Cell

VEGF-Induced Adult Neovascularization: Recruitment, Retention, and Role of Accessory Cells

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DOI 10.1016/j.cell.2005.10.036

SUMMARY

Adult neovascularization relies on the recruitment of circulating cells, but their angiogenic roles and recruitment mechanisms are unclear. We show that the endothelial growth factor VEGF is sufficient for organ homing of circulating mononuclear myeloid cells and is required for their perivascular positioning and retention. Recruited bone marrow-derived circulating cells (RBCCs) summoned by VEGF serve a function distinct from endothelial progenitor cells. Retention of RBCCs in close proximity to angiogenic vessels is mediated by SDF1, a chemokine induced by VEGF in activated perivascular myofibroblasts. RBCCs enhance in situ proliferation of endothelial cells via secreting proangiogenic activities distinct from locally induced activities. Precluding RBCCs strongly attenuated the proangiogenic response to VEGF and addition of purified RBCCs enhanced angiogenesis in excision wounds. Together, the data suggest a model for VEGF-programmed adult neovascularization highlighting the essential paracrine role of recruited myeloid cells and a role for SDF1 in their perivascular retention.

INTRODUCTION

Formation of new blood vessels in adult organs is fundamentally different from developmental neovascularization. Whereas developmental vasculogenesis relies on a reservoir of local angioblasts, adult neovascularization may utilize circulating endothelial progenitor cells (EPCs) mobilized from the bone marrow (BM) and eventually incorporated within the forming vasculature at a distant organ (Asahara et al., 1999; Asahara et al., 1997; Lyden et al., 2001; Rafii and Lyden, 2003). The relative contribution of circulating EPCs to adult organ and tumor vasculature is, however, highly variable, and may range from a minor (Machein et al., 2003; Peters et al., 2005; Rajantie et al., 2004; Wagers et al., 2002; Ziegelhoeffer et al., 2004) to a major contribution (Garcia-Barros et al., 2003; Hattori et al., 2001), presumably reflecting also differences in the genetic background, the organ (or tumor) involved (Ruzinova et al., 2003), and the nature of the angiogenic stimulus.

Recently, increased attention has been directed to other populations of BM-derived cells recruited to sites of ongoing angiogenesis but that do not function as EPCs. These cells might nevertheless contribute to neovessel formation (De Palma et al., 2003; Takakura et al., 2000) by a yet-unknown mechanism. The present study focuses on the angiogenic roles of these cells, collectively defined here as recruited bone marrow-derived circulating cells (RBCCs) and on their homing mechanisms.

Elucidating the angiogenic roles of RBCCs is of a particular significance in light of the need to reconcile the apparent limited contribution of EPCs with the apparent beneficial effect of autologous BM cells administered to ischemic tissues (Kalka et al., 2000; Kocher et al., 2001), findings that have already prompted clinical trials (Schachinger et al., 2004). In addition, understanding the unknown mechanisms underlying RBCC homing may provide potential new targets for augmenting or, conversely, attenuating angiogenesis.

Another unique aspect of adult neovascularization is that, unlike the hardwired program of developmental angiogenesis, it is usually triggered by either environmental stress (e.g., hypoxia) or by stochastic genetic changes in tumors. These incidental stimuli usually culminate in upregulated expression of VEGF. It is, therefore, mechanistically unclear how VEGF single-handedly orchestrates a complete angiogenic program, a task known to be executed by multiple and often

nonredundant factors. For example, the fact that VEGF is a relatively weak endothelial cell mitogen suggests that additional EC mitogens are likely to be recruited. The present study addresses the proposition that VEGF may recruit complementary proangiogenic activities, not only through local upregulation of proangiogenic genes but also through import of ''accessory'' cells that provide them. Accordingly, a mechanism must exist by which VEGF acts to recruit accessory cells and position them close to angiogenic vessels within the target organ.

To address these issues, we devised a transgenic system for conditional and reversible induction of VEGF in selected adult organs. The system has several advantages over previously employed systems of VEGF-induced neovascularization in the adult. First, as a ''clean'' genetic switch system, it circumvents confounding factors associated with other modes of VEGF delivery, such as stress, inflammatory, and immunological responses. Notably, it allows examining VEGF functions in the absence of hypoxia, an environmental factor recently shown to influence trafficking of hematopoietic cells (Ceradini et al., 2004). Second, the synchronous and robust reaction to VEGF enables to summon onto an organ of choice circulating cells in quantities allowing their isolation and subsequent characterization. Third, the possibility to switch off VEGF production at any desired schedule allows distinguishing roles of VEGF in initial recruitment of circulating cells from a role in maintaining incoming cells in the organ.

Here, we provide evidence that VEGF induces perivascular expression of the chemokine SDF1 that functions, in turn, to position RBCCs in this strategic location from which they act in a paracrine fashion to enhance in situ proliferation of resident, activated endothelial cells.

RESULTS

A Genetic System for Conditional and Reversible Induction of VEGF in Selected Adult Organs

To induce formation of new blood vessels in selected adult organs, a transgenic system in which VEGF expression is induced at will in the respective organ, steadily maintained for the desired duration, and subsequently can be switched off was designed. Briefly, transgenic mice expressing a tetracycline-regulated transactivator protein (tTA) exclusively in the myocardium or liver (driver lines) were mated with transgenic mice harboring a VEGF164-encoding transgene driven by a tetracycline-responsive promoter (responder line). Pups that inherited both transgenes were selected for modulating VEGF expression, whereas littermates that inherited only one of the two transgenes served as controls. The onset of VEGF induction in these animals and the duration of expression were tightly controlled by including or omitting tetracycline from the drinking water (''off'' and ''on'' modes, respectively; see Experimental Procedures for details). Previous studies from our laboratory indicated that in MHCα-tTA$^+$/tet-VEGF$^+$ animals, VEGF164 expression was strongly induced within 24 hr after withdrawal of tetracycline in most cardiomyocytes and in P_{LAP}-tTA$^+$/tet-VEGF$^+$ animals in most hepatocytes (Dor et al., 2002). Ongoing production of VEGF by the liver resulted in its accumulation in the serum to steady-state levels of 120 to 3000 pg/ml and when induced in the heart, to levels of 70 to 140 pg/ml. For comparison, control mice had only 45–100 pg/ml VEGF in their circulation. The apparent variability among individual mice with respect to the levels of circulating VEGF induced by the genetic switch enabled us to determine the threshold levels of circulating VEGF conducive for an ensuing angiogenic response. Using BrdU$^+$ proliferating endothelial cells as a readout for angiogenesis, a significant angiogenic response was already detected for the lower end of this range in the liver (see Figure S1 in the Supplemental Data available with this article online) as well as in the heart. Notably, these levels of circulating VEGF are comparable and even lower than the respective levels detected in cancer patients (Kraft et al., 1999; Salven et al., 1997), in neovascularization associated with acute myocardial infarction (Ogawa et al., 2000), or during wound healing (Infanger et al., 2004). Thus, we believe that findings obtained with the aid of this transgenic switch system are also valid for naturally triggered angiogenesis, as indeed extended below for wound-healing neovascularization.

VEGF Induces Homing of Circulating Myeloid Cells into the Respective Target Organ

Inducing VEGF expression in the adult heart or liver led to massive infiltration of circulating cells, specifically into the respective organ. Recruited cells could be readily distinguished morphologically from the resident cells of the respective organ (Figure 1A). Infiltration of circulating cells was already evident shortly after the onset of VEGF induction, clearly visible within the first 4 days, and notably, preceding emergence of new vessels. Thus the notion that these recruited cells may contribute to neovessel formation is at least temporally feasible.

To track the BM origin of recruited cells, the marrow of double-transgenic animals was reconstituted with genetically tagged cells prior to switching on VEGF expression. Specifically, BM cells derived from ROSA-βGal mice or, in other experiments, marrow derived from β-actin-eGFP mice were used to replace the marrow of irradiated double transgenic recipients (see Experimental Procedures for details and for analysis of chimerism). In the noninduced liver, only very few βGal- or eGFP-labeled cells were detected (data not shown), whereas following VEGF induction, the organ was heavily populated by BM-derived cells (Figures 1B and 1C), indicating that their accumulation in the liver is due to active recruitment by VEGF and not merely reflecting a natural turnover of organ resident BM-derived cells that have taken place since BM grafting.

Recruited RBCCs summoned to the organ by VEGF were only rarely incorporated within the endothelium, arguing against a significant contribution of EPCs to the newly acquired vasculature in this system (Figures 1B and 1C). Instead, RBCCs were mostly distributed around blood vessels with a clear affinity to reside in close proximity to vessels. This spatial relationship between RBCCs and endothelial cells suggested a paracrine angiogenic role for RBCCs rather than a role of serving as endothelial progenitors.

