Supporting Information

Purhonen *et al.* 10.1073/pnas.0710516105

SI Materials and Methods

Syngeneic Bone Marrow Transplantations. BM cells were collected by flushing femurs and tibias of the donor mice with 29-gauge needle into DMEM (Invitrogen) supplemented with 2 mM L-Glutamin, 100 units/ml penicillin, and 100 μg/ml streptomycin (Sigma–Aldrich). Unselected BM cells (7×10^6) were transplanted into corresponding syngeneic WT mice via tail vein injection. The recipients were irradiated 1 day before transplantation by a lethal dosage of 9.1 Gy. Mice were used for experiments 5–25 weeks after transplantation. As an extra precaution, the BM reconstitution of the chimeric GFP or DsRed.T3 BM mice was controlled by FACS analyses for GFP or DsRed.T3 on peripheral blood cells. The chimeras always had as much or more fluorescent peripheral blood cells than the transgenic donors, thus confirming successful reconstitution (data not shown). The Provincial State Office of Southern Finland approved all of the mouse experiments.

Parabiosis. For parabiosis experiments, APCmin mice (1) were surgically conjoined to mice expressing eGFP under the β-actin promoter to generate a common anastomosed circulatory system. Parabiosis was performed as previously described (2, 3) and in accordance with the guidelines established by Stanford University for the humane treatment of animals. Shortly, matching skin incisions were made from the olecranon to the knee joint of each mouse, and the s.c. fascia was bluntly dissected to create about 1/2 cm of free skin. The olecranon and knee joints were attached by a single 2-0 silk suture and tie, and the dorsal and ventral skins were approximated by staples or continuous suture. Chimerism of lymphoid cells in the Peyer's patch of the APCmin mice was used to monitor the 50% blood cell chimerism in the system (Fig. 5*A*).

Mobilization of BM-Derived Cell Populations. Age- and sex-matched (8 weeks, male) C57BL/6 and BALB/c mice were purchased from Harlan Nederland. 2×10^6 B16-F1 melanoma cells (ATCC) or PBS in a volume of 100 μl were inoculated s.c. into dorsal hindlimb of C57BL/6 mice ($n = 12$ in each group). Peripheral blood samples from a tail vein of each mouse were taken on days 0 (before inoculation), 3, 5, 7, 11, 14, and 17. On every sampling day, the tumor size (largest diameter) was measured; if the diameter of the tumor exceeded 20 mm, the animal was killed. Recombinant murine VEGF164 (Biosource), 10 μg in 100 μl of 0.1% BSA (Sigma–Aldrich), was administered to C57BL/6 mice ($n = 6$) by i.p. injection daily for 5 days. The dosing scheme (10 μg of VEGF/mouse/day i.p) and the mouse strain we used (C57BL/6) were the same as in the original work describing circulating EC progenitors (4). Control mice received 100 μl of 0.1% BSA according to the same schedule. Peripheral blood samples were taken on days 0 (before inoculation), 3, 5, and 7. Adenoviral vectors encoding for lacZ or murine VEGF164 (5) (10^9 PFU dose in 100 μl) were administered by tail vain injections into BALB/c mice. Adenovirus titers were measured with state-of-the-art methods according to international ARMWG standard adenovirus prep with methods suggested by the adenovirus standardization working group (6). Replication competent adenovirus (RCA) levels were measured in A546 cells with a standard viral cytopathic effect (CPE) assay (7). No measurable RCA was present in any of the used virus preps. The correct function of the AdVEGF164 batch used also was controlled on HeLa cells *in vitro* [supporting information (SI) Fig. S1]. HeLa cells (150,000 cells per ml) were infected with the AdVEGF164 or with the AdLacZ at multiplicity of infection of 0, 1, 5, or 50. After 48 h, the culture medium was assayed for mouse VEGF by using a mouse VEGF ELISA Kit (R&D Systems). The adenoviral vectors were used in the mouse experiments within the same concentration range as reported previously in papers describing the mobilization of VEGFR-2+ EC precursors (8–10). In the control group, 100 μl of PBS was administered. Peripheral blood samples were taken on days 0, 5, and 7. The virus dose used with the C57BL/6 mice was 10^8 PFU. Blood samples from C57BL/6 mice were taken on days 0 (before inoculation), 3, 5, 7, 14, and 21. The number of mice in each adenoviral treatment and control group was 6.

