Engraftment and Reconstitution of Hematopoiesis Is Dependent on VEGFR2-Mediated Regeneration of Sinusoidal Endothelial Cells

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SUMMARY

Myelosuppression damages the bone marrow (BM) vascular niche, but it is unclear how regeneration of bone marrow vessels contributes to engraftment of transplanted hematopoietic stem and progenitor cells (HSPCs) and restoration of hematopoiesis. We found that chemotherapy and sublethal irradiation induced minor regression of BM sinusoidal endothelial cells (SECs), while lethal irradiation induced severe regression of SECs and required BM transplantation (BMT) for regeneration. Within the BM, VEGFR2 expression specifically demarcated a continuous network of arterioles and SECs, with arterioles uniquely expressing Sca1 and SECs uniquely expressing VEGFR3. Conditional deletion of VEGFR2 in adult mice blocked regeneration of SECs in sublethally irradiated animals and prevented hematopoietic reconstitution. Similarly, inhibition of VEGFR2 signaling in lethally irradiated wild-type mice rescued with BMT severely impaired SEC reconstruction and prevented engraftment and reconstitution of HSPCs. Therefore, regeneration of SECs via VEGFR2 signaling is essential for engraftment of HSPCs and restoration of hematopoiesis.

INTRODUCTION

The functional and structural basis of blood cell development lies within the bone marrow (BM), consisting of several microenvironments including the osteoblastic niche (OBN). The OBN is believed to maintain the quiescence of stem cells. Although the phenotypic and functional attributes of the OBN are well defined (Arai et al., 2004; Calvi et al., 2003; Hattori et al., 2002; Heissig et al., 2002; Kiel et al., 2005; Yoshihara et al., 2007; Zhang et al., 2003), the identity of the vascular compartments in BM contributing to hematopoietic stem and progenitor cell (HSPC) homeostasis is unknown. During hematopoietic recovery after myelosuppression, HSPCs might interact with BM vasculature (Avecilla et al., 2004; Kiel et al., 2005; Kopp et al., 2005b; Takaku et al., 2000), a process that could be important for engraftment of HSPCs and reconstitution of hematopoiesis. However, as the phenotypic signature and molecular pathways involved in maintenance and regeneration of the BM vasculature are unknown, the role of neoangiogenesis in the regulation of HSPCs remains to be elucidated.

Currently, the BM vasculature is loosely identified as a network of thin-walled, fenestrated sinusoidal endothelial cells (SECs) and smooth-muscle-invested arterioles. SECs have a unique expression of adhesion molecules, allowing the trafficking and homing of HSPCs to the BM (Avecilla et al., 2004; Kiel et al., 2005; Kopp et al., 2005b; Takaku et al., 2000), a process that could be important for engraftment of HSPCs and reconstitution of hematopoiesis. However, as the phenotypic signature and molecular pathways involved in maintenance and regeneration of the BM vasculature are unknown, the role of neoangiogenesis in the regulation of HSPCs remains to be elucidated.

Studying the BM microvasculature has been hampered due to technical difficulties of working with calcified tissues. Furthermore, the angiogenic pathways regulating SEC maintenance and regeneration are unknown. Thrombospondins deployed from megakaryocytes were identified as negative regulators of BM SECs; however, the positive regulators of SECs are unknown (Kopp et al., 2005a). This hypothesis is reciprocal in that SECs provide not only a conduit for mature hematopoietic cells to the circulation but also a cellular platform whereby HSPCs differentiate and set the stage for hematopoietic reconstitution (Avecilla et al., 2004; Mohle et al., 1997; Rafii et al., 1994, 1995). However, this dependence is reciprocal in that SECs provide not only a conduit for mature hematopoietic cells to the circulation but also a cellular platform whereby HSPCs differentiate and set the stage for hematopoietic reconstitution (Avecilla et al., 2004; Mohle et al., 1997; Rafii et al., 1994, 1995).

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receptors, VEGFR2 and VEGFR3, in supporting BM angiogenesis has not been investigated.

Herein, we established reproducible techniques for staining decalcified BM sections and for performing polyvariate flow cytometric analyses on BM, allowing the definition of an angiogenic profile of BM vasculature distinguishing between arterial vessels and SECs. We show that VEGFR3 is selectively expressed by SECs, but not arterioles, permitting the tracking and quantification of SECs. We demonstrate that, although at steady state, VEGFR2 is dispensable for maintenance of SECs and HSPCs, after myelosuppressive insult, lack of VEGFR2 activation results in impaired regeneration of VEGFR3+ Sca1+/C0 SECs and HSPCs. Therefore, VEGFR2 is essential for orchestrating the engraftment and restoration of HSPC populations and reconstitution of hematopoiesis.

