors from the bone marrow and recruit them into tumor vasculature (10). Marrow cells were transduced with a MIG-FKBP/VEGFR-2 construct and then transplanted into lethally irradiated recipient mice. Six weeks after marrow transplant, mice were s.c. implanted with B6RV2 tumor cells and injected daily for 10 days with 10 mg/kg AP-20187 to dimerize the intracellular domain of VEGFR-2. Transplanted mice injected with the vehicle only were used as controls. We have previously shown that this strategy recapitulates VEGFR-2 signaling pathways in endothelial and bone marrow cells, increases myeloid progenitor activity, and expands the myeloid cell population ($CD11b^+$, Gr-1^+) in the bone marrow and peripheral blood of transplanted mice (10, 49). However, VEGFR-2 activation in marrow cells did not result in increased levels of VEGFR-2^+/VE-cadherin$^+$ endothelial progenitors in the bone marrow or peripheral blood of transplanted mice (Fig. 5, *B* and *C*), nor did it result in increased contribution of marrow-derived endothelial cells to the vasculature of B6RV2 tumors (Fig. 5*D*). It therefore appears that the VEGF/VEGFR-2 pathway may not be sufficient for the recruitment and/or expansion of endothelial progenitor cells.

Determination of the proportion of endothelial progenitors in human umbilical cord blood

Because incorporation of bone marrow-derived endothelial progenitors into tumor vasculature is such a rare event, it is likely that endothelial progenitors are present in exceedingly low numbers in the circulation. To determine the proportion of circulating endothelial progenitors in humans using a rich source of hemopoietic stem cells, we examined umbilical cord blood. $CD133^+$ cells, Lin^- cells, and total mononuclear cells from umbilical cord blood were cultured for up to 6 wk following serial plating of nonadherent cells over 3 days in endothelial cell medium supplemented with endothelial growth factors as previously described (4). Serial plating of nonadherent cells ensured that only transplantable endothelial progenitors and not mature endothelial cells were measured (4). Colonies of adherent cells were quantified after 3 wk and normalized to the total number of cells plated at the start of the assay. Endothelial colonies adopted a cobblestone morphology and stained for endothelial markers such as vWF and P1H12 (CD146) (Fig. 6, *A* and *B*). Analyses of endothelial colonies following expansion revealed that the cells express eNOS, Tie-1, Tie-2, and VEGFR-2 (Fig. 6, *C* and *D*), confirming their endothelial phenotype. Furthermore, these cells possessed the ability to form tubes in fibrin gels in response to FGF-2 (Fig. 6, *F* and *G*), confirming that they have the functional properties of mature endothelial cells. We observed a 16-fold enrichment of endothelial colonies in the CD133-purified cells over total mononuclear cells and a 2.7-fold increase over Lin^- cells (Fig. 6*E*), indicating that the $CD133^+$ cells are markedly enriched for endothelial progenitors, which is consistent with the observation that $CD133^+$/VEGFR-2^+ cells are