Blood Sampling, Cell Isolation, and Flow Cytometry. Blood samples were collected from tail vein by using Na-heparin capillaries (Heinz Herenz), and the collected blood was mixed with an equal amount of 0.5 M EDTA. Total WBCs were quantified by lysing the RBCs with 2% acetic acid, the cells were counted by using a Neubauer hemacytometer. Peripheral blood white cells (PBWCs) for flow cytometry were isolated by lysing the RBCs with Pharm Lyse lysing buffer (BD Biosciences PharMingen). Before staining, the cells were incubated with anti-mouse CD16/CD32 blocker (BD PharMingen) according to the manufacturer's instructions to reduce FcγII/III receptor-mediated antibody binding. The PBWCs were stained for 30 min at 4°C with isothiocynate (FITC)-conjugated anti-mouse CD11b or Sca-1, phycoerythrin (PE)-conjugated anti-mouse VEGFR-2, and allophycocyanin (APC)-conjugated anti-mouse CD117. The detection of VEGFR-2+ cells by the PE-conjugated anti-VEGFR-2 antibody clone Avas12α1 (PD PharMingen) was controlled by two-channel FACS analysis for VEGFR-2 and CD31 (APC-conjugated clone MEC 13.3) by using murine MS-1 endothelial cells (ATCC no. 2279; a kind gift from Jack L. Arbiser, Emory University School of Medicine, Atlanta) as positive control. All labeled monoclonal antibodies were purchased from BD PharMingen. After staining, the cells were washed twice with 1 X PBS containing 1% fetal bovine serum (PromoCell) and 0.1% sodium azide (Sigma–Aldrich) and fixed with 2% paraformaldehyde (PFA; Sigma–Aldrich). In analyses, dead cells and cell debris were excluded by gating the population according to the forward and side light scatters. The number of positive cells were compared with the number of cells positive in the staining with the IgG isotype controls (BD PharMingen) and determined with FACSAria flow cytometer (Becton Dickinson). Before each analysis, laser compensations were adjusted automatically with FACSDiva software version 4.1.2 (Becton Dickinson).

Induction of Angiogenesis in Mice by Using AdVEGF, Syngeneic B16 Melanoma Tumors, Spontaneous APCmin Tumors, or Matrigel Plugs. The BM-transplanted chimeric mice were injected s.c. in the ear with AdVEGF164 (2×10^8 PFU per injection per ear). After a minimum of 14 days, the mice were killed, and the ears were processed for immunohistochemistry. The B16-F1 melanoma cell line (ATCC) was maintained in DMEM supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (PromoCell). To induce tumor angiogenesis, the mice were injected s.c. with B16 cells (2×10^6 cells in 20 μl), the tumors were allowed to grow for 7–21 days, the mice were killed, and the tissues were processed for analyses. APCmin mice were parabiosed at 7–9 weeks of age (before the onset of tumorigenesis) and then killed at 22–24

weeks of age. Upon killing, intestinal adenomas were visually scored and parabiosed APCmin mice presented 20–55 adenomas per mouse. Matrigel plugs (400 μl per injection Basement Membrane Matrix; BD PharMingen) supplemented with recombinant murine VEGF164 (100 ng/ml; R&D Systems) were injected close to the dorsal midline of the ventral side of the mouse. The plugs were excised and processed for tissue analyses at several time points (from 1 day to 6 months) after injection.