RESULTS

Bone Marrow Vasculature Is Demarcated by VEGFR2*VEGFR3* SECs

For the first time, we were able to reproducibly immunophenotype BM vessels by modifying standard immunohistochemical (IHC) (see Figure S1 available online) and immunofluorescence (IF) protocols and employing polyvariate flow cytometry. We identified angiogenic signatures and the precise geometric localization of BM arterioles and SECs using a panel of antibodies (Abs) against vascular specific markers, including, but not limited to, VE-cadherin, VEGFR2, VEGFR3, CD31, MECA32, and Sca1 (Figure 1A; Table S1). Furthermore, we genetically tracked the expression of VEGFR2 and VEGFR1 in reporter mice, where the expression of gfp and lacZ are driven by the endogenous VEGFR2 promoter (VEGFR2-GFP mice) (Ema et al., 2006) (Figures 1B–1D) and VEGFR1 promoter (VEGFR1-lacZ mice) (Fong et al., 1999), respectively (Figure 1Ac).

Using this approach, we found that in steady-state conditions, the vasculature of femurs from C57BL/6J wild-type (WT) mice consists of small arterioles and capillaries that span the BM and supply radially and regularly distributed SECs (Figures 1A and 1B). SECs are an interconnected network of vessels arising from cortical capillaries and encompass the major BM vascular compartment that interacts with hematopoietic cells (Movie S1). At steady state, both arterioles and SECs were immunopositive for VE-cadherin, MECA32, VEGFR2, and CD31 (Figures 1A–1D; Table S1). β-galactosidase (βGal) staining of BM from VEGFR1-lacZ mice indicated that VEGFR1 is expressed in both arterioles and SECs (Figure 1Ac; Table S1). IHC of BM from WT mice (Figures 1Ae and 1Af) and confocal IF of BM from VEGFR2-GFP mice (Figure 1D) revealed that BM SECs are specifically VEGFR3+ and Sca1−, while arterioles are VEGFR3− and Sca1+. In order to exclude the possibility that VEGFR3+ vessels are of lymphatic origin, we stained femurs for the lymphatic marker LYVE1 and found that the BM is devoid of LYVE1+ lymphatics (data not shown).

Based on these studies, we designated an immunophenotypic signature for steady-state BM SECs as VE-cadherin+...
MECA32+CD31+VEGFR2+VEGFR3+Sca1− while BM arterioles were identified as VE-cadherin+MECA32+CD31+VEGFR2+VEGFR3−Sca1+ (Figure 1; Table S1). Therefore, differential expression of Sca1 and VEGFR3 can be used to identify BM arterioles and SECs.

VEGFR2 and VEGFR3 Expression in BM Is Restricted to SECs

Previous uncorroborated studies have suggested that non-EC populations might express VEGFR2 and VEGFR3. To resolve this issue, we used IF and polyvariate flow cytometry. Osteopontin (OPN) IF on BM from VEGFR2-GFP mice demonstrated that there is no overlap between VEGFR2-GFP+ cells and OPN+ osteoblasts (OBs) (Figure 1B). Co-IF on BM from VEGFR2-GFP mice shows that VEGFR2-GFP+ cells, but not OPN+ OBs, are CD31+ and VE-cadherin+VEGFR3+ (Figures 1B–1D). To confirm that VEGFR2 expression is restricted to SECs, we performed polyvariate flow cytometry on BM mononuclear cells from crushed and enzymatically processed femurs. We show that VEGFR2-GFP+ cells are comprised of CD31+VEGFR2+CD45−CD11b−TER119− SECs, lacking expression of hematopoietic markers (Figure 2A and data not shown). In order to prove that VEGFR3 is expressed exclusively by functional SECs, we injected WT mice with fluorophore-conjugated Isolectin GS-IB4 to identify functional and patent SECs. Using a similar polyvariate flow cytometric approach, we demonstrated that VEGFR3 is expressed on functional CD45−VE-cadherin−Isolectin GS-IB4+ SECs (Figure 2B). In addition, cKit+Lineage+Sca1+ HSPCs (KLS) were VEGFR3−, suggesting that VEGFR3 expression within the BM is restricted to SECs (Figure 2C).

Nonetheless, it is possible that subsets of hematopoietic repopulating cells may be VEGFR2+. To rule this out, we purified VEGFR2+GFP+lineage− cells from β-actin-GFP mice, where GFP is universally expressed, and transplanted these cells into lethally irradiated mice with a radioprotective dose of GFP−BM. After 4 weeks, PB was assessed for the presence of GFP+ cells. No GFP+ cells were detected in the PB from any of the transplanted mice.

Consistent with the previously published report (Haruta et al., 2001), VEGFR2+GFP+lineage− cells failed to repopulate lethally irradiated mice (Figure 2D), whereas VEGFR2−GFP+ cells reconstituted host hematopoiesis (data not shown). Moreover, we demonstrate that in Col2.3GFP reporter mice, where expression of GFP is driven by the 2.3 kb fragment of the collagen alpha 1 type I promoter and is restricted to OBs (Dacic et al., 2001), VEGFR2 and VEGFR3 were not expressed by GFP+ OBs (Figure 3D). Collectively, these data suggest that the expression of VEGFR2 and VEGFR3 is restricted to SECs, and even if VEGFR2 is expressed on a small population of non-SECs, it does not serve any important hematopoietic function. Therefore, interference with VEGFR2 or VEGFR3 signaling has no direct effect on the reconstitution of OBs or hematopoietic cells.
Irradiation Remodels SECs, but Not OBs