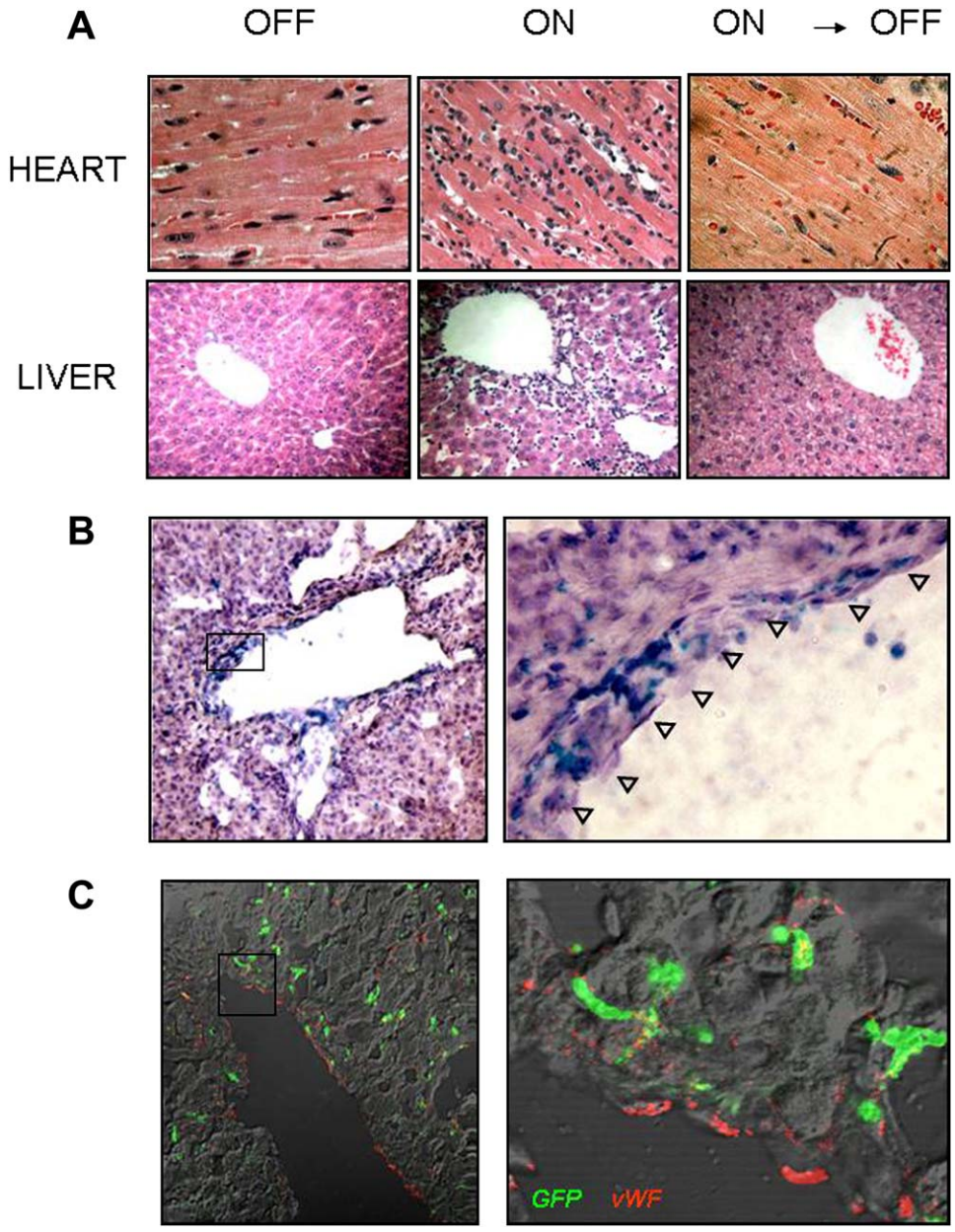

Figure 1. VEGF Recruits Circulating Hematopoietic Cells to the Organ from which It Emanates

VEGF expression was induced in the heart or liver of newborn or 4 weeks old mice, respectively, as described in the Experimental Procedures, and the respective organ was retrieved 2 weeks thereafter.

(A) H&E-stained sections of the myocardium and liver maintained in the VEGF "off" (left) or "on" (middle) modes. Right images are from mice in which VEGF expression was switched on for 2 weeks (at this time, mice had a level of circulating VEGF comparable to the mice shown in the middle lanes) and subsequently switched off for 2 weeks. Note the presence of recruited cells in the organ maintained in the "on" mode and their complete loss following VEGF withdrawal.

(B and C) The bone marrow of P_{LAP}-tTA/VEGF^{+} animals was replaced with genetically tagged marrow donated by either ROSA-βGal mice (B) or β-actin-EGFP transgenic mice (C) described in the Experimental Procedures. Following a complete BM reconstitution, VEGF expression was switched on for 2 weeks. Ten micrometer-thick liver cryosections were stained for β-galactosidase activity or viewed for GFP fluorescence (in sections also stained for vWF), respectively. Note that BM-derived cells are clustered around blood vessels. A higher magnification of the boxed area in (B) clearly shows that recruited cells assume a periendothelial location but that BM-derived cells are not incorporated within the endothelium (which is indicated by arrowheads). Likewise, in (C), GFP^{+} cells reside outside the vWF^{+} endothelium.

VEGF is known to induce mobilization of BM cells into the bloodstream, as reflected in a significant increase in WBC counts by circulating VEGF (Hattori et al., 2001). In our system, an 8-fold increase in WBC count was observed when VEGF was induced in the liver, but only a minor increase in WBC count was observed following a heart switch. This is likely because the levels of circulating VEGF resulting from a heart switch were significantly lower. Yet, even in the case where mobilization of BM cells by VEGF was not excessive, circulating cells were efficiently attracted to and accumulated within the target organ. Thus, we consider the role of VEGF in homing circulating cells to the target organ to be of a greater significance than its role in mobilizing cells into the circulation, a task known to be accomplished also by other cytokines (like GM-CSF).

To elucidate the cellular composition of RBCCs, we devised a procedure for their isolation in entirety after their arrival to the liver or heart. Two weeks after the onset of VEGF expression, sufficient amounts of RBCCs have already accumulated in the respective target organ to allow their isolation in sufficient quantities. Importantly, at this time there was no evidence for tissue inflammation or damage that might be associated with a prolonged exposure to VEGF. Advantage was then taken of the fact that recruited cells are loosely associated with the liver for their separation, without the need for proteolysis and for their further purification through banding on a Ficoll gradient. The yield of liver RBCCs was 1.5×10^7 cells per organ whereas only 1% to 10% that amount could be isolated from the noninduced liver using the same procedure. FACS analyses have shown that the vast majority of isolated cells are positive for the panhematopoietic marker CD45 (Figure 2A, left), positive for VEGF-R1 and negative for VEGF-R2 (data not shown), indicating that cells recruited by VEGF are predominantly hematopoietic in nature. In situ hybridization with a CD45-specific riboprobe corroborated the observation that RBCCs are retained close to the vessels from which they have apparently extravasated (Figure 2B).

Further analysis, using different hematopoietic cell markers, showed that 40% of $CD45^+$ RBCCs retrieved from the liver were also CD11b positive (data not shown).

Since VEGF can induce extra-medullary hematopoiesis in the liver (Hattori et al., 2001; our unpublished data) and, therefore, the retrieved cell population could have also contained cells engaged in active hematopoiesis, we extended a similar analysis to RBCCs retrieved from induced hearts. Like in the liver, 66% of $CD45^+$ cell recruited by VEGF to the heart were also CD11b positive (Figure 2A, right). Notably, B and T lymphocytes could not be detected in heart RBCCs, as evident by the lack of CD19 or CD3 expression (data not shown), suggesting that the major components in the RBCC population are myeloid cells.

To show that the perivascular positioning of RBCCs demonstrated above for CD45 cells, in general, specifically applies to myeloid cells, we bred onto the VEGF switch system a transgenic GFP reporter which is driven by the CX3CR1 promoter. Activity of this chemokine receptor promoter is restricted to mononuclear myeloid cells, including all circulating $CD116^+$ monocytes, and is essentially absent from cells of the lymphoid lineage (Geissmann et al., 2003; Jung et al., 2000). As shown in Figure 2C, CX3CR1/GFP-positive cells were indeed localized close to vessels in both the liver and heart. This localization has suggested that recruited mononuclear myeloid cells may act paracrinically on the adjacent endothelium and prompted experiments described below.

VEGF Induces Perivascular Expression of the Chemokine SDF1 that Is Responsible for Positioning RBCCs Close to Blood Vessels

To explain how VEGF alone may act to retain RBCCs in a perivascular position, we hypothesized that VEGF induces expression of a particular chemokine that functions to capture incoming cells expressing cognate receptors. As a first step, we determined the repertoire of chemokine receptors expressed in incoming cells by analyzing RNA extracted from isolated RBCCs on an Affymetrix gene array. Out of the several C-C and C-X-C type chemokine receptors expressed by these cells, we focused on CXCR4 because it was found to be expressed by the vast majority of $CD45^+$ cells that have entered the organ (Figure 3A) and since its obligatory ligand CXCL12 (also known as SDF1) was previously found to be important for recruiting hematopoietic stem cells to the liver (Kollet et al., 2003). We first verified that incoming RBCCs are indeed responsive to SDF1 using a transfilter migration assay. As shown in Figure 3B, RBCCs retrieved from the liver showed a strong chemotactic response to SDF1.

Next, we examined whether SDF1 is naturally induced downstream of VEGF and in what locations. Immunohistochemical analysis showed that switching on VEGF expression in either heart or liver resulted in induction of SDF1 protein, predominantly around blood vessels of the respective organ (Figure 3C) and to a lesser extent also in the endothelium (data not shown). Further, in situ mRNA hybridization for SDF1 indicated that perivascular cells are the producers of this secreted chemokine in the VEGF-induced organ (Figure 3D). In situ hybridization for SDF1 and CXCR4 in adjacent serial sections confirmed an intimate spatial relationship where incoming $CXCR4^+$ cells are retained as a halo around the ring of SDF1-expressing cells surrounding vessels (Figure 3D).

To better identify the SDF1-expressing cells, double immunostaining was performed using SDF1 antibodies in combination with fibroblastic or smooth muscle/pericyte markers. In the VEGF-induced heart, SDF1 protein mostly colocalized with α-smooth muscle actin (αSMA; Figure 4A, left panels) and with vimentin (data not shown), suggesting the fibroblastic or smooth muscle nature of SDF1-expressing cells.