Immunohistochemistry and Whole Mounts. For staining of whole mounts, all samples were fixed in 4% PFA; blocked with PBS buffer containing 5% serum (Vector Laboratories), 0.2% BSA, and 0.3% Triton-X (Sigma–Aldrich); and incubated with the primary antibodies for 2 days at room temperature. Autofluorescent cartilage was removed from the ears before fixing. The samples were washed and incubated with fluorochrome-conjugated secondary antibodies overnight at room temperature. Finally, the plugs were sliced, the ears were flattened, and the samples were mounted with antifading medium (Vectashield; Vector Laboratories). For immunohistochemistry of cryosections, samples were fixed for 1 h with 2% PFA and incubated in 20% sucrose/PBS overnight. After the cryopreservation, tissues were embedded in OCT compound (Tissue-Tek; Sakura Finetek Europe) and frozen at −70°C. Sections (8–20 μm) were stained with the primary antibodies overnight at 4°C and subsequently detected with fluorochrome-conjugated secondary antibodies for 30 min at room temperature. Finally, the sections were mounted with antifading medium (Vectashield). The primary antibodies used were rat anti-mouse CD31/PECAM-1 (BD PharMingen), rat anti-mouse CD105/endoglin (BD PharMingen), rabbit anti-mouse/human von Willebrand Factor (vWF; DAKO), and two rat anti-mouse VEGFR-2 antibodies: AVAS 12 α1 (BD PharMingen) and DC101 (ImClone Systems). The secondary antibodies used were Alexa594 anti-rat, Alexa594 anti-rabbit, Alexa633 anti-rat, and Alexa633 anti-rabbit (Molecular Probes). The correct detection of the endogenous GFP signal was controlled by also staining part of the samples with an anti-GFP-Alexa488 antibody (Molecular Probes). To verify that detected blood vessels are actually functional vessels, we used rhodamine-labeled *Ricinus Communis* -lectin (Vector Laboratories) as a marker of the blood perfusion (11). Lectin was injected as a single 500-μg dose i.v. to the tail vein 5–15 min before perfusion fixation with 4% PFA. The samples were analyzed with a Zeiss LSM510 laser scanning confocal microscope (Carl Zeiss) by using multichannel (sequential) scanning in frame mode and a 40× (NA = 1.3) Plan-Neofluar oil immersion objective (LSM 5 Software version 3.2). Single XY scans had an optical slice thickness of ≤1.0 μm. Additionally, the samples were analyzed and photographed with a Zeiss Axioplan 2 immunofluorescence microscope by using ×20 (NA = 0.5) and ×40 (NA = 0.75) Plan-Neofluar objectives, AxioCam Hrc camera, and Axiovision 4.3 software (Carl Zeiss).

β-Gal Staining of Matrigel Plugs, Tumor Tissues, and BM Cells. Matrigel plugs or s.c. tumors were placed into cold PBS and fixed [0.2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3), including 0.5 mM EGTA and 2 mM $MgCl_2$] for 30 min at room temperature. Subsequently, the samples were washed three times with washing buffer [0.1 M phosphate buffer (pH 7.3) containing 2 mM $MgCl_2$, 0.01% deoxycholic acid sodium salt, and 0.02% Nonidet P-40] for 15 min. Samples were stained overnight at 37°C in wash buffer containing 1 mg/ml X-gal, 2.12 mg/ml potassium ferrosyanide, and 1.64 mg/ml potassium ferrisyanide. After staining, the samples were washed with the wash buffer overnight at 4°C. Finally, the samples were fixed overnight with 4% PFA at 4°C. All reagents were form Sigma–Aldrich. BM cells of femurs and tibias were flushed and stained identically to the method described above except that before staining the cells (1.5×10^6 BM cells) were suspended in 350 μl of matrigel (BD Biosciences), and the matrigel cell suspension was left to solidify for 1 hour at 37°C. The matrigel plugs and tumors were analyzed and photographed with Leica MZFLIII fluorescence stereomicroscope with 12.5:1 zoom by using Leica DFC480 camera and Firecam software (Leica). The BM cells in matrigel were photographed with Zeiss Axiovert 135 inverted microscope by using LD Acroplan objectives 20× (NA = 0.4), 40× (NA = 0.6 corr), and 63× (NA = 0.75 corr) and a Hamamatsu digital camera (C4742–95). Before sectioning, the β-gal-stained matrigel and tumor samples were dehydrated through increasing concentrations of ethanol and cleared with xylene and mounted in paraffin wax. By using microtome, 20-μm sections were cut onto the SuperFrost microscope slides (O. Kindler) and dried overnight at 37°C. Subsequently, the samples were deparafinized, rehydrated, and stained in Certistain Nuclear fast red (Merck) for 2.5 min.