It has been speculated, but not corroborated, that the OBN and BM SECs are spatially separate and functionally distinct entities. To resolve this controversy, we employed VEGFR3 and VE-cadherin as specific markers of BM vasculature to determine the physical location of BM SECs in relation to OBs. Hematoxylin and eosin (H&E) and anti-VEGFR3 IHC demonstrated that BM SECs are always in close proximity or directly apposed to OBs (Figure 3A). Consistent with these results, in Col2.3GFP mice, both in the diaphyseal and metaphyseal marrows, GFP+ OBs reside in direct apposition to VEGFR3+ SECs (Figures 3B and 3C). Three-dimensional imaging confirms that OBs are invested by VEGFR3+ SECs (Movie S2). After treating Col2.3GFP mice with 650 rad, there is a disruption of VE-cadherin+ vessels, while the physical integrity of GFP+ OBs is maintained (Figures 3E and 3F). These data suggest that VEGFR3+ VEGFR2− OBs (Figure 3D) and VE-cadherin+VEGFR3+ BM SECs form a continuous compound cellular niche and there is no major regression of GFP+ OBs after irradiation.

Degree of Injury to SECs Is Dictated by the Extent of Myelosuppressive Injury

As shown in Figure 3F, irradiation induces a significant alteration in the BM vasculature. Thus, to define molecular pathways involved in BM SEC regeneration, we employed three models of myelosuppression. This allowed examination of the consequences of mild, moderate, and severe regression of SECs on reconstitution of hematopoiesis. First, we used the 5-fluorouracil (5FU, 250 mg/kg) model of myelosuppression, which induces mild regression of SECs (Figures 4A–4C). To induce moderate and severe vascular regression in the BM, we treated mice with sublethal (650 rad) or lethal (950 rad) irradiation, respectively. We observed an incremental loss of SECs in WT mice treated with 950 rad > 650 rad > 5FU as quantified by costaining with EC-specific markers (Figures 4A–4C). In support of these data, recently it has been shown that irradiation induces regression through nonapoptotic cell death followed by regeneration from surviving vascular cells (Li et al., 2008). The severe regression of SECs in mice irradiated with lethal 950 rad dose was rescued.
with BM transplantation (BMT) with $5 \times 10^5$ whole BM cells (950 rad + BMT) (Figure 4C), providing an ideal model to test the role of regeneration of SECs in reconstitution of hematopoiesis.

To categorize and quantify the severity and degree of damage to regenerating SECs in each model of myelosuppression, we devised a staging system (Figures 4B and 4C). At days 7 and 10 after treatment of mice with 5FU, 650 rad, or 950 rad with or without BMT, two types of abnormal SECs were evident. During the early recovery after 5FU and early and late phases of recovery after 650 rad, there was a predominance of type I vessels presenting as discontinuous or hemorrhagic vessels (Figures 4B and 4C). Although by day 10, the SECs in 5FU-treated mice were primarily nonhemorrhagic and nondiscontinuous; in mice treated with 650 rad and more prominently with 950 rad (without BMT rescue), SECs were discontinuous and hemorrhagic with denuded ECs from vessel walls, designated as type II regressed vessels (Figures 4B and 4C). There were no normal vessels present in the femurs of mice irradiated with 950 rad without BMT, and by day 10, there was no evidence of vascular regeneration of the regressed vessels (Figure 4C). However, in 950 rad + BMT-treated mice, there was significant vascular recovery with emergence of normal and type I vessels and significant reduction of type II regressed vessels (Figure 4C). These data suggest that BM SECs comprise malleable and dynamic vessels with the potential to regenerate and remodel into functional sinusoids. However, the molecular pathways driving the regeneration and assembly of regressed SECs are unknown. We hypothesized they may involve the activation of VEGF receptors, given their significant expression by BM SECs (Figures 1–3).
Although there is a slight, yet significant, downregulation of VEGFR3 expression during early stages of hematopoietic recovery, there is a robust upregulation of VEGFR2 expression on day 5 postirradiation, as determined by MFI of VEGFR2 and VEGFR3 expression on CD45+Cd0/Cd0 SECs (Figures S2Ba and S2Bb; Table S1). Furthermore, qPCR on BM from 5FU-treated mice demonstrated that VEGF-C and VEGF-A are significantly upregulated at varying times after myelosuppression (Figures S2Ca and S2Cb), guaranteeing activation of VEGFR2 during hematopoietic recovery. VEGF-A protein was upregulated in BM plasma from 650 rad-treated mice as compared to untreated animals (Figure S2D). These data suggest that VEGFR2 and VEGFR3 and their cognate ligands are present and variably expressed during hematopoietic recovery and may play a role in the reconstruction of regressed SECs without influencing the function of OBs.