Next, we wished to demonstrate that perivascular expression of SDF1 also accompanies pathophysiological neovascularization. To this end, specimens representing ongoing angiogenesis in the ischemic heart (experimentally induced by ligation of the left anterior descending coronary artery [LAD]), in a growing prostate tumor and during healing of excision wounds, were similarly analyzed for SDF1 and αSMA

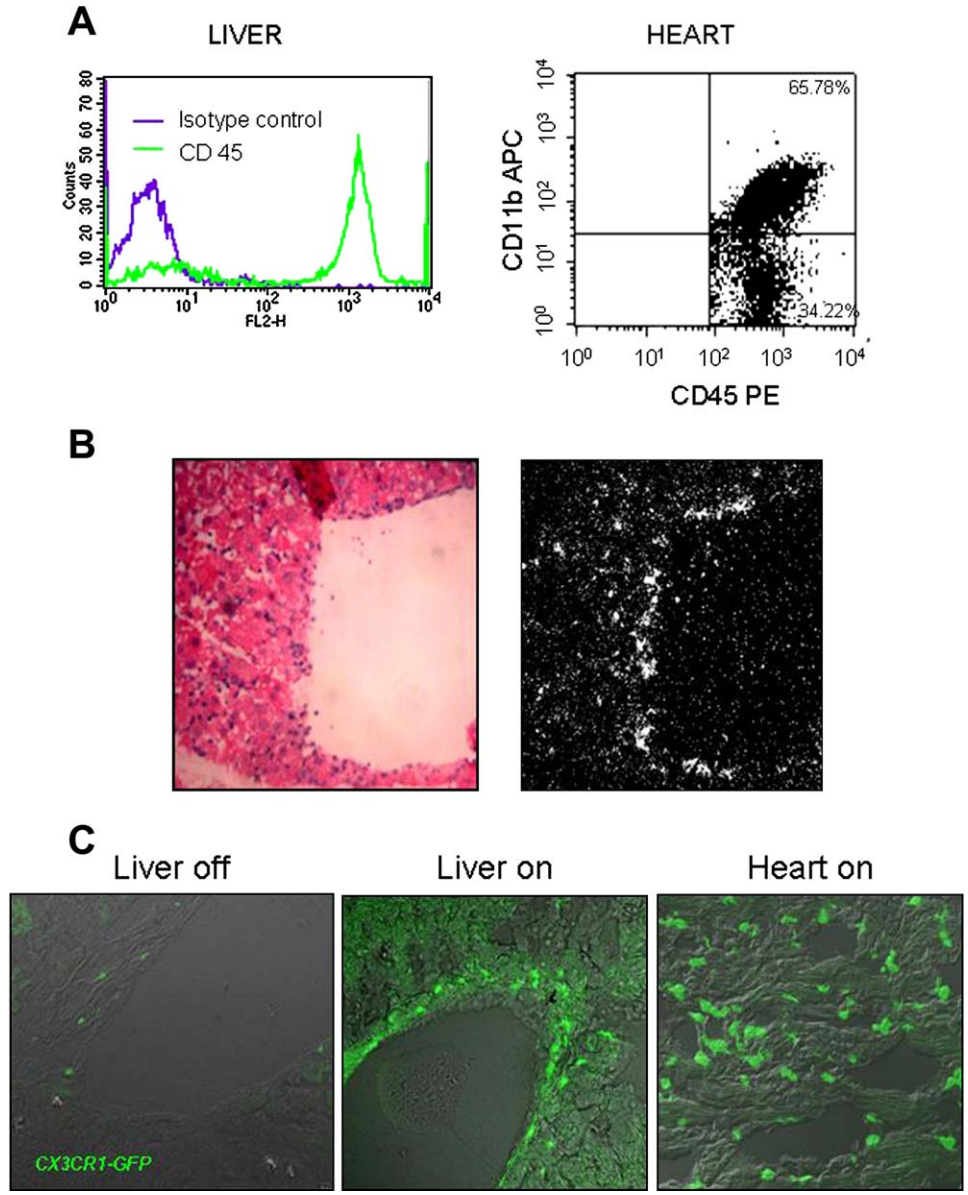

Figure 2. RBCCs, Mostly Myeloid Cells, Are Retained in a Perivascular Position

(A) (Left) Cells recruited to the liver following a 2 week VEGF switch were isolated as described in the text and analyzed by FACS for CD45 expression. (Right) Heart RBCCs were purified and sorted for CD45 and CD11b expression. The figure depicts $CD45^+$-gated cells showing that about 2/3 of them are also $CD11b^+$.

(B) In situ mRNA hybridization with a CD45-specific probe of the VEGF induced liver showing recruited $CD45^+$ surrounding a blood vessel. Left and right figures are brightfield and darkfield images, respectively.

(C) CX3CR1-GFP transgenic mice were bred with double-transgenic mice harboring either the liver- or heart specific-switchable system. VEGF expression was induced in triple-transgenic mice as described in Figure 1, and CX3CR1/GFP-expressing cells before or after inducing VEGF were visualized by GFP fluorescence. Note the perivascular positioning of recruited myeloid cells in both the induced liver and heart.

expression. As shown in Figure 4A, colocalized expression of SDF1 and αSMA in perivascular cells was indeed observed in all cases, indicating that perivascular induction of SDF1 is an integral part of adult angiogenesis in general. As evident in Figure 4A, with the exception of the VEGF genetic switch system, SDF1 expression was detected in additional sites besides its perivascular expression. This was anticipated, considering that SDF1 is known to be regulated by different cues (e.g., by hypoxia [Ceradini et al., 2004]). It was important, therefore, to determine whether in the pathophysiological settings too, SDF1 is regulated by VEGF. To this end, VEGF signaling was specifically inhibited during wound

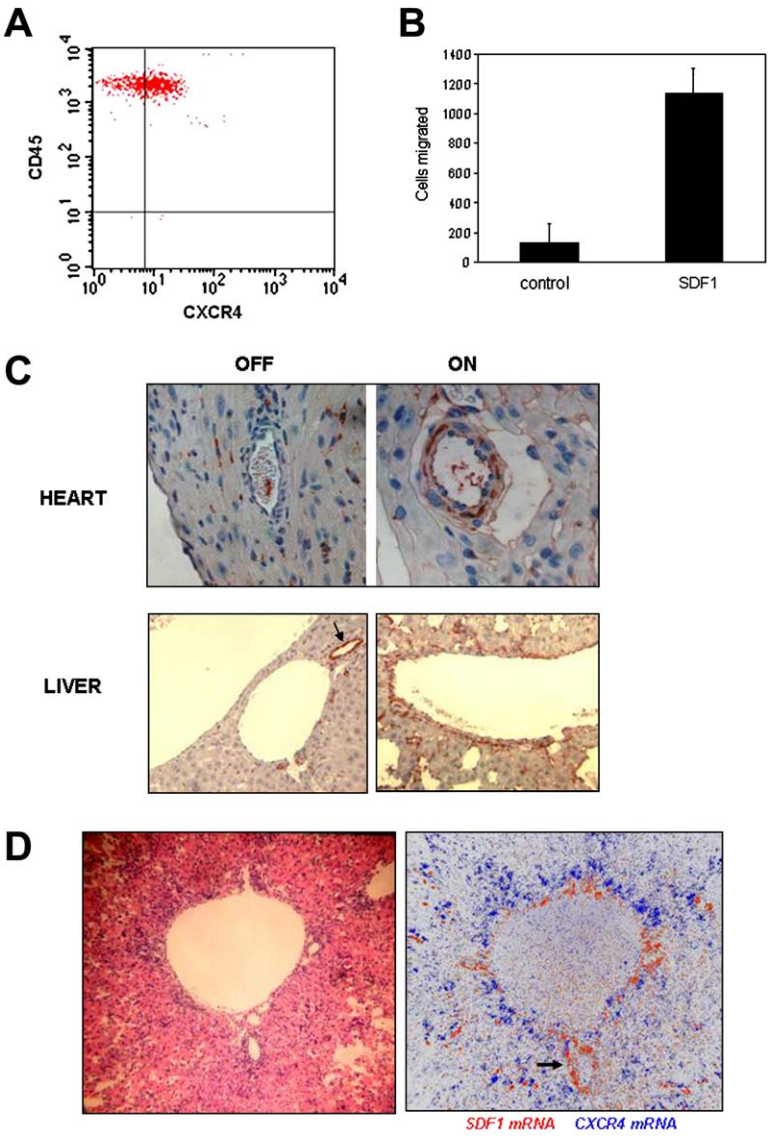

Figure 3. RBCCs Express CXCR4 and Are Responsive to SDF1 Induced by VEGF in Perivascular Cells

(A) FACS analysis of RBCCs retrieved from the liver, showing that the majority of $CD45^+$ cells are also positive for CXCR4.

(B) 2×10^5 RBCCs retrieved from the liver were placed in the upper chamber of a transfilter migration device. Cells migrated toward a serum-free control medium or toward a serum-free medium containing 250 ng/ml SDF1 protein were counted by FACS (number of cells/30 s).

(C) Immunostaining for SDF1 in the noninduced (*off*) and VEGF-induced (*on*) heart and liver. Note that VEGF induces SDF1, specifically in perivascular cells of either organ. In the liver, SDF1 is also constitutively expressed in the bile duct epithelium (arrow).

(D) In situ hybridization of SDF1α and CXCR4 mRNAs. Two serial sections of a VEGF-induced liver were hybridized with a SDF1- or a CXCR4-specific antisense riboprobe. The respective hybridization signals, visualized by darkfield illumination and pseudocolored in red (SDF1) or blue (CXCR4), were superimposed to produce the image on the right. Note that extravasated $CXCR4^+$ cells (clearly visible in the brightfield image on the left) are distributed as a halo around SDF1-expressing perivascular cells. Arrow points at the bile duct epithelium expressing SDF1 mRNA.

healing angiogenesis through transgenic induction of a chimeric protein that acts as a "VEGF trap" (see Experimental Procedures for details). Specimens were then retrieved at the midst of the wound healing process and were analyzed for SDF1 expression. SDF1 expression was strongly induced upon wounding the skin, but its upregulated expression was negated when the VEGF trap was activated (Figure 4B and a quantitative summary in Figure 7A). Importantly, inability to upregulate SDF1 in the wound in the face of VEGF suppression was specifically accounted for by the apparent inhibition of its perivascular expression (Figure 4C), suggesting that in wound angiogenesis, induction of perivascular SDF1 is downstream of VEGF.

Currently, it is not known whether induction of SDF1 expression in these cells by VEGF is direct or indirect. In vitro, at least, VEGF directly induced SDF1 mRNA in primary fibroblast cultures, but not in primary cultures of smooth muscle cells (Figure S2).