1. Moser AR, Pitot HC, Dove WF (1990) A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. *Science* 247:322–324.
2. Bunster E, Meyer RK (1933) An improved method of parabiosis. *Anat Rec* 339:339–343.
3. Wright DE, Wagers AJ, Gulati AP, Johnson FL, Weissman IL (2001) Physiological migration of hematopoietic stem and progenitor cells. *Science* 294:1933–1936.
4. Asahara T, *et al.* (1999) VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. *EMBO J* 18:3964–3972.
5. Vajanto I, *et al.* (2002) Evaluation of angiogenesis and side effects in ischemic rabbit hindlimbs after intramuscular injection of adenoviral vectors encoding VEGF and LacZ. *J Gene Med* 4:371–380.
6. Hutchins B, *et al.* (2000) Working toward an adenoviral vector testing standard. *Mol Ther* 2:532–534.
7. Dion LD, Fang J, Garver RI, Jr (1996) Supernatant rescue assay vs. polymerase chain reaction for detection of wild type adenovirus-contaminating recombinant adenovirus stocks. *J Virol Methods* 56:99–107.
8. Hattori K, *et al.* (2001) Vascular endothelial growth factor and angiopoietin-1 stimulate postnatal hematopoiesis by recruitment of vasculogenic and hematopoietic stem cells. *J Exp Med* 193:1005–1014.
9. Heissig B, *et al.* (2002) Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand. *Cell* 109:625–637.
10. Lyden D, *et al.* (2001) Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. *Nat Med* 7:1194–1201.
11. Chang YS, *et al.* (2000) Mosaic blood vessels in tumors: Frequency of cancer cells in contact with flowing blood. *Proc Natl Acad Sci USA* 97:14608–14613.