To test this hypothesis, WT mice, myelosuppressed with 5FU, 650 rad, or 950 rad + BMT, were treated with neutralizing monoclonal antibodies (mAb) against VEGFR2 (DC101) or VEGFR3 (mF4-31C1) (Prewett et al., 1999; Pytowski et al., 2005; Tammela et al., 2008). Treatment of 5FU myelosuppressed mice with mAb to either VEGFR2 or VEGFR3 had only marginal effect on delaying vascular recovery within the BM (data not shown). However, when 650 rad-treated mice were injected with mAb to VEGFR2, there was a significant reduction in HSPC reconstitution. On day 14 after irradiation, mice treated with mAb to VEGFR2, but not the control IgG group, showed significant thrombocytopenia (Figure 5A) and reduced number of spleen colony-forming units (CFU-S) (Figure 5B) and BM cell counts (Figure 5C). Defects in BM cytoarchitecture with a predominance of disorganized, leaky, and hemorrhagic (type I vessels) VE-cadherin+ SECs was observed in BM from VEGFR2 mAb-treated mice at 14 days posttreatment (Figure 5D). Additionally, there was
a profound decrease in KLS population (Figure 5E) and CFU-C (Figure 5F). These data suggest that blocking VEGFR2 signaling during BM regeneration after myelosuppression interferes with proper assembly of SECs, impairing hematopoiesis.

Conditional Deletion of VEGFR2 Gene in Adult Mice Does Not Affect Steady-State Hematopoiesis

Neutralizing mAb to VEGFR2 blocks only the paracrine/extrakinetic activation of VEGFR2. As VEGFR2 can be activated by both extrakine and intrakine loops in ECs (Lee et al., 2007), we generated mice with floxed VEGFR2 alleles to interrogate the role of complete VEGFR2 deficiency on the reconstitution of HSPCs in steady-state conditions and after myelosuppression in adult mice. We targeted exon 3 of the vegr2 gene, which does not contain any known regulatory elements, to generate VEGFR2<sup>e3loxP/e3loxP</sup> mice (Figures S3A and S3B). VEGFR2<sup>e3loxP/e3loxP</sup> mice were bred with the VEGFR2<sup>−/−</sup> line to generate VEGFR2<sup>e3loxP/+</sup> mice. These animals were crossed with ROSA-CreERT2 transgenic mice, to establish the ROSA-CreERT2<sup>+/+</sup> VEGFR2<sup>e3loxP/−</sup> experimental mice and ROSA-CreERT2<sup>+/+</sup> VEGFR2<sup>e3loxP/−</sup> controls (Figure S3C). Tamoxifen treatment of these mice yielded VEGFR2<sup>lox/−</sup> and control VEGFR2<sup>lox/−</sup> mice. VEGFR2<sup>lox/−</sup> mice have complete knockdown of VEGFR2 in BM as determined by real-time PCR of genomic DNA for VEGFR2 gene excision (Figure S3D) and IHC for VEGFR2 protein (Figure S3E). There were no defects in steady-state hematopoiesis in VEGFR2<sup>lox/−</sup> mice, with white blood cells (WBCs) and platelets remaining at normal levels several weeks after tamoxifen treatment (Figures 5G and 5H). Furthermore, at steady state, VEGFR3<sup>−/−</sup> SECs in VEGFR2<sup>lox/−</sup> mice were phenotypically normal (Figure 5Ga). This is consistent with results in which WT mice at steady state but chronically treated with mAb to VEGFR2 (or VEGFR3) did not show any hematological defects during and after treatment (Figure S4). These data suggest that, although during embryonic development deletion of VEGFR2 (Ema et al., 2006; Shalaby et al., 1995) results in angiogenic defects, at steady state conditional deletion of VEGFR2 in adult mice does not affect hematopoiesis.

Reconstitution of SECs and Hematopoiesis Is Impaired in Irradiated VEGFR2-Deficient Mice

To determine whether inhibition of both paracrine and intrakine VEGFR2 signaling is more effective in blocking re-generation of SECs and imparting a more severe hematopoietic impairment, VEGFR2<sup>lox/−</sup> mice were sublethally challenged with 650 rad. As compared to control mice, there was a significant delay in WBC (Figure 5Gb) and platelet (Figure 5Gc) recovery in VEGFR2<sup>lox/−</sup> mice, with persistent life-threatening pancytopenia on day 10 postirradiation. Furthermore, there was a severe defect in recovery of VEGFR3<sup>−/−</sup> SECs resulting in the appearance of type II regressed vessels (Figures 5H–S). These data suggest that loss of both VEGFR2 intrakine and extrakine signaling results in a considerable impairment in SEC regeneration and defects in hematopoietic reconstitution. Therefore, the extent of damage to SECs and the degree of VEGFR2 inhibition dictate the rate of reconstruction of SECs and hematopoietic recovery.