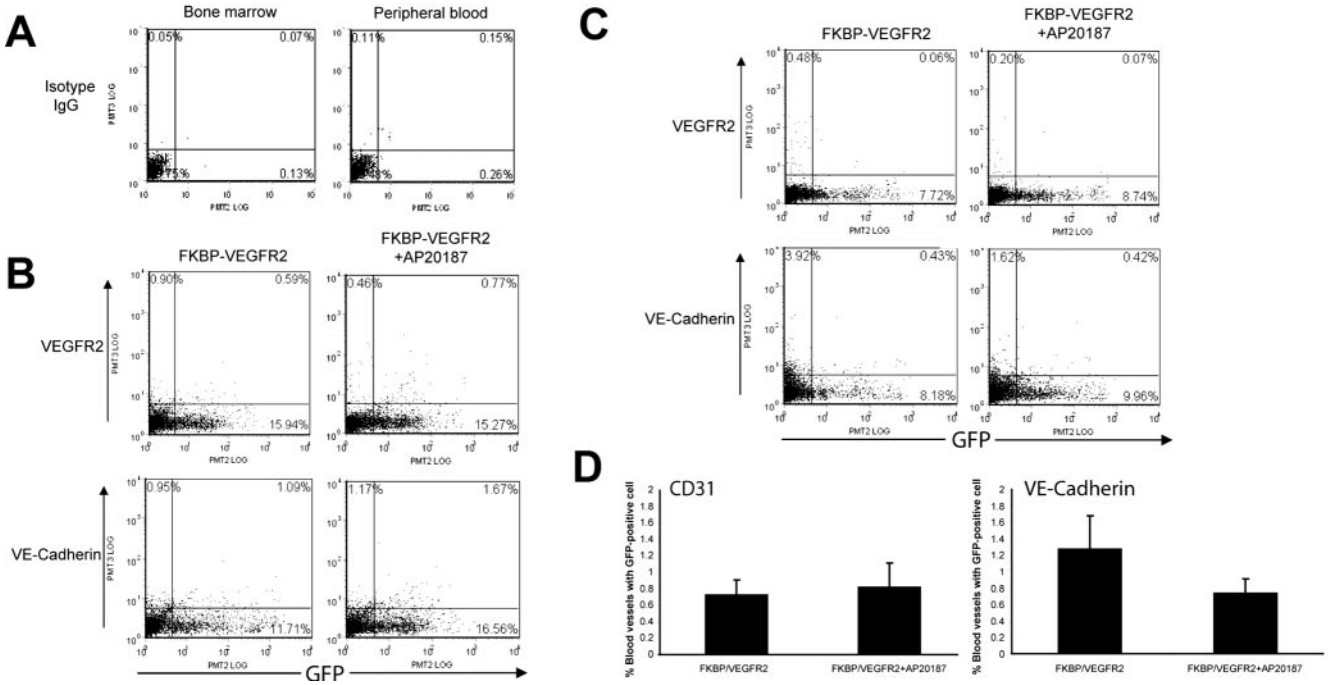

FIGURE 5. VEGFR-2 activation in marrow cells does not result in endothelial progenitor mobilization or recruitment into tumor vasculature. Mice transplanted with FKBP/VEGFR-2 were injected i.p. with AP-20187 or vehicle for 10 consecutive days. At the end of this period, bone marrow cells (*B*) and peripheral blood (*C*) were harvested and stained with VEGFR-2 or VE-cadherin to detect endothelial progenitor cells. Bone marrow and peripheral blood cells were stained with isotype Ig as control (*A*). *D*, B6RV2 tumor sections from the same mice were immunostained with either anti-CD31 or anti-VE-cadherin Abs to detect GFP^+ bone marrow cells incorporating into tumor vasculature. Data represent means ± SEM of GFP^+ cells per vessel. At least three sections per tumor and four tumors per group were analyzed.

associated with a population of endothelial progenitors (5). Nevertheless, the frequency of endothelial progenitors was <1 in 10^7 cells of the total mononuclear cell population, highlighting the rarity of this cell population.

Discussion

Classically, tumor neovascularization has been thought to be limited to angiogenesis (29). Prior research has demonstrated this process to be mediated through the release of angiogenic factors such as VEGF and bFGF (30). However, recent advances in vascular biology have led to a revisitation of this conventional concept. For example, it has been suggested that tumor cells themselves may generate vascular conduits that facilitate tumor perfusion independent of tumor angiogenesis (31). Other reports suggest that monocytes/macrophages are the initiators of angiogenesis by creating channels through proteolytic degradation of the matrix (32, 33). Bone marrow-derived endothelial progenitors have also been suggested to significantly contribute to neovascularization during tumor angiogenesis (3, 34, 35). However, the relative levels of contribution of endothelial progenitors to this process is unclear because there have been conflicting reports regarding this issue (2, 3, 36–38).

Our findings demonstrate that marrow-derived endothelial cells do integrate into the tumor microvasculature but at a very low level. The incorporation of marrow-derived endothelial cells into tumor blood vessels was quantitated to be $<1\%$ of CD31- or VE-cadherin-stained blood vessels. Our findings are consistent with recent reports that question the proposed major role and the biological significance of bone marrow-derived endothelial progenitors in tissue revascularization (37, 38). However, these results contrast with those of a previous report demonstrating that ~90% of blood vessels in B6RV2 tumors are composed of bone marrow-derived endothelial cells (3). Even though it is possible that different tumor implantation schedules may account for some of the differences observed in our studies, because the tumors were only grown for 10 days, it is very unlikely to be the only factor because other groups could not observe any significant contribution of endothelial progenitors to the vasculature of LLC and B6RV2 tumors grown for longer periods of time (2–3 wk) (8, 37). Therefore, this discrepancy may be due to different analytical methods such as the use of β-galactosidase instead of GFP as a marker for bone marrow-derived cells, the use of different mouse strains, or the use of different Abs to identify endothelial cells.

Several studies have suggested that transplanted marrow cells can differentiate into unexpected lineages, including myocytes, hepatocytes, and neurons (39–41). A potential problem, however, is that reports investigating such differentiation in vivo tend to conclude donor origin of transdifferentiated cells on the basis of the existence of donor-specific markers such as *lacZ*, GFP, or the Y chromosome (42, 43). Recent work reporting the potential of stem cells to fuse with different cell types such as hepatocytes and myocytes (44) has led to the suggestion that some of the transdifferentiation events reported for tissue-specific stem cells may in fact be a result of cell fusion (21). In this paper, we were able to rule out cell fusion using a novel strategy and demonstrate the presence of a bona fide marrow-derived endothelial cell progenitor.