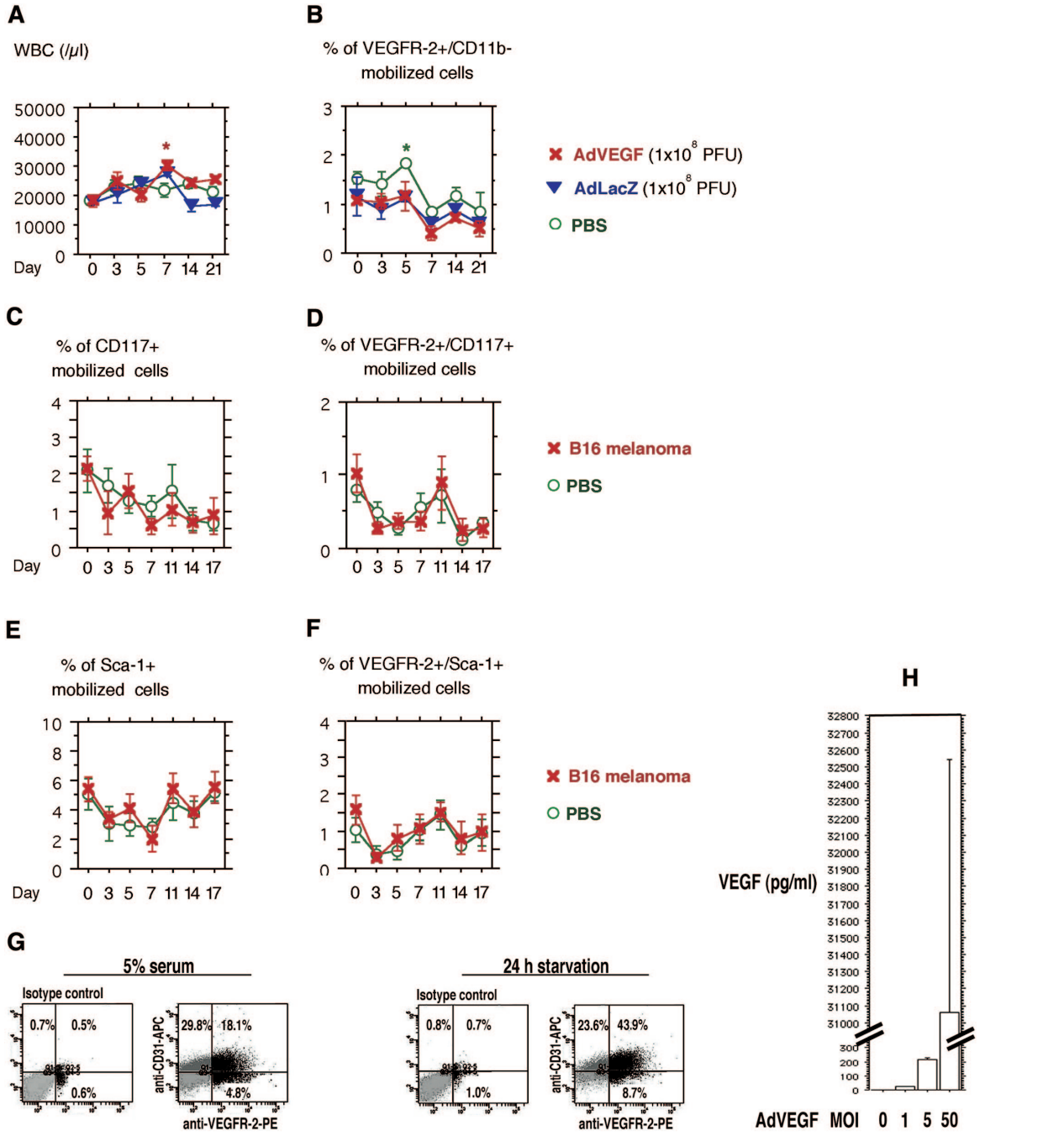

Fig. S1. Adenoviral VEGF delivery or tumors do not promote mobilization of putative EC precursors in C57BL/6 mice. The results are given as mean ± SE. The asterisks indicate statistical significance ($P < 0.05$). Day 0 (d0) shows baseline levels before injection or inoculation. The *x* axis of the graphs are not linear. (*A* and *B*) C57BL/6 mice received a single i.v. administration of AdVEGF (1×10^8 PFU; $n = 6$), AdLacZ (1×10^8 PFU; $n = 6$), or PBS (100 μl; $n = 6$) on day 0. Mobilized cells were isolated from the mice on days 0–21, counted, and analyzed by flow cytometry. Both AdLacZ and AdVEGF caused nonspecific mobilization of WBCs. (*C–F*) C57BL/6 mice were inoculated s.c. with B16 tumors (2×10^6 cells; $n = 12$) or PBS (100 μl; $n = 12$). Mobilized peripheral blood cells were isolated from the mice on days 0–17 and analyzed by flow cytometry. The percentages of mobilized circulating CD117+ cells, VEGFR-2+/CD117+ cells, Sca-1+ cells, and VEGFR-2+/Sca-1+ cells are shown. (*G*) The detection of VEGFR-2+ cells by the PE-conjugated anti-VEGFR-2 antibody clone Avas12α1 was controlled by two-channel FACS analysis for VEGFR-2 and CD31 by using murine MS-1 endothelial cells as a positive control. IgG isotype controls and the antibody stainings are shown. Strong up-regulation of the cell-surface expression of VEGFR-2 can be seen in the cells starved for 24 h. (*H*) The function of the AdVEGF164 batch was controlled on HeLa cells. HeLa cells (150,000 cells per ml) were infected with the murine AdVEGF164 virus by using the indicated multiplicity of infection (MOI). After 48 h, the culture medium was assayed for mouse VEGF by using mouse VEGF ELISA. The results are given as mean ± SE of six parallel experiments in each group. Note that the *y* axis is discontinuous. Infection with the AdLacZ at identical MOIs did not result in detectable mouse VEGF production by HeLa cells (mean 0 pg/ml for each group) (data not shown).

