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FAST TRACK

Endothelial progenitor cells do not contribute to tumor endothelium in primary and metastatic tumors

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Despite extensive research, the contribution of bone-marrow-derived endothelial progenitor cells (BM-EPC) to tumor angiogenesis remains controversial. In previous publications, the extent of incorporation of BM-EPCs into the endothelial cell (EC) layer in different tumor models has been reported as significant in some studies but undetectable in others. Here, we studied the differentiation of BM-EPCs and its contribution to tumor vessels in experimental and spontaneous lung metastasis (B16 melanoma and prostate carcinoma), in an autochthonous transgenic model of prostate tumorigenesis, in orthotopically implanted lung tumors [Lewis lung carcinoma (LLC)], in heterotopic subcutaneous models (LLC and C1 prostate carcinoma) growing in green fluorescent protein (GFP)-expressing bone marrow (BM) chimeras. Immunofluorescence was performed with a set of endothelial and hematopoietic markers and confocal microscopy was used to generate 3D reconstruction images. By performing rigorously conducted morphological studies, we found no evidence of BM-EPCs differentiation into tumor endothelium independently of tumor type, grade and organ site in primary and metastatic tumors. The vast majority of GFP^+ cells were trafficking leucocytes or periendothelial myeloid cells. To explore the possibility that local overexpression of vascular endothelial growth factor (VEGF) might increase the numbers of incorporated BM-EPCs, we analyzed tumors genetically manipulated to overexpress $VEGF_{164}$. Local VEGF production induces a massive infiltration of bone-marrow-derived cells, but did not lead to vessel wall integration of these cells. Collectively, these findings suggest that during tumor progression vascularization occurs primarily via classical tumor angiogenesis (*e.g.*, sprouting of pre-existing ECs), whereas BM-EPCs do not incorporate into the vessel wall to any significant extent.

Key words: endothelial progenitor cells; tumor vascularization; VEGF; angiogenesis; postnatal vasculogenenesis

The formation of new blood vessels is required for the growth and dissemination of cancer. Classically, tumor vascularization develops through the sprouting from existing vessels and co-option.[1] New evidence has shown that adult neovascularization may arise also from bone-marrow-derived endothelial progenitor cells (BM-EPCs) providing an alternative source of endothelial cells (ECs).[2–5] This concept has attracted considerable interest because BM-EPCs might represent a new target for pro- or anti-angiogenesis interventions. Besides BM-EPCs, different subsets of bone marrow (BM) cells are attracted to the tumor area, including tumor-associated macrophages (TAMs),[6] Tie2-expressing monocytes (TEMs),[7] neutrophils and mast cells.[8] The relationship between ECs, BM-EPCs, and these different subsets of myeloid cells are not completely understood. Although the hematopoietic myeloid cells might support tumor angiogenesis through paracrine mechanisms such as local expression of proangiogenic factors, BM-EPCs are thought to merge within the vessel wall, where they differentiate into ECs (a process defined as postnatal vasculogenesis).[2,3,9,10] However, an extensive variability regarding the contribution of postnatal vasculogenesis to the tumor vascularization has been described, with values ranging as high as 50% incorporated cells,[3,9,11,12] to undetectable numbers.[7,13–17] Recently, a major role of BM-EPCs has been proposed in models of pulmonary metastasis as critical regulators of the angiogenic switch.[18]

Initially, BM-EPCs were isolated from BM and peripheral blood[19] as cells expressing hematopoietic stem cell markers like CD34 or CD133[20] and for endothelial markers like vascular endothelial growth factor receptor-2 (VEGFR-2)[21] and VE-cadherin.[22] The absence of a specific marker, which identifies the BM-EPCs subpopulation certainly contributes to the wide discrepancy found in the different studies and have cast doubts about the role of BM-EPCs in tumor vascularization. This controversy may arise from the methodological difficulties to distinguish bone marrow-derived cells (BMDC) from intimately associated cells.[17,23] Another important source of variability is the choice of tumor model[24] and stages of tumor progression.[25] Several studies are based on subcutaneously growing tumors.[3,4] The rapid growth and development of the vascular network in these tumor models may not exactly resemble tumor growth in humans and the results obtained with such models may be not relevant to human malignancies. Data obtained from spontaneously arising murine tumors showed low levels of BM-EPCs[24] and a high variability of BMDC incorporation depending on tumor type and mouse strains used in the studies.[26]

Whereas the ability of VEGF to induce rapid mobilization of BMDC into the bloodstream[27,28] and to increase the number of circulating BM-EPCs[29–31] is well established, the role of VEGF in adult vasculogenesis still remains controversial. Although some reports suggest that local VEGF levels promote vasculogenesis,[2,30–32] other studies on VEGF-induced vascularization showed accumulation of myeloid cells, but no increase in the level of BM-EPCs vascular engraftment.[17,33,34] An extensive study using different VEGF sources as well as different BM chimeric mice could not demonstrate an increase in BM-EPCs contribution to endothelium.[17] Despite intensive work on this issue, the role of BM-EPCs contribution to tumor endothelium is still unresolved. Further studies are required to evaluate the BM-EPCs differentiation. In our study, by using confocal analysis and 3-dimensional reconstruction (3D) in different primary and metastatic tumor models, we provide evidence that independent of tumor histology, malignancy grade, organ site or local levels of VEGF, BM-EPCs do not differentiate into mature ECs and very rarely incorporate luminally into neovessels in untreated tumors.

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Material and methods

Cell lines and growth conditions

The transgenic model of the prostate tumor (TRAMP) epithelial cell line C1 has been established from the primary prostate tumors[35] and obtained generously from Dr. Pajong (Department of Radiotherapy, University of Freiburg Medical School). They were maintained as monolayer cultures in Dulbecco's modified Eagle medium (DMEM, Life Technologies) with 2 mM L-glutamine, supplemented with 5% fetal bovine serum (FBS, Life Technologies), 5% Nu-serum IV (Collaborative Biomedical Products), 5 μg/ml insulin and 25 U/ml penicillin/streptomycin.

Lewis lung carcinoma (LLC) cells and the murine melanoma cell line B16 were bought from American Type Culture Collection. Both cell lines were maintained as monolayer cultures in DMEM with 2 mM L-glutamine, supplemented with 10% FBS, 100 μg streptomycin and 100 U/ml penicillin.