Ongoing Stimulation of the VEGF-SDF1 Pathway Is Essential for Maintaining Imported RBCCs within the Organ

Results presented above indicated that VEGF recruits circulating cells to the organ where it is produced. We next examined whether a continued VEGF expression is required for retaining imported cells within the organ and also whether RBCC retention is mediated by SDF1. To this end, we induced VEGF expression for two weeks and then switched it off. While the liver or the heart contained an abundance of RBCCs by the end of the induction period, recruited cells were completely lost within a few days after terminating VEGF expression (Figure 1A). The loss of recruited RBCCs was also evident from a 10- to 100-fold reduction in the yield of RBCCs that could be isolated from livers in which VEGF expression has been switched off. In the organ in which VEGF has been switched off, physical loss of RBCCs was also manifested by a marked reduction in organ CXCR4

expression, the presumed consequence of the observed downregulation in SDF1 expression (Figure S3A). A role for SDF1 in RBCCs retention during wound healing was suggested by a similar coreduction in SDF1 and CXCR4 expression in the wound area upon activation of the VEGF trap (Figure 4B, quantified in Figure 7A).

To provide a definite proof that SDF1 is essential for homing and/or retention of incoming $CXCR4^+$ cells, we inhibited SDF1 signaling using AMD3100, a bicyclam that is extensively used as a CXCR4-specific inhibitor (reviewed in De Clercq [2003]). In contrast to VEGF-induced animals harboring a saline-releasing pump where CXCR4 mRNA was abundantly present, $CXCR4^+$-expressing cells could not be detected in the livers of VEGF-induced animals harboring an osmotic pump releasing AMD3100 (Figure S3B). Together, the data support a scenario where SDF1 functions downstream of VEGF to maintain these cells within the target organ.

Ectopic expression of SDF1 in the noninduced liver, achieved via adenovirus-mediated delivery of SDF1 cDNA, was not sufficient for homing of $CXCR4^+$ cells to the liver. Yet, ectopically expressed SDF1 partially prevented the loss of RBCCs already recruited to the liver by VEGF, a loss that would have otherwise taken place upon VEGF withdrawal (Figure S4). These findings suggest that SDF1 acts primarily as a RBCCs ''retainer'' and less, if at all, as a chemoattractant for these cells (see Discussion).

RBCCs Are Required for Angiogenesis via Enhancing In Situ Proliferation of Resident Endothelium

We next wished to determine whether VEGF-instructed recruitment of RBCCs is an integral component of adult organ neovascularization and, specifically, to examine whether these imported cells are essential for efficient neovascularization. First, the potential of RBCCs to promote sprouting angiogenesis ex vivo was examined. When placed atop collagen-embedded mouse aortic segments, RBCCs isolated from the induced liver induced extensive sprouting at a magnitude that even exceeded that induced by a recombinant VEGF protein (Figure 5A and quantification in Figure 5B). To show that this is not a peculiarity of cells isolated from the liver, RBCCs summoned by VEGF to the heart and similarly isolated were also examined. Purified heart RBCCs promoted vessel sprouting at a comparable efficiency to that of liver RBCCs (Figure 5B). These experiments were repeated using purified $CXCR4^+$ cells, essentially with the same results (data not shown). Further, a strong sprouting-promoting activity was attributed to the $CD11b^+$ cells purified to homogeneity from the total RBCC population through capturing on magnetic beads conjugated to anti-CD11b antibodies (Figure 5B). Medium conditioned by each of these cell populations was as efficient as the respective cells in supporting sprouting (Figure 5), suggesting that vessel wall endothelium is likely activated by proteins secreted by nearby RBCCs in a paracrine fashion.

The repertoire of proangiogenic activities elaborated by RBCCs remains to be determined. As a first step, the profile of RBCC-expressed genes was elucidated through subjecting RNA isolated from purified RBCCs to a gene-array analysis. Candidate genes known to encode secreted proteins are then tested with respect to the ability of their specific inhibitors to block RBCC-initiated sprouting. As an example, MMP9 is highly expressed by RBCCs and its blockade, using the specific inhibitor GM6001, strongly inhibited RBCCs-driven sprouting angiogenesis ex vivo (Figure 5B). Importantly, the predominant contribution of MMP9 is by RBCCs and not by cells that are natural inhabitants in the VEGF-induced organ (comparative Affymetrix array data, not shown [see Discussion]).

To provide a definite in vivo proof that RBCCs recruited by SDF1 contribute to organ neovascularization, cells were prevented from reaching and/or staying within the organ through implanting an AMD3100-releasing pump prior to switching on VEGF, and the consequences on organ neovascularization were determined. Briefly, hepatic expression of VEGF was induced in P_{LAP}-tTA^+/$VEGF^+$ littermates, half of which were implanted with an AMD3100-releasing pump and the other half with a saline-releasing pump. Fourteen days later, livers were retrieved and analyzed with respect to the following parameters: the level of transgenic VEGF induced in the organ, the content of $CXCR4^+$ cells populating the organ, and the magnitude of the angiogenic response. Blocking SDF1/CXCR4 interaction led to a significant inhibition of liver neovascularization (Figure 6). A pairwise comparison of two representative littermates is shown in Figure 6A. In both animals, the VEGF switch worked equally well, as evidenced by a comparable level of transgenic VEGF induced in the organ. Yet, the apparent failure to recruit $CXCR4^+$ cells in the AMD3100-treated animal was associated with a markedly reduced angiogenic response, as already suggested by a significant inhibition in VE-cadherin expression (Figure 6A). A compromised angiogenic response was clearly evident from comparing the elaborate network of blood-filled neovessels induced close to the surface of the control liver to the scarcity of induced vessels in the AMD3100-treated animal (Figure 6B). Inspection of histological sections confirmed a significant reduction in the number of large lumenized and sinusoidal structures observed in sections of the AMD3100-treated animals (Figure 6B). Data compiled for several litters (excluding only animals in which the level of induced circulating VEGF did not reach the set threshold) were quantified (Figure 6C). The control and AMD3100-treated groups were very similar with respect to the level of transgenic VEGF induced, but the treated group had on average only one-fifth the normal amount of $CXCR4^+$ cells recruited to the organ. Inhibition of neovascularization in the AMD3100-treated group was again evident from visual inspection of whole mounts and liver sections and was also reflected in a 2.5-fold reduction in VE-cadherin expression. It should be pointed out, however, that quantifying neovascularization in the liver through marker analysis is difficult due to the fact that in this highly vascularized organ, newly added vessels comprise a relatively small fraction of vessels. Moreover, some of the markers routinely used to mark vessels (e.g., CD31) are expressed on RBCCs. Therefore, to directly focus on the angiogenic endothelium, we resorted to direct

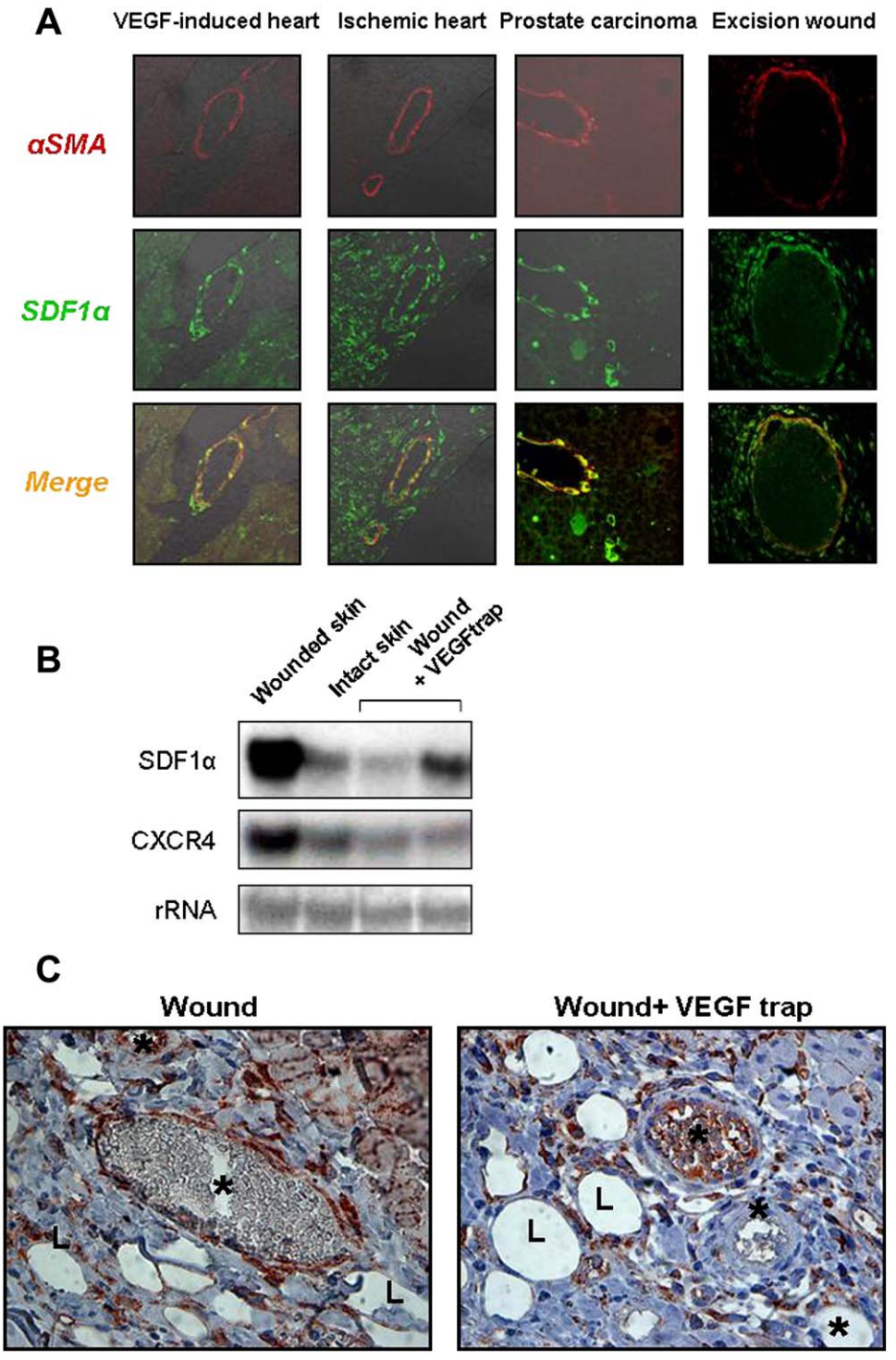

Figure 4. SDF1 Induction in αSMA$^+$ Perivascular Cells during Pathophysiological Angiogenesis Is VEGF Dependent

(A) Double immunostaining for αSMA (red) and SDF1 (green) in the following settings of angiogenesis: (1) myocardial angiogenesis induced by a genetic VEGF switch, (2) in the ischemic myocardium following experimental occlusion of the LAD coronary artery, (3) in a 22RV1 prostate carcinoma grafted into a SCID mouse, (4) in a skin excision wound 48 hr postwounding.