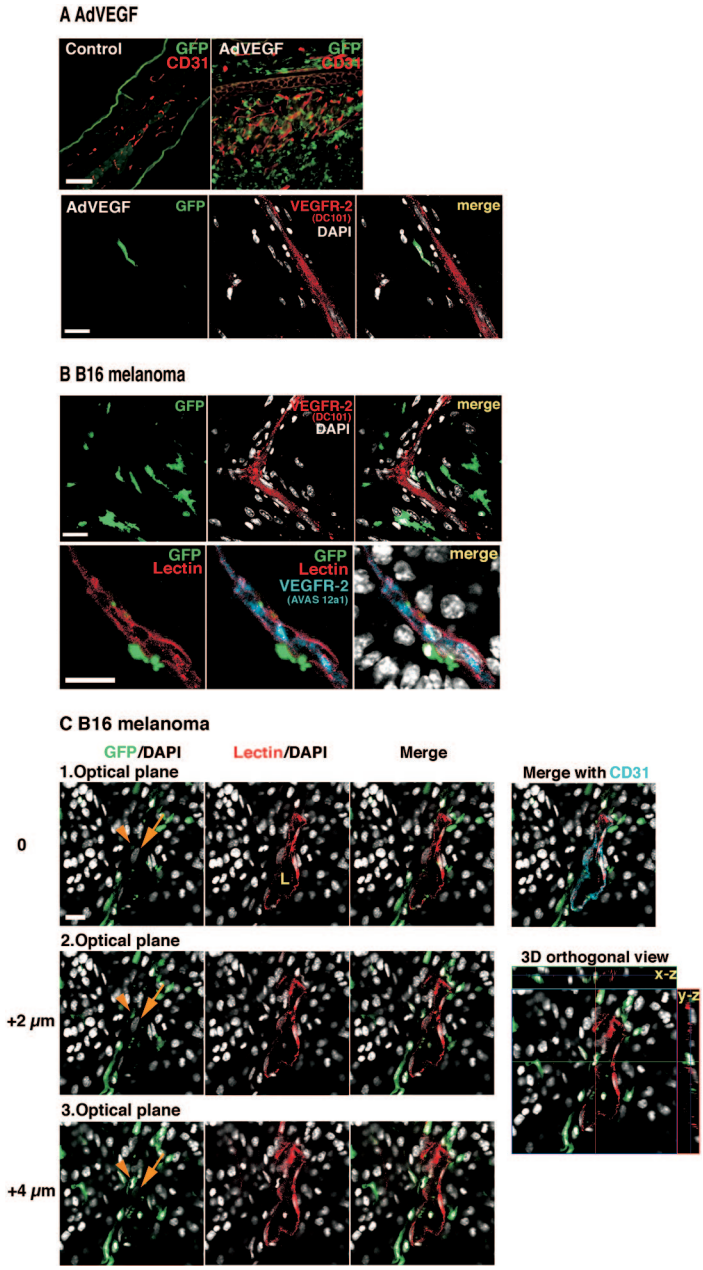

Fig. S2. All BM-derived cells recruited during VEGF- or tumor-induced angiogenesis are perivascular. ECs were detected against VEGFR-2 (antibody clones indicated), CD31, and by using Ricinus Communis-lectin perfusion. Transgenic GFP reporter is expressed in BM-derived cells. The nuclei are stained with DAPI (white) to recognize individual cells. (*A*) The s.c. injection of AdVEGF resulted in a robust enhancement of angiogenesis as demonstrated by CD31 staining of untreated and AdVEGF-injected ear tissues. High-resolution confocal scans of angiogenic VEGFR-2+ vessels demonstrate the perivascular location of BM-derived cells. (Scale bars: *Upper*, 100 μm; *Lower*, 20 μm.) (*B*) Angiogenesis was induced in the reconstituted hosts by B16 melanomas. Vascular ECs were stained for VEGFR-2 (upper, antibody clone DC101). Functionality of VEGFR-2+ vessels was verified by lectin perfusion (*Lower*). All BM-derived cells are perivascular in location. (Scale bar: 20 μm.) (*C*) Optical sectioning of a B16 melanoma tumor blood vessel lumen (indicated L) detected by lectin perfusion (red) and CD31 staining (blue). Three different optical planes (the distance between the planes is 2 μm) and representative 3D orthogonal projections (*x–z* and *y–z* axes) are shown. The sectioning demonstrates the close proximity of the BM-derived cells (arrowheads) and vascular ECs (arrows). (Scale bar: 20 μm.)