GP + E86 virus producer cells encoding for the full length mouse $VEGF_{164}$ cDNA[36] were grown in DMEM containing 10% FCS and 0.2 mg/ml G418 (Calbiotech). LLC cells overexpressing $VEGF_{164}$ were established by retrovirus infection as described.[34] Twenty-four hours after infection, cells were incubated with medium containing 0.4 mg/ml G418 for selection. Colonies were harvested 14 days after cultivation in selection medium, expanded and assayed for VEGF expression. Characterization of VEGF overexpressing clones were performed as described.[15,34]

Animals

TRAMP mouse in a C57Bl/6 background (kindly provided by Dr. Norman M. Greenberg, Baylor Medical College, Houston, TX) is a transgene mouse carrying the −426/+28 fragment of the rat probasin gene fused to the SV40 T antigen.[37] This transgenic strain develops spontaneously an androgen-dependent prostate cancer at age of 18–30 weeks.

C57Bl/6J mice obtained from a colony of Animal Care of the University of Freiburg were used for implantation of syngeneic LLC, B16 or TRAMP C1 cells.

All procedures involving mice were conducted in accordance with protocols reviewed and approved by the local institutional Animal Care Committee.

Bone marrow transplantation

As donor strain we used C57Bl/6-Tg (ACTB-EGFP) 1Osb/J, which carries the enhanced green fluorescent protein (EGFP) driven by the universal chicken β-actin promoter and cytomegalovirus intermediate enhancer. All cell types in these animals express EGFP. BM cells were harvested aseptically by flushing the tibias and femurs of adult animals. Male TRAMP mice (12) and C57Bl/6J mice (20) aged between 6 and 11 weeks were lethally irradiated with 9 Gray of a cobalt source. After lethal irradiation, recipient mice received 5×10^6 unfractionated GFP-BM cells injected into the lateral tail vein.

Hematopoietic engraftment analysis

Donor cell engraftment efficiency was determined by FACS analysis. BM samples were collected at the time of autopsy as described earlier. Blood samples were obtained from tail vein before tumor implantation and from heart when the mice were sacrificed. BM cells were labeled with lineage-specific antibodies conjugated to PE (BD PharMingen, San Diego, CA) (Mac-1, CD45, CD117 and Flk-1). Samples were examined in 2-color FACS using Cellquest software (Becton Dickinson, Heildelberg, Germany). Engraftment was accessed by the percentage of GFP^+ cells in blood samples and in BM.

Tumor implantation

Six months after bone marrow transplantation (BMT), BM chimeric C57Bl/6J mice were injected s.c. with 2×10^6 TRAMP C1 cells or syngeneic LLC cells, resuspended in 100 μl of iced Matrigel (Becton-Dickinson, San Jose, CA). Intrapulmonary implantation of syngeneic LLC cells into the lung was performed as described.[38] For melanoma lung metastatic model, 3×10^5 B16 cells in 100 μl PBS were injected into the lateral tail vein.

Tumor preparation and immunohistochemistry

TRAMP mice were sacrificed at various time points (aged 24–40 weeks) after BMT. Prostate tumors and tumor-infiltrated seminal vesicles with macroscopic signs of infiltration as well as organs with macroscopically visible metastasis (lung and liver) were collected for histological analysis. S.c. TRAMP C1 tumors ($n = 3$) were explanted after 4.5 weeks and s.c. LLC tumors ($n = 6$) after 3 weeks. The lungs of mice implanted with LLC cells ($n = 4$) were removed at day 12 and the lungs from animals with B16 metastases ($n = 3$) were harvested after 2 weeks. The mice were anesthetized and perfused transcardially with 2% paraformaldehyde (PFA) in PBS. Organs were explanted, postfixed in 2% PFA overnight at 4°C and cryoprotected with overnight soaking in 0.5 M sucrose at 4°C. They were snap frozen in liquid-nitrogen-cooled 2-methylbutane. Cryosections (16–30 μm) were stained with the following antibodies: Anti-mouse CD45 (rat, Cymbus Biotechnology, 1:100), anti-mouse F4/80 (rat, Serotec, 1:100), anti-mouse CD31 (rat, BD Biosciences, 1:100), anti-mouse CD105 (rat, Southern Biotech, 1:100), anti-human von Willebrand Factor (rabbit, DAKO, Denmark, 1:120), anti-VE-Cadherin (rat, BD Biosciences, 1:50), anti-alpha SMA, used to identify pericytes (mouse, Sigma, 1:500), anti-GFP (rabbit, Molecular Probes,1:200) and anti-VEGFR-2 (anti-mouse flk-1, goat, R&D Systems, 1:10). Antigen retrieval was performed using Proteinase K (20 μg/ml) in TE buffer (pH 8.0) at 37°C for 15–30 min and following 5–10 min of incubation at room temperature. Sections were blocked for nonspecific antibody binding with 2% normal goat serum (except for anti-flk-1, which was blocked with 1% bovine serum albumin in PBS), followed by incubation with primary antibodies. After 3 washes with PBS, sections were incubated with AlexaFluor555-, AlexaFluor 546-or AlexaFluor647-conjugated goat anti-rat, goat anti-rabbit or donkey anti-goat immunoglobulins (all Molecular Probes, 1:800). Controls were performed by omitting the primary antibody or by using unspecific immunoglobulins. After nuclei staining with DAPI sections were mounted. For some applications, anti-CD45 and anti-alpha SMA antibody were directly labeled with AlexaFluor647 following the manufacturer's instructions (Monoclonal Antibody Labeling, Invitrogen).

Confocal and 3-dimensional reconstruction

Tissue sections were analyzed using confocal microscopes (Leica TCSNT and Leica TCS SP2AOBS). GFP was imaged with excitation at 495 nm and emission at 510 nm, AlexaFluor555 with excitation at 547 nm and emission at 572 nm and AlexaFluor647 was imaged with excitation at 650 nm and emission at 670 nm. DAPI staining was imaged with excitation at 345 nm and emission at 455 nm. Micrographs were acquired using 20× or 40× oil-immersion objective lens. For 3-dimensional reconstruction, data were analyzed using Imaris 5.2.2 imaging software.