(B and C) Wounded skin tissue was removed at 48 hr postwounding and divided symmetrically in two halves. One was used for extracting RNA (B) and the other for preparing tissue sections (C).

measurements of endothelial cell proliferation. BrdU was injected into mice with an ongoing heart or liver VEGF switch, and the respective organ was retrieved 3 hr later. Proliferating cells were then visualized by BrdU immunohistochemistry. In both organs, extensive endothelial cell proliferation took place almost exclusively in endothelial cells residing in the vessel wall (Figure S5). To examine whether CXCR4 inhibition will negatively impact on proliferation of vessel-wall endothelium, BrdU pulse experiments were also performed in the presence of AMD3100. CXCR4 inhibition and the resultant failure to retain recruited cells led to a 3-fold reduction in the number of vessels that contained one or more BrdU-positive cells and nearly 8-fold reduction in the total number of proliferating endothelial cells (Figure 6C). These results indicate that RBCCs are required for efficient proliferation of vessel wall endothelium.

To substantiate that the VEGF-SDF1-RBCCs assisted angiogenesis connection also operates during wound healing angiogenesis, the natural process was manipulated in three different ways. Namely, inhibition of VEGF signaling through the use of a VEGF trap (some representative results were already presented in Figure 4), blocking SDF1 signaling through the use of AMD3100, and administration of isolated RBCCs. As summarized in Figure 7A, VEGF suppression abrogated upregulated expression of SDF1 in the wound, inhibited CXCR4 expression, and completely blocked angiogenesis (here, VE-cadherin was used as a vascular readout). SDF1 blockade by AMD3100 similarly inhibited CXCR4 and angiogenesis. Conversely, injection of purified RBCCs to the wound area significantly augmented neovascularization. These results indicate that RBCCs recruited by VEGF via SDF1 are instrumental also to wound healing angiogenesis.

DISCUSSION

This study focuses on the poorly understood function of circulating cells recruited to sites of ongoing neovascularization in the adult. While infiltration of circulating cells to angiogenic sites has been observed frequently, the notion that BM-derived cells are actually required for adult neovascularization (De Palma et al., 2003) needed to be substantiated. We use the term RBCCs to include all types of recruited cells and focused on the function of RBCCs that are not EPCs.

Here, we resorted to a transgenic system designed for intrinsic induction of VEGF without external insults like hypoxia, inflammation, and tissue injury in order to provoke recruitment of circulating cells. With the aid of this system, we showed that VEGF alone is sufficient to instruct homing of hematopoietic cells onto the organ from which it emanates. Together with evidence that these cells play an indispensable role in neovessel formation, findings reported here establish that VEGF-induced recruitment of RBCCs is an integral component of adult neovascularization.

The relative contribution of BM-derived EPCs to adult neovasculatures is currently debated and appears to greatly depend on the experimental system studied. In our system, representing adult organ neovascularization, the contribution of EPCs to neovessels was very low. Results of the bone marrow transplantation experiments (Figures 1B and 1C) were consistent with results of the BrdU pulse labeling experiments (Figure S5), indicating that the major source for new endothelial cells is their expansion via in situ proliferation of vessel-wall endothelium. It could be argued, nevertheless, that BrdU-positive endothelial cells may represent preintegrated EPCs with a high proliferation capacity. This was not compatible, however, with the failure to detect strings of βGal^+ or GFP^+ endothelial cells (i.e., ECs of a BM origin) in the neovascularized organ.

The precise composition of the RBCCs population, presumed to be heterogeneous, was not fully determined. Particularly, marker combinations typifying perspective EPCs were not thoroughly examined, considering the very low EPC contribution. Quantitatively, analysis of the entire RBCCs population, retrieved from two different organs, has clearly identified $CD45^+/CD11b^+$ myeloid cells as the largest RBCCs component. The current confusion regarding the nature of circulating cells recruited to angiogenic sites results in part from a lack of clear criteria for their isolation from the bloodstream. For example, cells isolated from the human circulation and defined by some investigators as EPCs were shown by others as mostly nonproliferating cells expressing monocytes/macrophage markers (Rehman et al., 2003). Our study, on the other hand, has used the target organ as a functional filter to isolate the angiogenically relevant cells, thereby circumventing the need to rely on marker expression.

Monocytes are not only equipped with a battery of chemokine receptors but may directly respond to VEGF chemotaxis by virtue of their VEGF-R1 expression (Barleon et al., 1996). Notably, VEGF-R1-positive cells were also found to be abundantly corecruited with EPCs to angiogenic tumors (Lyden et al., 2001). It appears that RBCCs are mostly naturally circulating mononuclear myeloid cells endowed with the capacity to secrete proangiogenic activities (Figure 5). This argues that RBCCs' capture and positioning might be of a greater significance than their BM mobilization.

The paracrine mode of RBCCs action necessitates a mechanism to retain incoming cells nearby the endothelium upon which they act. Findings reported here disclosed a mechanism by which RBCCs are retained close to blood vessels, namely, induction by VEGF of perivascular SDF1 expression that functions to capture incoming $CXCR4^+$ cells. Extending the observation of perivascular SDF1 induction to the major settings of natural angiogenesis and, in particular,

(B) Northern blot analysis for SDF1 and CXCR4 mRNAs in the intact skin, wounded skin, and wounded skin of mice in the presence of a chimeric soluble VEGF-R1 protein (VEGF trap). The soluble VEGF-R1 protein was induced 7 days before wounding and was present in the circulation during the healing process at concentrations of 2062 pg/ml or 1150 pg/ml (for the respective left and right lanes).
(C) Immunostaining for SDF1. Blood vessels were marked by asterisks and lipid droplets marked by L. Note the loss of perivascular SDF1 in the face of VEGF inhibition.

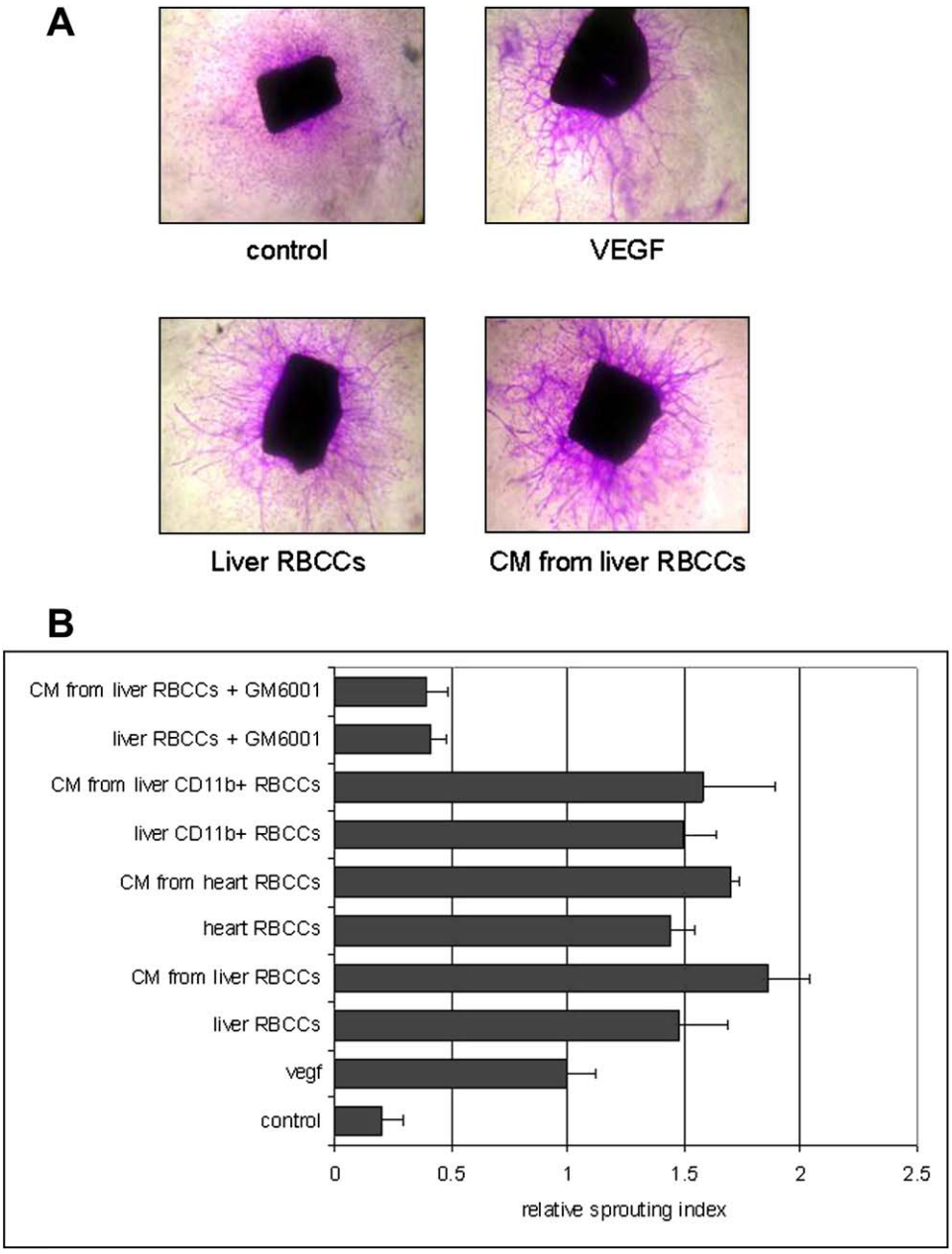

Figure 5. RBCCs Retrieved from VEGF-Induced Heart and Liver Promote Sprouting Angiogenesis Ex Vivo

Mouse aortic segments were embedded in collagen and overlaid with either a serum-free control medium or a serum-free medium containing 10 ng/ml VEGF, or 6×10^4 RBCCs retrieved from the indicated organ with or without further fractionation, or overlaid with 500 μl serum-free medium conditioned overnight by these cells. Capillary sprouts were visualized by crystal violet staining (representative examples are shown in (A). Total length of capillary sprouts was measured; a relative sprouting index was calculated as described in the Experimental Procedures and is presented relative to VEGF-induced sprouting (B). The MMP inhibitor GM6001 was added at a concentration of 25 μM.

demonstrating that perivascular SDF1 induction during wound healing is VEGF dependent (Figure 4), indicated that SDF1 upregulation is an integral part of a general program set in motion by VEGF to localize RBCCs close to angiogenic vessels (see model in Figure 7B).