VEGFR2 Is Essential for Engraftment and Reconstitution of HSPCs after Severe Myelosuppression

To evaluate the significance of VEGFR2 activation on regeneration of SECs and restoration of HSPC function after myelosuppression, we studied the effect of varying VEGFR2 inhibition in a HSPC transplantation model. We employed the 950 rad + BMT mouse model since, in this model, transplantation of HSPCs induces rapid regeneration of the type II-regressed SECs (Figure 4C) allowing interrogation of the role of VEGFR2 inhibition in mediating SEC regeneration and HSPC engraftment. WT mice were lethally irradiated with 950 rad followed by transplantation with HSPCs and were treated with low-dose (400 µg/dose, thrice weekly) and high-dose (800 µg/dose, thrice weekly) mAb to VEGFR2. In mice treated with 950 rad + BMT in combination with high dose mAb to VEGFR2, there was a significant inhibition in hematopoietic recovery, resulting in demise of the mice (n = 10, p < 0.05) (Figure 6A). The majority of mice treated with high dose, but not low dose, mAb to VEGFR2 succumbed to BM suppression due to persistent thrombocytopenia and leukopenia (Figure 6B). Treatment of mice with high dose mAb to VEGFR2 prevented rebound splenomegaly (Figure 6C) and abrogated the regeneration of normal VE-cadherin<sup>+</sup> vessels as the BM was primarily populated with discontinuous, hemorrhagic, denuded, regressed type II SECs (Figures 6D and 6D).

Using polyparative flow cytometry, we quantitatively assessed the number of regressed VEGFR3<sup>−/−</sup> SECs in the 950 rad + BMT mice treated with anti-VEGFR2 mAb. On day 13 after 950 rad + BMT treatment, the absolute number of VEGFR3<sup>−/−</sup>Sca1<sup>−/−</sup> SECs, quantified by the number of VEGFR2<sup>−/−</sup>VEGFR3<sup>−/−</sup>VE-cadherin<sup>−/−</sup> CD31<sup>−</sup>Sca1<sup>+</sup>CD45<sup>+</sup>CD11b<sup>+</sup>Ter119<sup>−</sup> SECs, was diminished in mice treated with high-dose mAb to VEGFR2, but not low-dose mAb treatment (Figure 6E) or controls. The failure of hematopoietic recovery in mice treated with high dose mAb to VEGFR2 was due to a profound decrease in total hematopoietic cells and lineage<sup>−</sup> (Lin<sup>−</sup>) cells (Figures 6F and 6G). In particular, the KLS population was reduced to a virtually undetectable level (Figure 6H). Although control mice had 30,744 KLS cells, high-dose anti-VEGFR2-treated mice had only 135 KLS, and low-dose anti-VEGFR2 treated mice had 3,568 KLS cells (Figure 6H). These data suggest that inhibition of VEGFR2, selectively expressed by SECs but not OBs prevents regeneration of damaged SECs, thereby interfering with engraftment of HSPCs and replenishment of KLS population leading to hematopoietic failure in an OB-independent manner (Figure 7).

Blocking VEGFR3 in mice treated with 950 rad + BMT does not affect regeneration of BM vasculature but interferes with the proper remodeling of neovessels, leading to the formation abnormal predominantly type I vessels (Figure SSE) along with a significant decrease in the Lin<sup>−</sup> and CFU-C populations (Figures S5B and S5D). In 950 rad + BMT mice treated with mAb to VEGFR3, there is a trend in reduction of KLS population at day 10 (Figure SSC). Thus, after severe myelosuppressive insult, extrakine activation of the VEGF-C/VEGFR3 signaling pathway is not essential for regeneration of type II regressed vessels, but instead regulates proper patterning of re-generating VEGFR2<sup>−/−</sup> SECs, thereby accelerating progenitor cell (CFU) expansion, thereby fine-tuning hematopoietic reconstitutition.
DISCUSSION

The first evidence illustrating a critical role for SECs in the regulation of hematopoiesis was the finding by our group that revascularization of the BM after myelosuppression fully restored thrombopoiesis in thrombopoietin-deficient mice (Avecilla et al., 2004). These data demonstrated that regeneration of functional SECs is sufficient for lineage-specific differentiation of hematopoietic progenitors. However, uncertainties in spatial location, angiogenic determinants, and phenotypic attributes of BM vasculature have evaded functional definition due to technical barriers of working with calcified tissues and the nonstatic nature of the hematopoietic system (Kopp et al., 2005b, 2006). Using technical advances in BM preparation, we provide comprehensive phenotypic and functional data establishing a molecular signature of BM SECs at steady state and during hemangiogenic regeneration. We demonstrate that at steady state, BM SECs, representing the predominant vascular surface area in BM, are a continuous network of interconnected VE-cadherin+VEGFR2+VEGFR3+Sca1+ vessels, while smooth muscle invested arterioles are VE-cadherin+VEGFR2+VEGFR3−Sca1+. We show that the extent and rate of SEC regeneration after myelosuppression dictate the degree of hematopoietic recovery. Severe myeloablation induced by 650 or 950 rad irradiation does not significantly disrupt OBs, but leads to regression of VEGFR3+VEGFR2+Sca1− SECs, and the magnitude of the regression correlates with the severity in hematopoietic failure. Delay in VEGFR2-dependent recovery of SECs results in failure of HSPC expansion, leading to life-threatening pancytopenia. These data indicate that timely VEGFR2-dependent reassembly...
of SECs after severe myeloablative regression is critical for reconstitution of HSPCs and hematopoiesis. We demonstrate that virtually every OB is in close apposition to SECs establishing a compound OB-SEC niche. The close association of SECs with OBs suggests that targeting VEGFR2 and VEGFR3 may interfere with hematopoiesis through a non-SEC-mediated mechanism. Indeed, the expression of VEGFR2 and VEGFR3 might not be limited to vascular cells within BM (Gerber et al., 2002; Haruta et al., 2001). To address this issue, we tracked VEGFR2+ SECs in VEGFR2-GFP mice and OBs in Col2.3GFP mice to show that VEGFR2 is restricted to SECs and not OBs or other non-EC BM compartments. In agreement with Haruta et al. (2001), we demonstrate that even if a subset of VEGFR2+ cells contains an unrecognized hemangiogenic cell type, it does not serve a hematopoietic function, as transplantation of VEGFR2+ cells fails to engraft lethally irradiated mice. Therefore, targeting VEGFR2 will only interfere with the regeneration of the BM SECs, but not OBs or other non-ECs in the BM.