Quantification of GFP infiltration

Cryosections of different tumors retrieved from GFP-transplanted mice were analyzed with the fluorescence microscope DMRX (Leica, Heerbrug, Switzerland) using a 20× objective lens and documented with the imaging system IM1000 (Leica). Quantification of GFP^+ cell infiltration was carried out using ImageJ software.

Results

In the spontaneous prostate tumor model, BMDC do not differentiate into vascular cells

To determine the participation of adult BM-EPCs to tumor neovascularization in an autochthonous transgenic model of prostate carcinoma, we transplanted unfractionated GFP-BM cells into

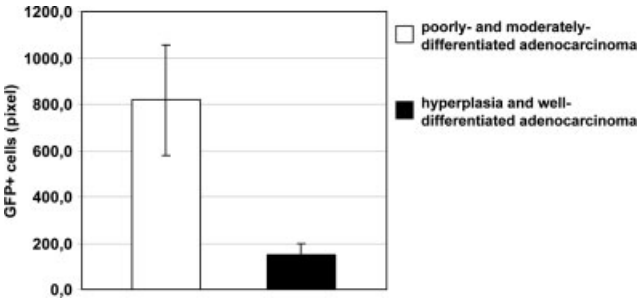

FIGURE 1 – Recruitment of BMDCs increases in an autochthonous model of prostate cancer in tumors of more malignant grade. BMDC recruitment was quantified in the spontaneous prostate tumors. Poorly and moderately differentiated tumors ($n = 3$) were compared with hyperplasia and well-differentiated prostate tumors ($n = 4$). At least 10 sections per animal were analyzed. Quantification of pixels corresponds to GFP^+ cells. Histograms represent mean value. Error bars represents standard deviation.

lethally irradiated male TRAMP recipients, thus creating GFP-TRAMP chimeric mice.

To evaluate the efficiency of engraftment of donor cells, we quantified the number of GFP^+ in peripheral blood (before tumor implantation and at the time of autopsy) and in the BM cells when animals were sacrificed. FACS analysis of BM cells and peripheral blood confirmed stable and complete chimerism. Only chimeric mice showing BM-chimerism >85% of GFP^+ cells in peripheral blood (similar levels of GFP^+ cells was observed in peripheral blood of GFP transgenic donor mice) were included in this study (data not shown).

Animals were sacrificed at different ages (ranging from 24 to 40 weeks) so that tumors in different developmental stages could be explanted. We observed a delay in prostate tumor growth after lethal irradiation. Mice sacrificed at 16 or 20 weeks did not show any pathological tissue in the prostate and were not included in this study. Tumors were classified in hyperplasia, well-differentiated, moderately or poorly differentiated adenocarcinomas according to histology patterns of TRAMP (for review: http://thegreenberglab.fhcrc.org/research/research_tools.html).

As expected a high number of GFP^+ BMDC were recruited to the tumors. The detection of GFP endogenous signals was controlled by staining some tumor probes with an anti-GFP. In PFA fixed tissues, we observed that the detection of endogenous GFP signals matched the detection using an anti-GFP antibody (data not shown). We determined the effect of the tumor grade on BMDC recruitment in the spontaneous prostate tumor model. A quantitative analysis (Fig. 1) demonstrated that the recruitment of BMDC was significantly increased in poorly and moderately differentiated prostate tumors compared with prostate tumors of well-differentiated grade and preneoplastic hyperplasia. We focused on the BM-EPCs cells that directly contribute to the neovascularization. For this purpose, we undertook an extensive immunological characterization by means of confocal microscopy followed by 3D reconstruction analyses. We investigated around 100 tumor vessels per tumor grade in serial sections using a set of hematopoietic and endothelial specific markers. As shown in Figures 2*a* and 2*b*, all infiltrating BMDC were positive for the hematopoietic marker CD45. Remarkably, in poorly differentiated prostate carcinomas, very few vessels (<1%) showed luminal incorporation of GFP^+ cells. However, these cells did not express EC markers such as CD31, CD105, von Willebrand Factor, Flk-1 and VE-cadherin but in contrast retained the hematopoietic differentiation, which is determined by the expression of CD45 (Figs. 2*c*, 3*a* and 3*b*).

To explore whether vasculogenesis is site-dependent, we analyzed subcutaneously isografted C1 TRAMP cells into syngeneic GFP BM chimeras (Fig. 2*d*). Again abundant BMDCs were recruited close to the vessel wall, but did not participate in its formation by physical integration.

The vast majority of GFP^+ cells expressed the macrophage marker F4/80. No colocalization of cells with endothelial markers could be found, independently of tumor grade, therefore indicating that tumor endothelium was derived from the host (Fig. 3*b*).

No incorporation of BMDC in different primary and metastatic lung tumor tissues

Next, we determined whether different tumor types growing orthotopically in lung tissue might lead to different rates of incorporation and transdifferentiation of BMDC into ECs. Chimeric mice transplanted with GFP + BM were either implanted intrapulmonary with LLC cells (Fig. 4*a* and Supporting Information Figure S1A) or implanted intravenously with the murine melanoma cell line B16 (Fig. 4*b*). In addition, we examined spontaneous lung metastasis in a TRAMP mouse (37 weeks old) with a poorly differentiated prostate adenocarcinoma (Fig. 4*c*). As shown in Figure 4, we could not detect BMDC incorporation into the EC layer in any of these lung tumors. Some GFP^+ cells showed an elongated shape and were closely related to the vascular lumen. However, by using 3D reconstruction, we determined that these cells are located outside the EC layer (Fig. 4*c*).

Effect of local VEGF expression on BMDC recruitment to sites of tumor neovascularization

To investigate the recruitment of BMDC by VEGF overexpression, GFP chimeric C57Bl/6 mice were injected s.c. with LLC-VEGF or mock-transfected LLC cells. Tumors were explanted 3 weeks postimplantation. The quantification of BMDC recruitment was performed on cryosections costained with DAPI. The results revealed a significant increase of BMDC in animals implanted with LLC-VEGF compared with the GFP infiltration in mock-transfected tumors (Fig. 5*a*). Although increased VEGF levels enhanced the number of BMDC in the subcutaneously implanted LLC tumors, we could not detect any contribution of marrow-derived cells to tumor endothelium (Fig. 5*b*).