Experiments reported here show that ongoing VEGF signaling is essential to maintain incoming $CXCR4^+$ cells within the target organ. Since SDF1 is also a chemoattractant for these cells (Figure 3) it is plausible that SDF1 operates in both homing of CXCR4-expressing cells as well as for their retention within the organ. Alternatively, homing might be mediated by other factors, like VEGF, a possibility supported by findings that blocking VEGF-R1 reduces the number of recruited perivascular cells in tumors (Hattori et al., 2001).

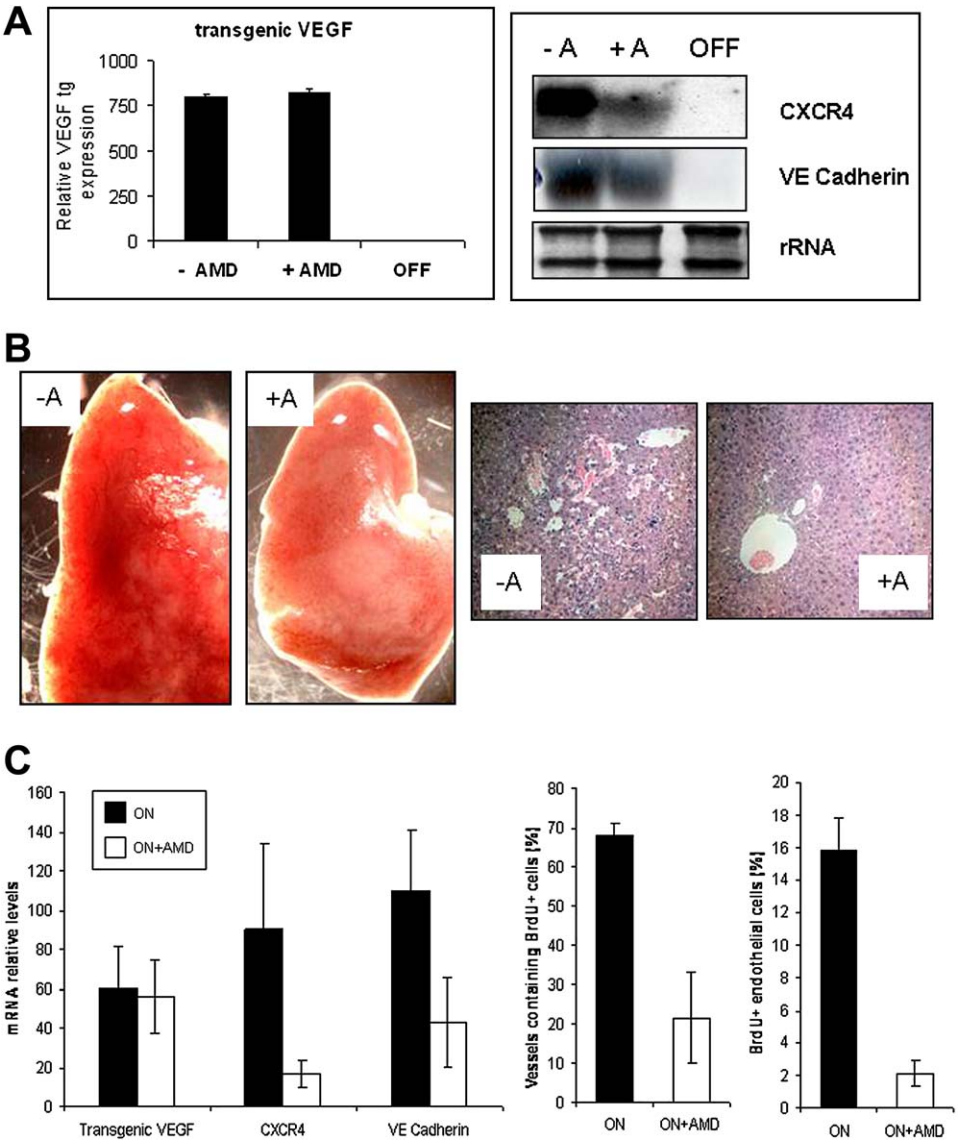

Figure 6. SDF1 Blockade Inhibits VEGF-Induced Neovascularization via Reducing In Situ Proliferation of Endothelial Cells

Mice were implanted with a control, saline-releasing osmotic pump or with an AMD3100-releasing osmotic pump at the onset of inducing VEGF expression in the liver. Two weeks later, the liver was excised and analyzed as indicated.

(A) A pairwise comparison of two representative littermates. Top left: Semiquantitative RT-PCR using primers detecting transgenic (but not endogenous) VEGF. Note a comparable level of VEGF induced in mice implanted with a control pump (−A) or an AMD3100 pump (+A). Top right: Northern blot for CXCR4 and VE cadherin.

(B) Left: Top view of the liver showing a dense network of superficial vessels induced by VEGF in the control animal that is strongly inhibited in the AMD3100-treated animal. Right: H&E-stained sections of the same liver lobe shown above showing that most of the deeper plexus of VEGF-induced neovessels are inhibited by AMD3100. The large channels seen in the right image are sections through the normal portal triad (PT).

(C) Data compiled from nine mice implanted with a control pump and 11 mice implanted with an AMD3100 pump (comprising all double-transgenic animals in three consecutive litters in which a successful VEGF switch was evidenced by >200 pg/ml of circulating VEGF). Animals were implanted with either a control pump or an AMD3100-releasing pump at the time of switching on hepatic VEGF expression. Quantification of transgenic VEGF was by semiquantitative PCR normalized to L19 RNA and of CXCR4 and VE-cadherin by densitometry of Northern blots. For measurements of $BrdU^+$ cells, BrdU was injected 3 hr before organ retrieval. Data shown are from three animals in each treatment group (two high-power field sections for each animal) counting a total of 116 and 63 vessels, respectively (identified as cells lining the lumen of erythrocyte-containing structures).

On its own, however, a chemotactic response to VEGF could not explain the perivascular localization of recruited cells, considering the uniform distribution of VEGF throughout the organ. Another possibility is that RBCCs passively extravasate the VEGF-activated endothelium and thus the main requirement is for SDF1-mediated retention. Thus, it

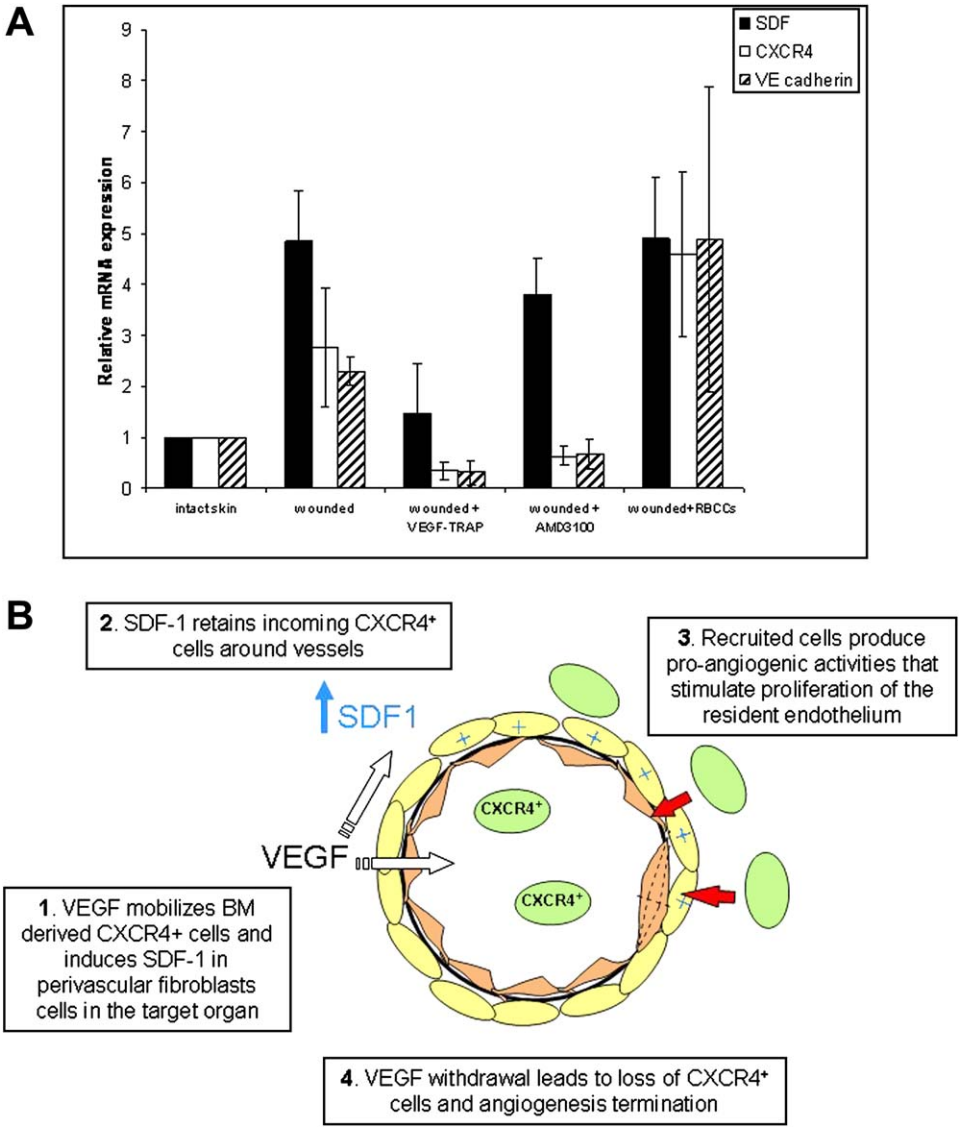

Figure 7. The VEGF-SDF1-RBCC-Assisted Angiogenesis Connection Is Operative during Wound Healing

(A) Skin wounds were performed as described above and were manipulated as follows: without further manipulation (n = 5); A VEGF trap was activated as described in Figure 4B (n = 4); for SDF1 blockade, an osmotic pump releasing AMD3100 was implanted prior to wounding as described above (n = 4); for RBCCs administration, 1.10^5 cells retrieved from a VEGF-induced liver were suspended in 100 μl PBS and injected s.c. close to the wound, at 30 min post-wounding (n = 9). RNAs were extracted from tissues retrieved at 48 hr after wounding and were subjected to a Northern blot analysis with the indicated probes. Hybridization signal intensities were measured and compared with those obtained with intact skin RNA (cohybridized on the same blot) that was given a value of 1.