At steady state, conditional deletion of VEGFR2 in adult mice has no observable effect on the integrity of BM SECs, while after irradiation, there is a failure in BM SEC regeneration when VEGFR2 signaling is impaired. These data compliment the report of Lee et al. that demonstrates the constitutive deletion of VEGF-A autocrine signaling in surviving adult mice manifested no apparent hematological defects (Lee et al., 2007). As Lee et al. did not evaluate the role of VEGF-A/VEGFR2 autocrine loop after myelosuppression, it remains to be determined whether the intrakine VEGF-A pathway plays a dominant role in the regulation of hematopoietic recovery. Nonetheless, we show that a mAb specific for the extracellular domain of VEGFR2 blocks engraftment and abrogates reconstitution of HSPCs after irradiation, indicating that VEGFR2 extrakine activation contributes to the engraftment and restoration of hematopoiesis. Collectively, our data propose that both paracrine and autocrine activation of VEGFR2 plays a seminal role in the reassembly of functional SECs after myelosuppression.

How does myelosuppression switch quiescent VEGFR2-indepen endent SECs to initiate a VEGFR2-dependent neoangiogenic program? The release of pro- and antiangiogenic factors by hematopoietic cells might dictate not only the quiescent state but also the regeneration potential of SECs. For example, myelosuppression may induce OBs and megakaryocytes to secrete VEGF-A (Mohle et al., 1997). Once hematopoietic recovery is complete, mature cells, such as polyploid megakaryocytes, release antiangiogenic factors, including thrombospondins (Kopp et al., 2006), which suppress angiogenesis temporizing any further increase in hematopoietic activity.

In this study, we distinguished SECs from BM arterioles. Given the low numbers of VEGFR3 Scal+ arterioles, it is difficult to assess the contribution of arterioles to hematopoiesis. However, as arterioles may participate in regulating homing of transplanted HSPCs, identifying the pathways involved in sustaining their stability may shed light on homing receptors that capture circulating HSPCs. The angiogenic factors required for maintenance

Figure 7. Schema Depicting the Effect of VEGFR2 and VEGFR3 Inhibition in the Generation of Type I and Type II Vessels and Reconstitution of HSPCs

Interfering with VEGFR2 signaling in mice treated with potent myelosuppressive insults, such as 650 to 950 rad, results in significant impairment in the reconstruction of regressed SECs, while inhibition of VEGFR3 results in a defect in patterning of SECs. VEGFR2 inhibition impairs BMT-induced regeneration of SECs resulting in the failure of engraftment and reconstitution of HSPCs. Regeneration of SECs after 5FU may be driven by VEGFR2-independent but VEGFR1- and Tie2-dependent regeneration of hemangiogenic cells (Hattori et al., 2002; Kopp et al., 2006a).
and regeneration of BM arterioles are not known and require further investigation.

Until now, BM vasculature has been defined by its morphological attributes and there are no consistent molecular definitions of the vascular compartments within BM. Arterioles and SECs have been interchangeably referred to as the BM vascular niche without a unified approach to define their functional contribution to hematopoeisis. As such, our data will not only resolve the confusion pervading the BM angiogenesis, but also sets forth a mechanistic language to define and identify the relevant BM vasculature compartments. Quantification of VEGFR2+VEGFR3+Sca1+ SECs will provide predictive surrogate biomarkers to assess responses to antiangiogenic therapy and evaluate the degree of HSPC and hematopoietic reconstitution after myelosuppression.

Accumulating evidence suggests that the vasculature within each organ is heterogeneous and is tailored to serve the physiological requirements of that organ. Here, we show that BM SECs represent a network of malleable vessels requiring reciprocal interaction with hematopoietic cells for rapid VEGFR2-dependent regeneration, orchestrating engraftment and replenishment of HSPCs. We also set forth the concept that selective targeting of SECs via VEGFR2 inhibition, which does not affect the physical integrity of the OBs, is sufficient to block hematopoietic recovery, suggesting that OBs most likely do not play a significant role in hematopoietic reconstitution after myeloblation. Therefore, therapeutic administration of angiogenic factors may provide a means to accelerate reconstitution of stabilized functional VEGFR2+VEGFR3+Sca1− SECs, thereby avoiding fatal complications associated with chemotherapy and irradiation induced pancytopenia.