Discussion

The integration of BM-EPCs into tumor vessels is not only of biological interest *per se* because convincing evidence for a functional incorporation may give rise to novel therapeutical concepts. The clinical relevance of BM-EPCs in the context of human cancer has been supported by the studies of Peters *et al.*[12] In this study, the authors showed a vascular incorporation of BMDC ranging between 2 and 12% in tumor tissues from patients submitted to gender-mismatched BM transplantation. In clinical and preclinical settings, it has been proposed that circulating BM-EPCs can be used as surrogate marker to help monitor antiangiogenic approaches.[23,39] Nevertheless, the ability of BM-EPCs to form blood vessels remains controversial. In several studies, though the contribution of BM-EPCs to tumor vascularization has been shown to be significant, in other reports their existence is considered artifactual rather than physiological.[3,13,16,17,40,41] One source of variability is the use of different methods for distinguishing between "true" vessel incorporation versus intimate perivascular location. The use of a high-resolution sequential confocal scanning capable of visualizing single cells constitutes an important tool to disclose the relationship of BMDCs and ECs in the vasculature.[13,17,42] In this study, we demonstrated that 3D reconstruction of multichannel microscopy pictures is required to accurately identify luminal incorporation and distinguish superimposition from true colocalization. Our data provide further evidence that BMDC do not differentiate into mature endothelial or mural cells.

Other potential reasons for the conflicting reports may arise from the tumor models used and the organ site studied. Ruzinova *et al.*[24] showed that the angiogenic response of xeno- or isografted tumors generated by tumor cell inoculation is very different from

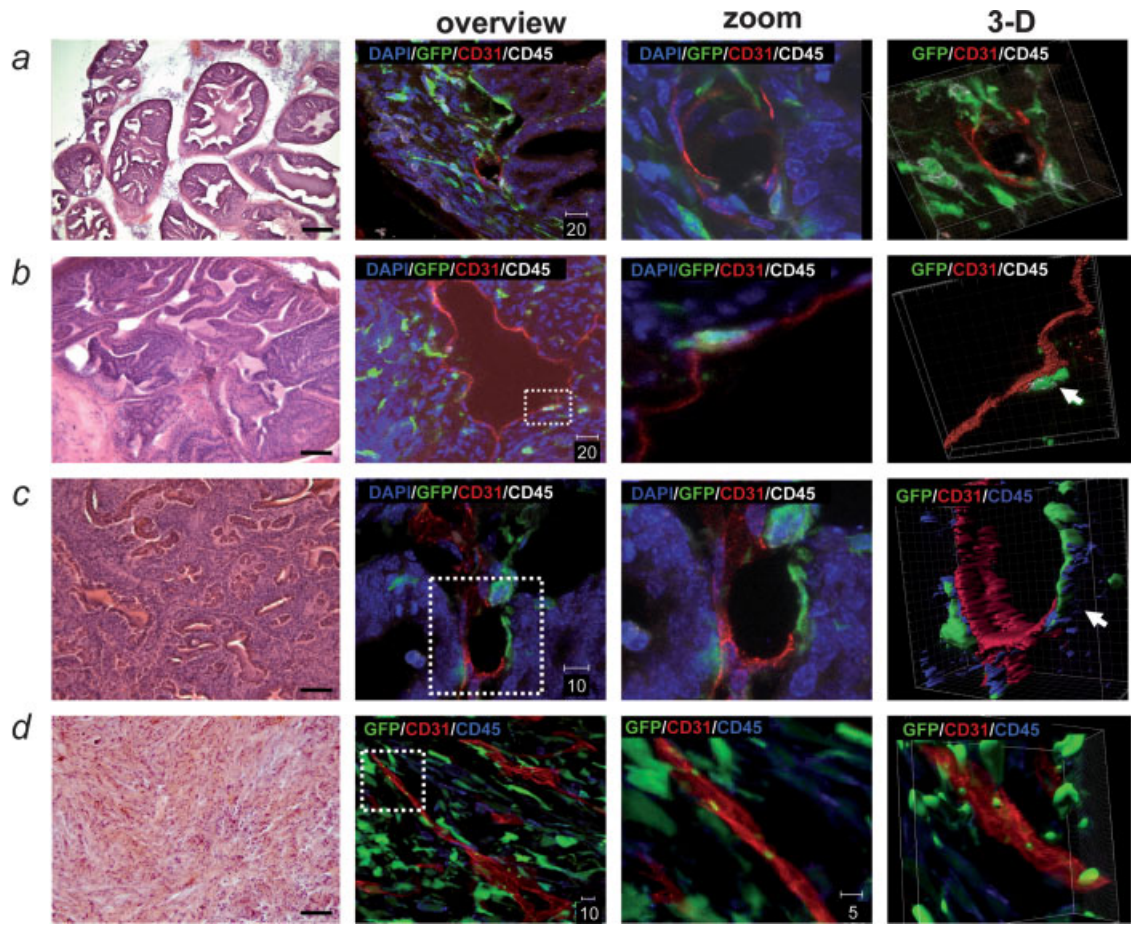

FIGURE 2 – BM-EPCs do not differentiate into vascular phenotype in the TRAMP transgenic model of the prostate tumor. Tumor tissues were stained with DAPI, CD31 and CD45. Seventy vessels were analyzed from every different tumor grade using high-resolution multichannel confocal scanning of thick sections to generate 3D visualization. Representative section of a prostate hyperplasia (*a*) of a well-differentiated prostate adenocarcinoma (*b*), of a poorly differentiated adenocarcinoma (*c*) and of a subcutaneously implanted prostate C1 tumors (*d*). A high magnification of the boxed area (zoom) and 3D reconstruction (projection) show that $GFP^+/CD45^+$ cells are closely related to $CD31^+$ endothelial cells (*b*, arrow), but do not participate in tumor endothelium. In poorly differentiated adenocarcinomas, we observed rare incorporation of $GFP^+/CD45^+$ into the vascular lumen (*c*, arrow). Scale bars on HE-stained tissue images: 100 μm.