Although others have described the presence of vWF^+ and $CD31^+$ marrow-derived cells lining the retinal vasculature of mice

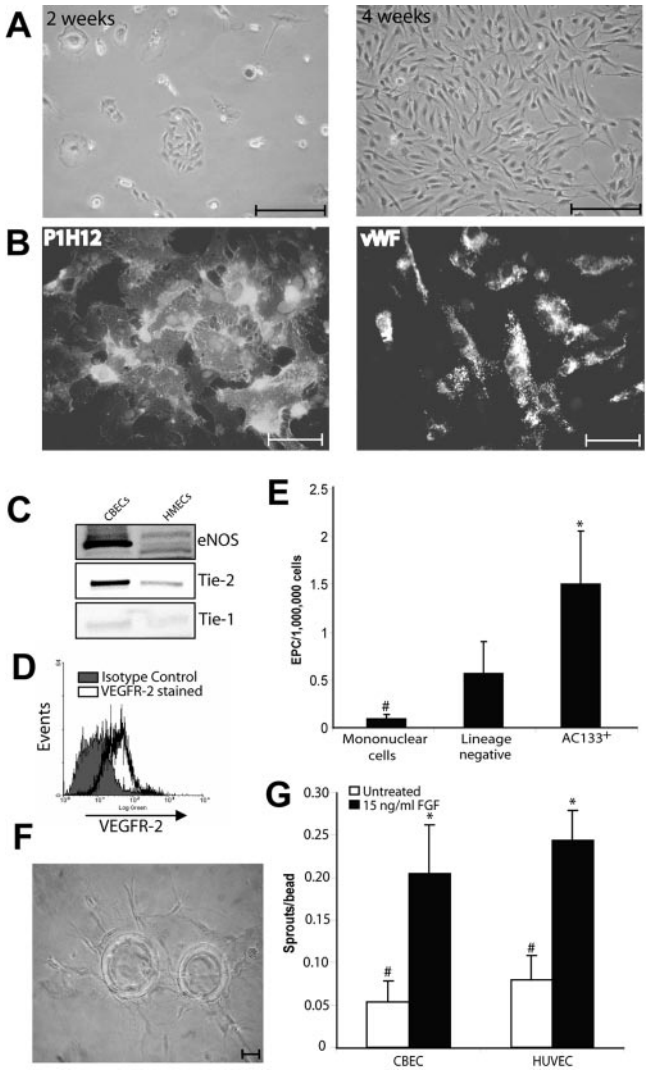

FIGURE 6. The endothelial progenitor is a rare cell in human umbilical cord blood and can give rise to mature, functional endothelial cells. Cells from umbilical cord blood ($CD133^+$, Lin^-, total mononuclear cells) were plated in medium supplemented with VEGF-A and bFGF. *A*, Phase contrast micrographs of endothelial colonies, which appeared 3–4 wk after plating. Scale bars represent 100 μm. *B*, Cells from the endothelial colonies stained positive for the endothelial markers vWF and P1H12. *C*, cells expressed eNOS, Tie-1, and Tie-2 by immunoblotting. *D*, Cells expressed surface VEGFR-2, as determined by flow cytometry. Original magnification, $\times$1000. Scale bars represent 25 μm. Four weeks after plating, colonies were quantified by counting the total number of $P1H12^+$ colonies and expressed as the total number of endothelial progenitors normalized to the original number of cells plated for each subset of mononuclear cells (*E*). Data in *E* represent the average of 7–12 independent experiments for each subset of mononuclear cells. *F*, CBEC form tubes in fibrin gels in the presence of FGF-2. *G*, Sprout formation for CBEC and HUVEC was quantitated after 3 days of incubation by counting the number of tube-like structures per microcarrier bead (sprouts per bead). *, Significant difference compared with #.