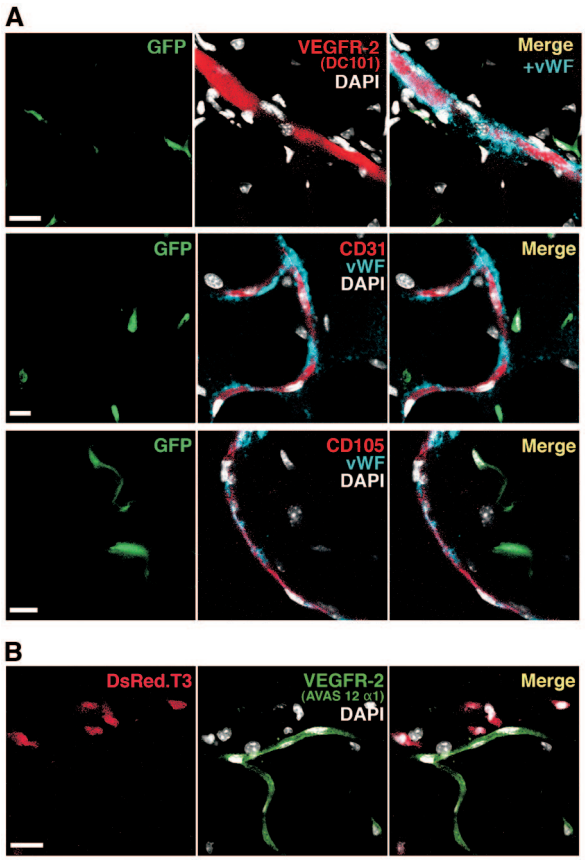

Fig. S3. No BM-derived vascular ECs are detected in matrigel plugs from chimeric mice engrafted with GFP- or DsRed-tagged BM. All blood vessels observed within the plugs must be novel. Multichannel confocal scans of whole mounted plugs are shown. No BM-derived vascular ECs by using antibodies against CD31, CD105, vWF, or VEGFR-2 (antibody clone indicated) were detected. The nuclei are stained with DAPI (white) to recognize individual cells. (Scale bars: 20 μm.) (*A*) Plugs implanted for 2 weeks in mice engrafted with GFP-tagged BM. (*B*) Plug implanted for 1 week in mice engrafted with DsRed.T3-tagged BM.