that of spontaneously arising tumor models. Also, their demand for BM-EPCs to initiate and maintain tumor angiogenesis may be distinct. Li *et al*.[25] have reported that the contribution of BM-EPCs into the vasculature of the TRAMP tumor model varies depending on the tumor grade, with increased recruitment of BM-EPCs cells into blood vessels of poorly differentiated prostate carcinoma. The authors estimated the amount of BM-EPC contribution by X-gal staining in LacZ BM transplants. Our studies confirm that an increase in BMDC infiltration is observed in more malignant prostate tumors. However, we did not observe a colocalization between GFP and endothelial markers, neither in nascent (hyperplasia) nor in large end-stage tumors (infiltrative adenocarcinomas). Possibly, the X-gal detection by light microscopy might result in overestimation of BM-derived vessels. To distinguish BM-EPCs from local vessel-derived ECs several studies have used tagged BM cells expressing a reporter gene under the Tie-2 promotor. Tie-2 is expressed by ECs, but also by hematopoietic cells. Therefore, Tie-2 is not a reliable indicator of the endothelial phenotype, hence determining donor cells differentiation requires colocalization with other markers.[7,15] However, by using a universal promoter to drive the expression of GFP, we cannot completely exclude that the high amount of GFP^+ cells infiltrating the tumor tissue might mask rarely incorporated BM-EPCs.

We observed only very few incorporated donor BMDC expressing the panhematopoietic marker CD45 in more malignant prostate carcinoma. Whether BM-EPCs express CD45 is still controversial. On the basis of FACS blood analysis, some authors have defined BM-EPCs as $CD45^{dim}$ $CD34^+VEGFR\text{-}2^+CD133^+$.[39] So far, a clear definition of the marker profile of BM-EPCs is largely missing. As proposed by Conejo-Garcia *et al*.,[32] leucocytes might undergo endothelial-like specialization under the influence of VEGF-A. Given the fact that in this study evidence for true endothelial incorporation was very rare (in $<1\%$ of the vessels), we consider that this process might be biological irrelevant during tumor progression. However, we cannot exclude in this study that BM-EPCs might play an essential role in the neovascularization of relapsing tumors after the administration of vascular disrupting agents, as proposed by Shaked *et al*.[4]

More recently, the contribution of BM-EPCs to metastatic tumors became a matter of debate in the literature. Whereas Gao *et al*.[18] have demonstrated that BM-EPCs are critical regulators of the angiogenic switch in mouse models of pulmonary metastasis, Duda *et al*.[26] using flow cytometry of cell suspensions from B16 and LLC lung metastasis observed only a minimal incorporation of BM-EPCs in genetically unmodified syngeneic C57Bl6 animals. Another reason for the discordant findings may result from the intrinsic dependence of tumor vessels on BM-EPCs in different histological types or stage of tumor development. By examining 3 different lung orthotopically growing tumors, we did

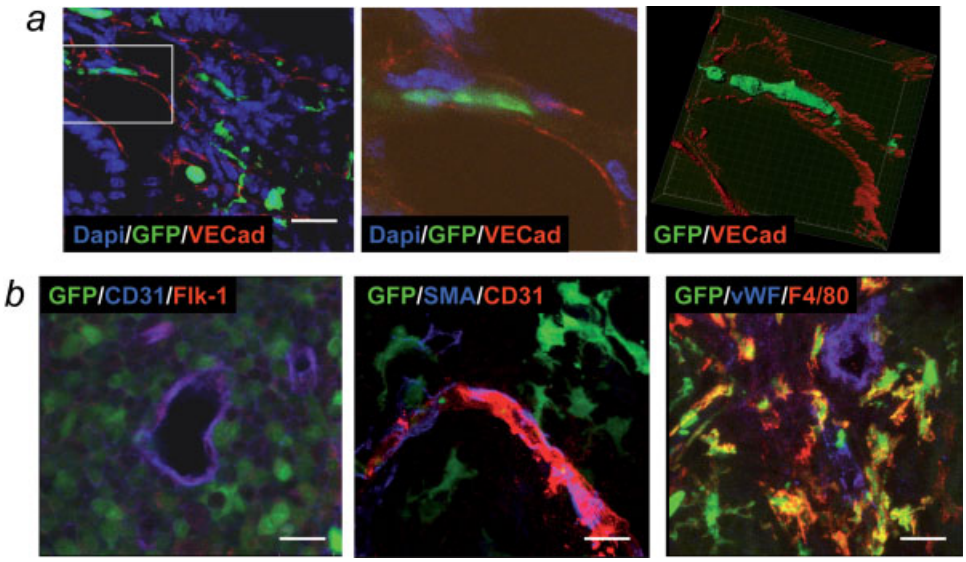

FIGURE 3 – Representative section of a prostate tumor stained for VE-Cadherin showing a GFP^+ cell closely apposed to the vascular wall (*a*). Representative pictures of different tumors showing no colocalization of GFP with Flk-1 or with SMA. The vast majority of GFP^+ cells are expressing the macrophage marker F40/80 (*b*). Scale bars: 20 μm.