transplanted with a single hemopoietic stem cell, the caveat regarding these markers, which are also expressed on hemopoietic cells, applies (25). In the present study, we show that tumors implanted into mice transplanted with a single hemopoietic stem cell display rare marrow-derived VE-$cadherin^+$ cells incorporating into the vasculature. The use of VE-cadherin as a specific marker for endothelial cells demonstrates definitively that these marrow-derived cells are endothelial, thereby confirming the existence of an adult hemangioblast in vivo. Even though the proportion of endothelial progenitor incorporation into tumor microvasculature was very low, these results confirm the existence of cells with hemangioblastic potential in postnatal bone marrow and are in accordance with recent studies that reported the existence of hemangioblasts in adult bone marrow (25, 38). It is interesting to note that we observed the same proportion of bone marrow-derived cells incorporated in the tumor vasculature of mice transplanted with either whole bone marrow cells or a single hemopoietic stem cell. This not only confirms that endothelial progenitors are of hemopoietic origin, but also allows us to rule out a mesenchymal origin of endothelial progenitors in our model; tumor incorporation of endothelial progenitors would have been expected to be higher in whole bone marrow transplants compared with single hemopoietic stem cells if mesenchymal stem cells present in the bone marrow were a source of endothelial progenitors. Moreover, marrow-derived endothelial cells were detected in B6RV2 tumors implanted in serially transplanted animals, confirming the long-term repopulating capacity of an endothelial progenitor or precursor cell. It is of interest to mention that GFP^+ cells could be detected in the skeletal muscle and participate in muscle regeneration in these same mice (14), which shows that this precursor is not limited to the hemopoietic and endothelial lineages.

It is likely that the differentiation of cells with hemangioblastic activity toward the endothelial lineage is a relatively rare event, which would make endothelial progenitors a rare cell population in comparison to other hemopoietic mononuclear cells. We show here that endothelial progenitors are extremely rare among umbilical cord blood mononuclear cells, consisting of <1 in 10^7 cells. The proportion of endothelial progenitors that we observed here was much lower than that previously reported (5, 6). However, in contrast to quantification using CD34, VEGFR-2, and CD133 positivity by flow cytometry as in the previous reports, we determined the proportion of endothelial progenitors using a functional differentiation assay. Moreover, we only considered late-outgrowth endothelial colonies and not early-outgrowth populations, which likely include mature endothelial cells that can be found in the circulation (4). The cell surface markers CD34, CD133, and VEGFR-2, which were used to detect endothelial precursors, are all present on other cell subsets in blood and bone marrow cells and could therefore explain the higher proportion of endothelial progenitors observed in previous papers (5, 6). Endothelial progenitors are enriched in the $CD133^+$ population of mononuclear cells, which also marks a population of primitive hemopoietic stem cells (45). However, whether $CD133^+$ cells comprise a population of adult hemangioblasts remains to be demonstrated at the clonal level. Furthermore, it is not possible to state whether the hemangioblast itself is a rare cell in the adult or whether the differentiation of this cell into an endothelial precursor, rather than a hemopoietic precursor, is a rare event in vivo and significantly rarer than the estimated frequency of hemopoietic stem cells in cord blood (46).

VEGF and its receptor VEGFR-2 have been shown to be critical for the differentiation and proliferation of endothelial progenitors in vitro (47). Furthermore, increased levels of VEGF in mice in vivo have been associated with mobilization of endothelial progenitors through activation of VEGFR-2 (26). Our results indicate that overexpression of VEGF-A by tumor cells, although resulting in increased neovasculature, does not induce an increase in the levels of incorporation of marrow-derived endothelial cells into the tumor vasculature. The low frequency of bone marrow-derived

endothelial progenitors incorporating into newly formed capillaries observed in our model possibly indicate that VEGF-A levels secreted by tumors may not be sufficient to recruit circulating endothelial progenitors. Furthermore, we found that activation of VEGFR-2 in bone marrow cells was not sufficient to expand or mobilize VEGFR-2^+/VE-cadherin$^+$ endothelial progenitors, nor did it increase the levels of incorporation of endothelial progenitors into the tumor vasculature, even though VEGFR-2 dimerization in this system is able to expand and mobilize a population of Gr-1^+/CD11b^+ myeloid cells (49). This may indicate a requirement for the presence of other VEGF receptors to expand and/or mobilize endothelial progenitors or suggests that VEGFR-2 activation is not sufficient to mobilize endothelial progenitor cells. It is also possible that VEGF-induced activation of VEGFR-2, even though it does not induce mobilization of endothelial progenitors per se, can promote the expansion and mobilization of myeloid cells such as macrophages, which in turn can actively promote tumor growth through their close interaction with tumor endothelium and the release of proangiogenic cytokines such as bFGF, IL-8, and platelet-derived growth factor (48).

Our findings demonstrate that there is an adult hemangioblast that can differentiate into endothelial precursor cells but that incorporation into the tumor microvasculature is a rare event and VEGF stimulation does not enhance this process. Understanding the factors that regulate contributions from primitive hemopoietic stem cells and their circulating progenitors to new vessel formation may ultimately provide additional ways to influence the process of neovascularization, which may prove beneficial in treating vascular-related diseases.

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Disclosures

The authors have no financial conflict of interest.

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