**EXPERIMENTAL PROCEDURES**

**Animals**

C57BL/6J (WT) and C57BL/6-Tg (ActeGFP)10sb/J mice were obtained from Jackson Laboratories (Bar Harbor, ME). VEGFR1-lacZ heterozygous mice were a gift from Dr. Guo-Hua Fong (Fong et al., 1999). Floxed VEGFR2 (VEGFR2flox/flox) mice were generated by Dr. Thomas Sato. Col2.3GFP mice were obtained from Dr. David Rowe (Dacic et al., 2001). VEGFR2-GFP mice were from Dr. Janet Rossant (Ema et al., 2006; Shalaby et al., 1995). Animal experiments were performed with the Institutional Animal Care and Use Committee of Weill Cornell Medical College using age- and sex-matched animals.

**BM and Organ Histology and IHC**

Tissues were fixed in 2%–4% paraformaldehyde (PFA), Femurs were decalcified using Decalcifying Solution (Richard-Allian Scientifc, MI) and frozen in OCT Compound (Sakura Finetek, CA) or embedded in paraffin. Paraffin sections were stained with hematoxylin and eosin (H&E, Histoserv, MD). For detection of VE-cadherin, MECA32, VEGFR2, and VEGFR3, paraffin sections were antigen-retrieved using Target Retrieval Solution (DAKO, CA). In some cases, IHC for VEGFR2 was performed on frozen sections. After endogenous peroxidase and nonspecific protein block (5% BSA, 10% donkey serum, and 0.02% Tween-20), primary Abs were incubated overnight at 4°C as follows: anti-VEGFR3 mAb (AFL4, 2 μg/ml, BD Pharmingen, CA), anti-pan EC antigen mAb (MECA32, 10 μg/ml, BD Pharmingen), anti-VE-cadherin polyclonal Ab (pAb, 1 μg/ml, R&D Systems, MN), anti-VEGFR2 pAb (T014, 10 μg/ml, gift from Dr. Phil Thorpe), and anti-VEGFR2 mAb (Avas12x1, 10 μg/ml, BD Pharmingen). After secondary pAb and streptavidin horseradish peroxidase incubations (Jackson IR, PA), staining was developed with DAB+ or AEC+ (DAKO) and briefly counterstained in Mayer’s hematoxylin (DAKO).

**VEGFR1-lacZ Staining**

Adult VEGFR1-lacZ mice were perfused with 0.2% glutaraldehyde. Femurs were postfixed for 4 hr at 4°C, decalcified in 10% EDTA, cryoprotected, and sectioned in OCT at 12 μm. Sections were incubated in X-Gal staining solution at 37°C for 12–16 hr and counterstained with Nuclear Fast Red (Biomeda, CA).

**BM IF and Detection of GFP**

WT, VEGFR2-GFP, and Col2.3GFP mice were perfused with 4% PFA. Femurs were postfixed, decalcified in 10% EDTA, cryoprotected, and snap frozen in OCT. Sections (12–30 μm) were blocked (5% donkey serum/0.3% Triton X-100) and incubated in primary Ab: anti-VEGFR3 mAb (mF4:31C1, 10 μg/ml, ImClone Systems, NY, or AFL4, 4 μg/ml, anti-VE-cadherin pAb (2 μg/ml), and anti-Sca1/Ly-6A/E mAb (D7, 10 μg/ml, BD). After incubation in fluorophore-conjugated secondary antibodies (1 μg/ml, Jackson IR), sections were counterstained with TOPRO3, DAPI, or Propidium Iodide (Molecular Probes/Invitrogen, CA).

**Flow Cytometric Analyses, Identification, and Quantification of BM SECs and Arterioles**

Purified mAbs were conjugated to Alexa Fluor dyes or DsRed per manufacturer’s protocols (Molecular Probes/Invitrogen). Whole BM cells from WT, VEGFR2-GFP, and Col2.3GFP animals were mechanically denuded of muscle/connective tissue, crushed in a sterile mortar, and digested in collagenase, while aggregating at 37°C for 45 min. Stained cells were analyzed on LSRII-SORP (BD). Data was processed with FACSDiva 6.1 software (BD). Doublets were excluded by FSC-W × FSC-H and SSC-W × SSC-H analysis, single stained channels were used for compensation, and fluorophore minus one (FMO) controls were used for gating. Fluorescent IgG controls were used to exclude nonspecific Ab binding. mAbs were purchased from BD except where noted: VE-cadherin (BV13, ImClone); VEGFR2 (mF4-31C1); VEGFR2 (DC101, ImClone, and Avas12x1); Sc1 (E13-161.7, C145, 30-F11), and CD11b (M1/70); TER119 (TER119); CD31 (MEC13.3); cKIt (ACK45); Gr-1 (RB6-8C5); CD41 (MWE0330); CD3 (17A2); and CD45 B220 (RA3-6B2).