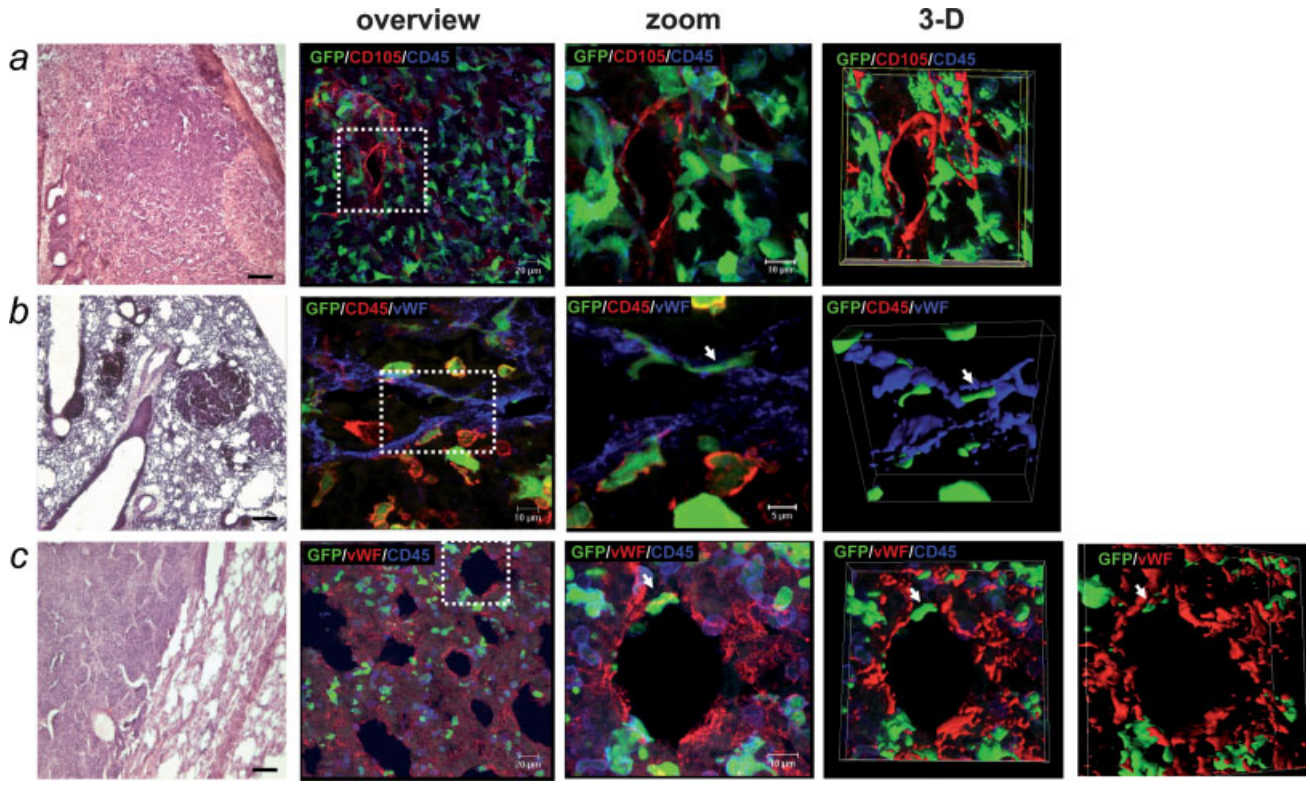

FIGURE 4 – No incorporation of BM-EPCs in different tumor types growing in lung tissue. Lethal irradiated C57Bl/6 mice were transplanted with GFP^+ BM cells and implanted with LLC cells (*a*) or B16 melanoma cells (*b*) 6 months after bone marrow transplantation. Spontaneously arising lung metastases were retrieved from a TRAMP mouse with a poorly differentiated adenocarcinoma (*c*). Several $GFP^+/CD45^+$ cells were closely apposed to the endothelium (arrows). Three-dimensional reconstruction of confocal images disclosed that the GFP^+ cell is lying outside the endothelial layer (*c*, white arrows). Scale bars on HE-stained tissue images: 100 μm.

not detect BMDC that differentiated into endothelium. We studied LLC and B16 tumors at day 12 and day 14, respectively, thus matching the time points for initiation of the angiogenic switch mediated by BM-EPCs in early tumors as postulated by Gao *et al*. Thus, regardless of the histological type, our data indicate that vasculogenesis in primary and metastatic tumors is marginal to undetectable.

Finally, we evaluated the effect of a local release of VEGF on BM-EPCs contribution to tumor vasculogenesis. Several previous studies have demonstrated a positive role of VEGF on BM-EPCs

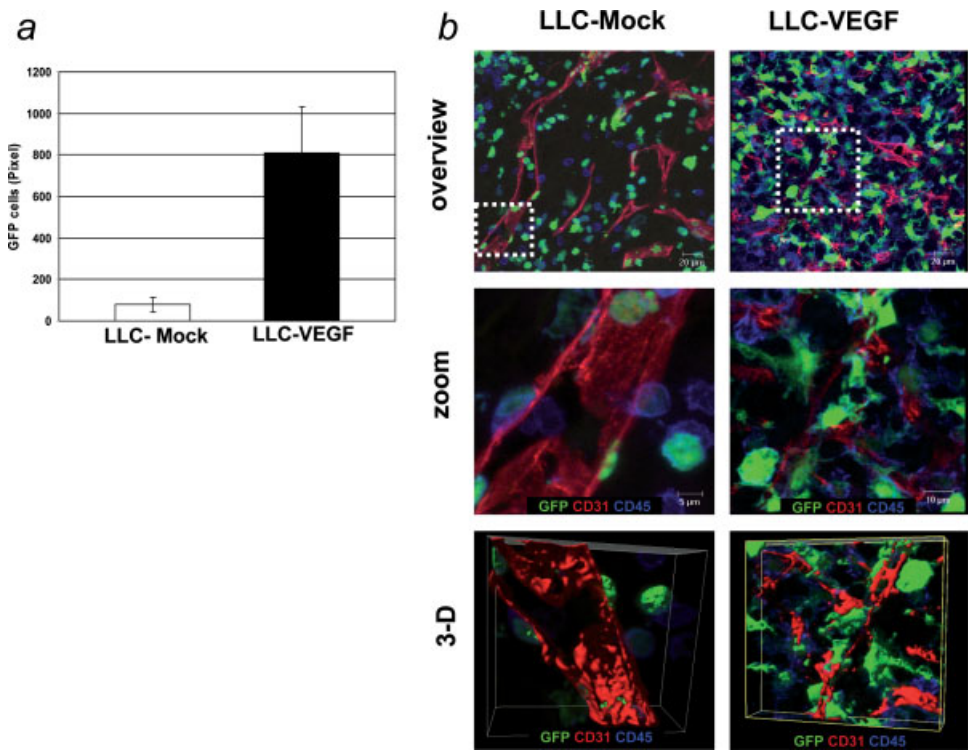

FIGURE 5 – Local levels of VEGF increase BMDC infiltration in tumor tissue but do not increase BM-EPCs incorporation. (*a*) LLC-overexpressing $VEGF_{164}$ (LLC-VEGF) or mock-transfected LLC (LLC-mock) cells were implanted in GFP-BM chimeras. For analysis of the GFP area fraction (in pixels), tumor sections (at least 10 sections/animal) were visualized at 20× magnification and quantified using Image J software. Error bars represent standard deviations. The histogram represents mean values of GFP^+ cells (percentage in pixel) in LLC tumors. Error bars represent the standard deviation. (*b*) Confocal images of sections stained with CD31 and CD45 show no incorporation of GFP^+ cells into tumor endothelium and no colocalization of GFP^+ cells and endothelial markers.