(B) A model for a program induced by VEGF for the recruitment and paracrine localization of angiogenic accessory cells.

is possible that RBCCs in our liver system extravasate through sinusoidal endothelial cells but are retained mostly around large veins. The action of SDF1 as a RBCCs retainer is also consistent with our finding that ectopic SDF1 expression partially prevents RBCC loss upon VEGF withdrawal (Figure S3) and with findings that the apparent increase in angiogenesis by exogenous SDF1 is only manifested in the presence of another insult like hypoxia (Abbott et al., 2004).

Modulations in SDF1 expression may greatly effect trafficking of hematopoietic cells into and away from the bone marrow (Peled et al., 1999) and to specific microdomains therein (Sipkins et al., 2005). Also, SDF1 plays a role in targeting $CXCR4^+$ metastatic cells to organs that produce it (Muller et al., 2001) and to control trafficking of circulating cells to hypoxic sites (Ceradini et al., 2004). A recent study has shown that recruitment of EPCs to breast carcinomas is mediated in part by SDF1 secreted by carcinoma-associated fibroblasts (Orimo et al., 2005). Ours is the first study, however, to show that SDF1 functions downstream of VEGF to retain RBCCs.

Findings reported here highlight a novel principle in VEGF-induced neovascularization, namely, a dual source of proangiogenic activities that complement VEGF. That is, ''imported'' paracrine activities contributed by RBCCs and activities induced by VEGF locally. Ongoing experiments attempt categorizing proangiogenic activities according to their respective source. In these experiments, the transcriptome of purified RBCCs is compared with whole organ RNAs at the ''off'' and ''on'' modes. An example for a RBCCs-contributed activity is MMP9, which has been shown to be an important component of angiogenesis (Bergers et al., 2000; Figure 5B). Examples for genes upregulated solely in the organ resident cells are multiple components of the PDGF and *Notch* pathways (data not shown). It can be further speculated that RBCCs are reprogrammed by VEGF and/or the organ milieu for more efficient secretion of proangiogenic proteins. This intriguing possibility is currently investigated through comparing expression profiles of $CD11b^+$ cells isolated from the circulation with that of $CD11b^+$ cells retrieved from the heart of the same VEGF-induced animal.

Finally, experiments reported here showing that inhibition of SDF1 impairs organ neovascularization suggest that the SDF1/CXCR4 system might be harnessed as a new target for antiangiogenesis. Conversely, the findings provide a mechanistic rationale for using SDF1 (Yamaguchi et al., 2003) or cells engineered to express $CXCR4^+$ to augment therapeutic angiogenesis.

EXPERIMENTAL PROCEDURES

Transgenic Mice and Conditional Modulations of VEGF Expression and Signaling

Heart-specific induction was achieved using a transgenic driver line in which tTA expression is driven by a myosin heavy chain-α heart specific promoter (MHCα; Yu et al., 1996). Liver-specific induction was obtained by using a driver line in which tTA expression is driven by a C/EBPβ (CCAAT/enhancer binding protein β) promoter (also known as liver-activator protein or PLAP; Kistner et al., 1996). The responder tet-VEGF164 transgenic line was previously described (Dor et al., 2002). The responder tet-VEGF TRAP transgenic line encodes a tetracycline-inducible protein composed of an IgG1-Fc tail fused to the extracellular domain of VEGF-R1 (corresponding to amino acid residues 1 to 631 in human VEGF-R1).

Induction of VEGF or of a soluble VEGF-R1 (''VEGF trap'') by tetracycline withdrawal and its shut-off by tetracycline addition were carried out as previously described (Dor et al., 2002). VEGF protein levels in sera of induced mice were measured using mouse VEGF ELISA kit (Oncogene Inc.), and levels of circulating VEGF trap protein were determined by a soluble VEGF-R1 ELISA kit (R&D Systems).

Isolation and Analysis of Recruited Cells

Circulating cells recruited to the liver of 8- to 10-week-old mice following VEGF induction were isolated by filtrating through 100 Mesh (Sigma) and purified by density gradient centrifugation with Histopaque-1077 (Sigma).

Circulating cells recruited by VEGF to the heart of 4- to 5-week-old mice were isolated by digesting the perfused heart tissue with collagenase D (Roch 1088858) for 1 hr at 37ºC and centrifugation at 1200 rpm for 7 min. For further fractionation of $CD45^+$ cells, $CXCR4^+$ cells, or $CD11b^+$ cells, cells were reacted with the respective antibodies and purified (to 96% purity) through capture on anti-PE antibody-coated micromagnetic beads (Miltenyi Biotec). Antibodies used were as follows: anti-mouse CD45 (PE-labeled, clone 30-F11 Pharmingen and APC-labeled, clone 104 e-Bioscience), mAb against mouse CXCR4 (PE- or FITC-labeled, clone 2B11 Pharmingen), mAb against mouse CD11b (PE-labeled, clone M1/70 Pharmingen, M1/70 e-Bioscience), and isotype antibody controls.

Bone Marrow Transplantation Experiments

Double-transgenic mice (double-positive for Ly 5.1 and Ly 5.2) were exposed to a single lethal dose of 10 Gy total body irradiation (TBI) from a Gamma cell 40 by a Gamma beam Cesium 137 source (produced by NDS Canada) at a 0.95 Gy/min dose rate. On the following day, the mice were inoculated intravenously with 5×10^6 donor BM cells originating from B6.129S7-Gtrosa26 mice (single-positive for Ly 5.2) or from C57BL/6-Tg (ACTbEGFP010sb mice). At 30 days posttransplant, the level of donor-type chimerism was >89%, as evidenced by FACS analysis of the donor single-positive marker (Ly 5.2) or of EGFP expression. This was also confirmed by β-galactosidase staining or EGFP expression observation of the hematopoietic organs (bone marrow, spleen, and thymus). VEGF expression was then switched on and the liver was retrieved 2 weeks thereafter.

Aortic Ring Sprouting Assay

Thoracic aortas were dissected from 8- to 10-week-old male mice, the adventitia was removed, and 0.5 mm ''rings'' were embedded in collagen as described by Licht et al. (2003). The collagen was then overlaid with either a medium alone (BIO-MPM-1) or a medium containing the tested factor or cells or their conditioned medium, and the plates were incubated at 37ºC in a humidified 5% CO_2 atmosphere with triweekly medium replacement. The MMP inhibitor GM6001 (Chemicon CC1010) was added at a concentration of 25 μM. After 7 days, the rings were fixed in 4% formaldehyde for 24 hr, followed by staining with crystal violet (0.02%). Micrographs of representative rings were taken using a digital camera and morphometric analysis of sprouting was performed on four rings manually using Image-J software according to Nissanov et al. (1995).

AMD3100 Administration

AMD3100 (a generous gift from AnorMED Inc.) was dissolved in 0.1M $NaHCO_3$ (pH 7.4), and a total of 5 mg per mouse was continuously delivered with the aid of a subcutaneous miniosmotic pump (Alzet) over a period of 14 days, starting 24 hr prior to switching on VEGF expression. Control animals were similarly implanted with saline releasing pumps.

LAD Ligation and Myocardial Angiogenesis

Myocardial infarction was induced as previously described (Chimenti et al., 2004).

Wound Healing Angiogenesis

NOD-SCID mice were anesthetized (Ketamine 50 mg/ml and xylazine 5 mg/ml, i.p.) and a 1 cm long, full thickness incision was made on the dorsal skin. Incisions were closed by cyanoacrylate glue and examined 48 hr later. Half of the wound area was processed for RNA analysis; the other half was fixed in 4% buffered paraformaldehyde and used for histological analysis.

Other Methods

For other methods, see Supplemental Experimental Procedures.

Supplemental Data

Supplemental Data include five figures and Supplemental Experimental Procedures and will be found with this article online at http://www.cell.com/cgi/content/full/124/1/175/DC1/.

ACKNOWLEDGMENTS

We thank Drs. Amnon Peled, Tamar Licht, Anat Globerman, and Dalit May for their valuable contributions and the Israel Science Foundation and the European Vascular Genomic Network (EVGN) for financial

support. M.G. was supported by a postdoctoral fellowship from the Israel Cancer Research Fund (ICRF) and from the Ministry of Science, Israel.

Received: September 12, 2004
Revised: August 11, 2005
Accepted: October 7, 2005
Published: January 12, 2006

REFERENCES

Abbott, J.D., Huang, Y., Liu, D., Hickey, R., Krause, D.S., and Giordano, F.J. (2004). Stromal cell-derived factor-1alpha plays a critical role in stem cell recruitment to the heart after myocardial infarction but is not sufficient to induce homing in the absence of injury. Circulation *110*, 3300–3305.

Asahara, T., Murohara, T., Sullivan, A., Silver, M., van der Zee, R., Li, T., Witzenbichler, B., Schatteman, G., and Isner, J.M. (1997). Isolation of putative progenitor endothelial cells for angiogenesis. Science *275*, 964–967.

Asahara, T., Masuda, H., Takahashi, T., Kalka, C., Pastore, C., Silver, M., Kearne, M., Magner, M., and Isner, J.M. (1999). Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. Circ. Res. *85*, 221–228.