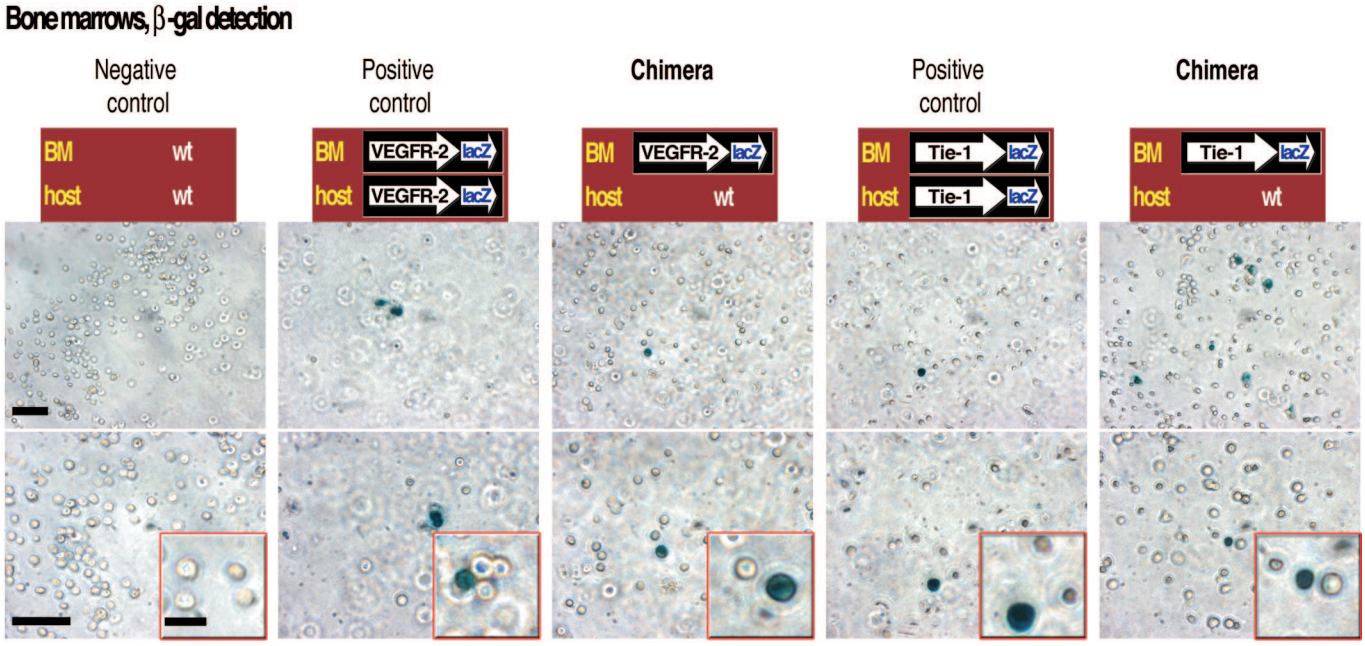

Fig. S4. Analyses for the presence of β-gal+ cells in the BM of transgenic or chimeric lacZ-tagged mice. At the end of the experiments with the EC-specific lacZ-tag models, the BM cells were examined for the presence of β-gal expression. The BMs from the transgenic C57BL/6J-Kdrtm1Jrt and CD-1/129Sv-tie^{lcz} donor mice contained constant, low numbers of β-gal+ cells. Identically, the BMs from the chimeric recipients contained constant, low numbers of β-gal+ cells, confirming the engraftment of VEGFR-2+ or Tie-1+ cells from the BM transplants, respectively. (Scale bar: 50 μm.) (*Insets*) Higher-magnification views of the BM cells. (Scale bar: 20 μm.)

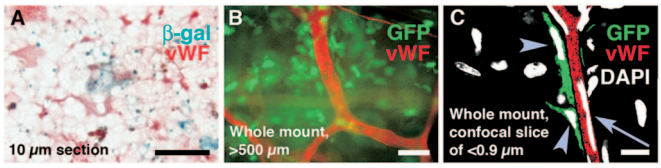

Fig. S5. Comparison of microscopic methods for marker gene detection in vasculature. (*A*) Immunohistochemical staining for von Willebrand Factor (vWF, red) in a tissue section (thickness, 10 μm) from chimeric mice engrafted with lacZ-tagged Rosa26 BM. Abundant β-gal+ cells (blue) originating from BM can be seen. However, it is not possible to determine whether the β-gal+ cells are endothelial or periendothelial. (Scale bar: 50 μm.) (*B*) Immunofluorescence microscopy of tissues from chimeric mice engrafted with GFP-tagged BM (green) and stained for vWF (red). The use of whole-mounted tissues (thickness, $>$500 μm) allows for the visualization of the 3D vascular network. (Scale bar: 50 μm.) (*C*) A thin optical slice (thickness $<$0.9 μm) from high-resolution multichannel (sequential) confocal scan of whole-mounted tissues. Visualization of the nuclei of the cells by using DAPI (white) provided a superb means to distinguish individual cells and tell apart the vascular ECs (arrow) and periendothelial cells (arrowheads). (Scale bar: 10 μm.)