incorporation into the vasculature.[2,30–32] In contrast, a recent study demonstrated the lack of BM-EPCs incorporation after VEGF stimulation.[17] Similarly, even though Ziegelhoeffer *et al.*[42] demonstrated that BM-EPCs number increased in peripheral blood after VEGF treatment, they did not observe marrow-derived cell incorporation into the vascular wall. In our study, quantitative analysis of BM-derived GFP^+ cells revealed a dramatic increase in tumor inflammatory infiltration, but again no incorporation of BM-EPCs into tumor vessels was observed. These results confirm previous findings from our laboratory showing that although VEGF enhances the recruitment of BMDC to the tumor tissue, these cells do not function as BM-EPCs.[34] In line with these results, Grunewald *et al.*[33] did not find any BMDC incorporation into the vasculature when VEGF is locally overexpressed in different organs supporting a paracrine key role of the BM circulating cells in the tumor neovasculogenesis.

The dispute on the functional role of BM-EPCs is matter of >50 reports in the recent literature (for review see Ref. 39,43). Using distinct methodologies, the existence of BM-EPCs has been established in certain models and disproved in others. We did not observe any relevant contribution of BM-EPCs in different types of primary and metastatic tumors using carefully conducted morphological studies. Therefore, we conclude that vascularization of tumor relies on the host ECs and not on the differentiation capacity of BM-EPCs.