Barleon, B., Sozzani, S., Zhou, D., Weich, H.A., Mantovani, A., and Marme, D. (1996). Migration of human monocytes in response to vascular endothelial growth factor (VEGF) is mediated via the VEGF receptor flt-1. Blood *87*, 3336–3343.

Bergers, G., Brekken, R., McMahon, G., Vu, T.H., Itoh, T., Tamaki, K., Tanzawa, K., Thorpe, P., Itohara, S., Werb, Z., and Hanahan, D. (2000). Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. Nat. Cell Biol. *2*, 737–744.

Ceradini, D.J., Kulkarni, A.R., Callaghan, M.J., Tepper, O.M., Bastidas, N., Kleinman, M.E., Capla, J.M., Galiano, R.D., Levine, J.P., and Gurtner, G.C. (2004). Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1. Nat. Med. *10*, 858–864.

Chimenti, S., Carlo, E., Masson, S., Bai, A., and Latini, R. (2004). Myocardial infarction: animal models. Methods Mol. Med. *98*, 217–226.

De Clercq, E. (2003). The bicyclam AMD3100 story. Nat. Rev. Drug Discov. *2*, 581–587.

De Palma, M., Venneri, M.A., Roca, C., and Naldini, L. (2003). Targeting exogenous genes to tumor angiogenesis by transplantation of genetically modified hematopoietic stem cells. Nat. Med. *9*, 789–795.

Dor, Y., Djonov, V., Abramovitch, R., Itin, A., Fishman, G.I., Carmeliet, P., Goelman, G., and Keshet, E. (2002). Conditional switching of VEGF provides new insights into adult neovascularization and pro-angiogenic therapy. EMBO J. *21*, 1939–1947.

Garcia-Barros, M., Paris, F., Cordon-Cardo, C., Lyden, D., Rafii, S., Haimovitz-Friedman, A., Fuks, Z., and Kolesnick, R. (2003). Tumor response to radiotherapy regulated by endothelial cell apoptosis. Science *300*, 1155–1159.

Geissmann, F., Jung, S., and Littman, D.R. (2003). Blood monocytes consist of two principal subsets with distinct migratory properties. Immunity *19*, 71–82.

Hattori, K., Dias, S., Heissig, B., Hackett, N.R., Lyden, D., Tateno, M., Hicklin, D.J., Zhu, Z., Witte, L., Crystal, R.G., 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.

Infanger, M., Schmidt, O., Kossmehl, P., Grad, S., Ertel, W., and Grimm, D. (2004). Vascular endothelial growth factor serum level is strongly enhanced after burn injury and correlated with local and general tissue edema. Burns *30*, 305–311.

Jung, S., Aliberti, J., Graemmel, P., Sunshine, M.J., Kreutzberg, G.W., Sher, A., and Littman, D.R. (2000). Analysis of fractalkine receptor CX(3)CR1 function by targeted deletion and green fluorescent protein reporter gene insertion. Mol. Cell. Biol. *20*, 4106–4114.

Kalka, C., Masuda, H., Takahashi, T., Kalka-Moll, W.M., Silver, M., Kearney, M., Li, T., Isner, J.M., and Asahara, T. (2000). Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization. Proc. Natl. Acad. Sci. USA *97*, 3422–3427.

Kistner, A., Gossen, M., Zimmermann, F., Jerecic, J., Ullmer, C., Lubbert, H., and Bujard, H. (1996). Doxycycline-mediated quantitative and tissue-specific control of gene expression in transgenic mice. Proc. Natl. Acad. Sci. USA *93*, 10933–10938.

Kocher, A.A., Schuster, M.D., Szabolcs, M.J., Takuma, S., Burkhoff, D., Wang, J., Homma, S., Edwards, N.M., and Itescu, S. (2001). Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac function. Nat. Med. *7*, 430–436.

Kollet, O., Shivtiel, S., Chen, Y.Q., Suriawinata, J., Thung, S.N., Dabeva, M.D., Kahn, J., Spiegel, A., Dar, A., Samira, S., et al. (2003). HGF, SDF-1, and MMP-9 are involved in stress-induced human CD34+ stem cell recruitment to the liver. J. Clin. Invest. *112*, 160–169.

Kraft, A., Weindel, K., Ochs, A., Marth, C., Zmija, J., Schumacher, P., Unger, C., Marme, D., and Gastl, G. (1999). Vascular endothelial growth factor in the sera and effusions of patients with malignant and nonmalignant disease. Cancer *85*, 178–187.

Licht, T., Tsirulnikov, L., Reuveni, H., Yarnitzky, T., and Ben-Sasson, S.A. (2003). Induction of pro-angiogenic signaling by a synthetic peptide derived from the second intracellular loop of S1P3 (EDG3). Blood *102*, 2099–2107.

Lyden, D., Hattori, K., Dias, S., Costa, C., Blaikie, P., Butros, L., Chadburn, A., Heissig, B., Marks, W., Witte, L., et al. (2001). Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. Nat. Med. *7*, 1194–1201.

Machein, M.R., Renninger, S., de Lima-Hahn, E., and Plate, K.H. (2003). Minor contribution of bone marrow-derived endothelial progenitors to the vascularization of murine gliomas. Brain Pathol. *13*, 582–597.

Muller, A., Homey, B., Soto, H., Ge, N., Catron, D., Buchanan, M.E., McClanahan, T., Murphy, E., Yuan, W., Wagner, S.N., et al. (2001). Involvement of chemokine receptors in breast cancer metastasis. Nature *410*, 50–56.

Nissanov, J., Tuman, R.W., Gruver, L.M., and Fortunato, J.M. (1995). Automatic vessel segmentation and quantification of the rat aortic ring assay of angiogenesis. Lab. Invest. *73*, 734–739.

Ogawa, H., Suefuji, H., Soejima, H., Nishiyama, K., Misumi, K., Takazoe, K., Miyamoto, S., Kajiwara, I., Sumida, H., Sakamoto, T., et al. (2000). Increased blood vascular endothelial growth factor levels in patients with acute myocardial infarction. Cardiology *93*, 93–99.

Orimo, A., Gupta, P.B., Sgroi, D.C., Arenzana-Seisdedos, F., Delaunay, T., Naeem, R., Carey, V.J., Richardson, A.L., and Weinberg, R.A. (2005). Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. Cell *121*, 335–348.

Peled, A., Petit, I., Kollet, O., Magid, M., Ponomaryov, T., Byk, T., Nagler, A., Ben-Hur, H., Many, A., Shultz, L., et al. (1999). Dependence of human stem cell engraftment and repopulation of NOD/SCID mice on CXCR4. Science *283*, 845–848.

Peters, B.A., Diaz, L.A., Polyak, K., Meszler, L., Romans, K., Guinan, E.C., Antin, J.H., Myerson, D., Hamilton, S.R., Vogelstein, B., et al. (2005). Contribution of bone marrow-derived endothelial cells to human tumor vasculature. Nat. Med. *11*, 261–262.

Rafii, S., and Lyden, D. (2003). Therapeutic stem and progenitor cell transplantation for organ vascularization and regeneration. Nat. Med. *9*, 702–712.

Rajantie, I., Ilmonen, M., Alminaite, A., Ozerdem, U., Alitalo, K., and Salven, P. (2004). Adult bone marrow-derived cells recruited during

angiogenesis comprise precursors for periendothelial vascular mural cells. Blood *104*, 2084–2086.

Rehman, J., Li, J., Orschell, C.M., and March, K.L. (2003). Peripheral blood ''endothelial progenitor cells'' are derived from monocyte/macrophages and secrete angiogenic growth factors. Circulation *107*, 1164–1169.

Ruzinova, M.B., Schoer, R.A., Gerald, W., Egan, J.E., Pandolfi, P.P., Rafii, S., Manova, K., Mittal, V., and Benezra, R. (2003). Effect of angiogenesis inhibition by Id loss and the contribution of bone-marrow-derived endothelial cells in spontaneous murine tumors. Cancer Cell *4*, 277–289.

Salven, P., Manpaa, H., Orpana, A., Alitalo, K., and Joensuu, H. (1997). Serum vascular endothelial growth factor is often elevated in disseminated cancer. Clin. Cancer Res. *3*, 647–651.

Schachinger, V., Assmus, B., Britten, M.B., Honold, J., Lehmann, R., Teupe, C., Abolmaali, N.D., Vogl, T.J., Hofmann, W.K., Martin, H., et al. (2004). Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction: final one-year results of the TOPCARE-AMI Trial. J. Am. Coll. Cardiol. *44*, 1690–1699.

Sipkins, D.A., Wei, X., Wu, J.W., Runnels, J.M., Cote, D., Means, T.K., Luster, A.D., Scadden, D.T., and Lin, C.P. (2005). In vivo imaging of specialized bone marrow endothelial microdomains for tumour engraftment. Nature *435*, 969–973.

Takakura, N., Watanabe, T., Suenobu, S., Yamada, Y., Noda, T., Ito, Y., Satake, M., and Suda, T. (2000). A role for hematopoietic stem cells in promoting angiogenesis. Cell *102*, 199–209.

Wagers, A.J., Sherwood, R.I., Christensen, J.L., and Weissman, I.L. (2002). Little evidence for developmental plasticity of adult hematopoietic stem cells. Science *297*, 2256–2259.

Yamaguchi, J., Kusano, K.F., Masuo, O., Kawamoto, A., Silver, M., Murasawa, S., Bosch-Marce, M., Masuda, H., Losordo, D.W., Isner, J.M., and Asahara, T. (2003). Stromal cell-derived factor-1 effects on ex vivo expanded endothelial progenitor cell recruitment for ischemic neovascularization. Circulation *107*, 1322–1328.

Yu, Z., Redfern, C.S., and Fishman, G.I. (1996). Conditional transgene expression in the heart. Circ. Res. *79*, 691–697.

Ziegelhoeffer, T., Fernandez, B., Kostin, S., Heil, M., Voswinckel, R., Helisch, A., and Schaper, W. (2004). Bone marrow-derived cells do not incorporate into the adult growing vasculature. Circ. Res. *94*, 230–238.