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References

1. Carmeliet P. Angiogenesis in health and disease. Nat Med 2003;9:653–60.
2. Asahara T, Takahashi T, Masuda H, Kalka C, Chen D, Iwaguro H, Inai Y, Silver M, Isner JM. VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. EMBO J 1999;18:3964–72.
3. Lyden D, Hattori K, Dias S, Costa C, Blaikie P, Butros L, Chadburn A, Heissig B, Marks W, Witte L, Wu Y, Hicklin D, et al. Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. Nat Med 2001;7:1194–201.
4. Shaked Y, Ciarrocchi A, Franco M, Lee CR, Man S, Cheung AM, Hicklin DJ, Chaplin D, Foster FS, Benezra R, Kerbel RS. Therapy-induced acute recruitment of circulating endothelial progenitor cells to tumors. Science 2006;313:1785–7.
5. Takahashi T, Kalka C, Masuda H, Chen D, Silver M, Kearney M, Magner M, Isner JM, Asahara T. Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. Nat Med 1999;5:434–8.
6. Lin EY, Nguyen AV, Russell RG, Pollard JW. Colony-stimulating factor 1 promotes progression of mammary tumors to malignancy. J Exp Med 2001;193:727–40.
7. De Palma M, Venneri MA, Galli R, Sergi SL, Politi LS, Sampaolesi M, Naldini L. Tie2 identifies a hematopoietic lineage of proangiogenic monocytes required for tumor vessel formation and a mesenchymal population of pericyte progenitors. Cancer Cell 2005;8:211–26.
8. 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;6:409–21.
9. Spring H, Schuler T, Arnold B, Hammerling GJ, Ganss R. Chemokines direct endothelial progenitors into tumor neovessels. Proc Natl Acad Sci USA 2005;102:18111–6.
10. Nolan DJ, Ciarrocchi A, Mellick AS, Jaggi JS, Bambino K, Gupta S, Heikamp E, McDevitt MR, Scheinberg DA, Benezra R, Mittal V. Bone marrow-derived endothelial progenitor cells are a major determinant of nascent tumor neovascularization. Genes Dev 2007; 21:1546–58.
11. Garcia-Barros M, Paris F, Cordon-Cardo C, Lyden D, Rafii S, Haimovitz-Friedman A, Fuks Z, Kolesnick R. Tumor response to radiotherapy regulated by endothelial cell apoptosis. Science 2003; 300:1155–9.
12. Peters BA, Diaz LA, Polyak K, Meszler L, Romans K, Guinan EC, Antin JH, Myerson D, Hamilton SR, Vogelstein B, Kinzler KW, Lengauer C. Contribution of bone marrow-derived endothelial cells to human tumor vasculature. Nat Med 2005;11:261–2.
13. De Palma M, Venneri MA, Roca C, Naldini L. Targeting exogenous genes to tumor angiogenesis by transplantation of genetically modified hematopoietic stem cells. Nat Med 2003;9:789–95.
14. Taverna D, Moher H, Crowley D, Borsig L, Varki A, Hynes RO. Increased primary tumor growth in mice null for beta3- or beta3/beta5-integrins or selectins. Proc Natl Acad Sci USA 2004;101:763–8.
15. Machein MR, Renninger S, Lima-Hahn E, Plate KH. Minor contribution of bone marrow-derived endothelial progenitors to the vascularization of murine gliomas. Brain Pathol 2003;13:582–97.
16. Gothert JR, Gustin SE, van Eekelen JA, Schmidt U, Hall MA, Jane SM, Green AR, Gottgens B, Izon DJ, Begley CG. Genetically tagging endothelial cells in vivo: bone marrow-derived cells do not contribute to tumor endothelium. Blood 2004;104:1769–77.
17. Purhonen S, Palm J, Rossi D, Kaskenpaa N, Rajantie I, Yla-Herttuala S, Alitalo K, Weissman IL, Salven P. Bone marrow-derived circulating endothelial precursors do not contribute to vascular endothelium and are not needed for tumor growth. Proc Natl Acad Sci USA 2008;105:6620–5.
18. Gao D, Nolan DJ, Mellick AS, Bambino K, McDonnell K, Mittal V. Endothelial progenitor cells control the angiogenic switch in mouse lung metastasis. Science 2008;319:195–8.
19. Asahara T, Murohara T, Sullivan A, Silver M, van der ZR, Li T, Witzenbichler B, Schatteman G, Isner JM. Isolation of putative progenitor endothelial cells for angiogenesis. Science 1997;275:964–7.
20. Gehling UM, Ergun S, Schumacher U, Wagener C, Pantel K, Otte M, Schuch G, Schafhausen P, Mende T, Kilic N, Kluge K, Schafer B, et al. In vitro differentiation of endothelial cells from AC133-positive progenitor cells. Blood 2000;95:3106–12.
21. Peichev M, Naiyer AJ, Pereira D, Zhu Z, Lane WJ, Williams M, Oz MC, Hicklin DJ, Witte L, Moore MA, Rafii S. Expression of VEGFR-2 and AC133 by circulating human CD34(+) cells identifies a population of functional endothelial precursors. Blood 2000;95:952–8.
22. Nishikawa SI, Nishikawa S, Hirashima M, Matsuyoshi N, Kodama H. Progressive lineage analysis by cell sorting and culture identifies FLK1+VE-cadherin+ cells at a diverging point of endothelial and hemopoietic lineages. Development 1998;125:1747–57.
23. Shaked Y, Bertolini F, Man S, Rogers MS, Cervi D, Foutz T, Rawn K, Voskas D, Dumont DJ, Ben David Y, Lawler J, Henkin J, et al. Genetic heterogeneity of the vasculogenic phenotype parallels angiogenesis: implications for cellular surrogate marker analysis of antiangiogenesis. Cancer Cell 2005;7:101–11.
24. Ruzinova MB, Schoer RA, Gerald W, Egan JE, Pandolfi PP, Rafii S, Manova K, Mittal V, Benezra R. Effect of angiogenesis inhibition by Id loss and the contribution of bone-marrow-derived endothelial cells in spontaneous murine tumors. Cancer Cell 2003;4:277–89.
25. Li H, Gerald WL, Benezra R. Utilization of bone marrow-derived endothelial cell precursors in spontaneous prostate tumors varies with tumor grade. Cancer Res 2004;64:6137–43.
26. Duda DG, Cohen KS, Kozin SV, Perentes JY, Fukumura D, Scadden DT, Jain RK. Evidence for incorporation of bone marrow-derived endothelial cells into perfused blood vessels in tumors. Blood 2006;107:2774–6.
27. Hattori K, Dias S, Heissig B, Hackett NR, Lyden D, Tateno M, Hicklin DJ, Zhu Z, Witte L, Crystal RG, Moore MA, Rafii S. Vascular endothelial growth factor and angiopoietin-1 stimulate postnatal hematopoiesis by recruitment of vasculogenic and hematopoietic stem cells. J Exp Med 2001;193:1005–14.
28. Moore MA, Hattori K, Heissig B, Shieh JH, Dias S, Crystal RG, Rafii S. Mobilization of endothelial and hematopoietic stem and progenitor cells by adenovector-mediated elevation of serum levels of SDF-1, VEGF, and angiopoietin-1. Ann NY Acad Sci 2001;938:36–45.
29. Kalka C, Masuda H, Takahashi T, Gordon R, Tepper O, Gravereaux E, Pieczek A, Iwaguro H, Hayashi SI, Isner JM, Asahara T. Vascular endothelial growth factor(165) gene transfer augments circulating endothelial progenitor cells in human subjects. Circ Res 2000; 86:1198–202.
30. 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;99:11951–6.
31. Li B, Sharpe EE, Maupin AB, Teleron AA, Pyle AL, Carmeliet P, Young PP. VEGF and PlGF promote adult vasculogenesis by enhancing EPC recruitment and vessel formation at the site of tumor neovascularization. FASEB J 2006;20:1495–7.
32. Conejo-Garcia JR, Benencia F, Courreges MC, Kang E, Mohamed-Hadley A, Buckanovich RJ, Holtz DO, Jenkins A, Na H, Zhang L, Wagner DS, Katsaros D, et al. Tumor-infiltrating dendritic cell precursors recruited by a beta-defensin contribute to vasculogenesis under the influence of Vegf-A. Nat Med 2004;10:950–8.
33. Grunewald M, Avraham I, Dor Y, Bachar-Lustig E, Itin A, Jung S, Chimenti S, Landsman L, Abramovitch R, Keshet E. VEGF-induced adult neovascularization: recruitment, retention, and role of accessory cells. Cell 2006;124:175–89.
34. Kerber M, Reiss Y, Wickersheim A, Jugold M, Kiessling F, Heil M, Tchaikovski V, Waltenberger J, Shibuya M, Plate KH, Machein MR. Flt-1 signaling in macrophages promotes glioma growth in vivo. Cancer Res 2008;68:7342–51.
35. Foster BA, Gingrich JR, Kwon ED, Madias C, Greenberg NM. Characterization of prostatic epithelial cell lines derived from transgenic adenocarcinoma of the mouse prostate (TRAMP) model. Cancer Res 1997;57:3325–30.
36. Sasaki M, Wizigmann-Voos S, Risau W, Plate KH. Retrovirus producer cells encoding antisense VEGF prolong survival of rats with intracranial GS9L gliomas. Int J Dev Neurosci 1999;17:579–91.
37. Greenberg NM, DeMayo F, Finegold MJ, Medina D, Tilley WD, Aspinall JO, Cunha GR, Donjacour AA, Matusik RJ, Rosen JM. Prostate cancer in a transgenic mouse. Proc Natl Acad Sci USA 1995;92:3439–43.
38. Doki Y, Murakami K, Yamaura T, Sugiyama S, Misaki T, Saiki I. Mediastinal lymph node metastasis model by orthotopic intrapulmonary implantation of Lewis lung carcinoma cells in mice. Br J Cancer 1999;79:1121–6.
39. Bertolini F, Shaked Y, Mancuso P, Kerbel RS. The multifaceted circulating endothelial cell in cancer: towards marker and target identification. Nat Rev Cancer 2006;6:835–45.
40. Zentilin L, Tafuro S, Zacchigna S, Arsic N, Pattarini L, Sinigaglia M, Giacca M. Bone marrow mononuclear cells are recruited to the sites of VEGF-induced neovascularization but are not incorporated into the newly formed vessels. Blood 2006;107:3546–54.
41. 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;85:221–8.
42. Ziegelhoeffer T, Fernandez B, Kostin S, Heil M, Voswinckel R, Helisch A, Schaper W. Bone marrow-derived cells do not incorporate into the adult growing vasculature. Circ Res 2004;94:230–8.
43. Rafii S, Lyden D. Therapeutic stem and progenitor cell transplantation for organ vascularization and regeneration. Nat Med 2003;9:702–12.