For quantification of SECs, bones were mechanically prepared as above and the number of SECs was quantified by costing with conjugated antibodies to VEGFR2, VEGFR3, VE-cadherin, CD31, CD45, CD11b, Ter119, and Sca1. Number of SECs equals the number of VEGFR2+VEGFR3+VE-cadherin+CD31+Sca1+CD45+CD11b+ Ter119+ cells. VEGFR2+VEGFR3+CD31+Sca1+CD45+CD11b+ Ter119+ cells were scored as arterioles and were not counted in the total number of SECs. Intensity of VEGFR2 and VEGFR3 expression was determined by calculating the Mean Fluorescent Intensity (MFI) with FACSDiva software.

**Isolation and Transplant of VEGFR2+Lin+ and VEGFR2+Lin- Population**

Total BM from C57BL/6-Tg (ActeGFP)10sb/J mice was magnetically depleted of mature cells (Milenyi Biotech, CA). The VEGFR2− fraction from the Lin− population was sorted using FACS Aria flow sorter (BD). Five-thousand VEGFR2Lin− cells were transplanted into lethally irradiated (850 rad) WT mice with a radioprotective dose of 1 × 106 WT total BM cells. Control VEGFR2Lin− GFP+ cells were also transplanted. After 4 weeks, the number of circulating GFP+ cells in PB from transplanted mice was evaluated by flow cytometry.

**Myelosuppression Models**

Mice received one of three different myelosuppression strategies: (1) a single intravenous (i.v.) injection of 5-fluorouracil (5FU, 250 mg/kg body weight); (2) 850 rad irradiation; or (3) 950 rad irradiation, either alone or followed by i.v. administration of 5 × 106 whole BM cells (BMT). Where indicated, cohorts of mice were treated with 400 μg/dose or 800 μg/dose of neutralizing mAb to VEGFR2 (DC101), 800 μg/dose of neutralizing mAb to VEGFR3 (mF4-31C1), or control IgG (Jackson IR) intraperitonally (i.p.) thrice weekly until the time points indicated in the text or until moribund.

**Image Acquisition and Image Analysis**

Histological and IHC images of BM sections were captured with AxioCam and AxioVision software (Zeiss, NY) mounted on Olympus BX51 microscope (Olympus America, NY). IF images were captured on AxioVision LSM510Meta.
confocal microscope (Zeiss). Selected images were rendered with Volocity 4 3D imaging software (Improvision, MA). Digital images were analyzed for SEC (VE-cadherin* and/or VEGFR3*) integrity as described in figure legends using ImageJ (NIH, MD) and Adobe Photoshop (Adobe, San Jose, CA). Type I and type II vessels were assessed and quantified by visual examination of three 200× images per femur, spanning the BM cavity. Discontinuous or hemorrhagic SECs were scored as type I abnormal SECs. Discontinuous, hemorrhagic, and denuded vessels were scored as type II regressed vessels. VEGFR3 Scalc+ vessels or vessels that were phenotypically apparent arteries due to smooth muscle investment were not included as normal, type I, or type II SECs.

Peripheral Blood Analysis
Retro-orbital peripheral blood (PB) was collected using microhematocrit capillary tubes (Fisher Scientific, PA). WBC counts with differential, hematoglobin, calculated hematocrit, and platelets were obtained using Bayer Advia 120 Multi-Species Hematology Analyzer (Bayer HealthCare, NY) with multispecies software (Bayer).

Isolation and Analysis of cKit+ lineage Scalc+/KLS Population
Total BM cells were isolated from myelosuppressed WT mice treated with anti-VEGFR2 mAb, anti-VEGFR3 mAb, or control IgG and were enriched using Lineage Cell Depletion Kit (Miltenyi), yielding >95% purity. Lineage cells were stained with anti-Scalc1 (D7, BD) and anti-cKit (2B8, BD), and analysis was performed on LSRII SORP using FACSDiva 6.1 software for data processing. Doubles were excluded by FSC-W × FSC-H and SSC-W × SSC-H analysis, and single-stained channels were used for compensation.

CFU-S Assay
Spleens from mice treated with anti-VEGFR2 or control were removed at day 12–14 after myelosuppression, fixed in Bouin’s solution (Sigma Aldrich, MO), briefly washed in PBS, and weighed, and CFU-S were counted and/or imaged on a Zeiss Discovery stereo microscope (Zeiss).

Generation of Inducible VEGFR2 Knockout Mice
Exon 3 of vegfr2 was targeted to generate the VEGFR2e3loxP/e3loxP mice (Figure S3). VEGFR2e3loxP/e3loxP mice were bred with VEGFR2fl/fl mice. VEGFR2fl/fl mice were bred with ROSA-CreERT2 transgenic mice to establish the ROSA-CreERT2:VEGFR2fl/fl line (and controls ROSA-CreERT2VEGFR2loxP/loxP). These mice were treated with tamoxifen at a dose of 5 mg/150 ml sunflower oil (Sigma) i.p. for 6 days interrupted for 3 days after the third dose. After 3 days of respite, the fourth dose was reinstituted for an additional 3 days. Mice were allowed to recover a minimum of 15 days before experiments commenced.

Statistical Analyses
Results were analyzed using Student’s t test. p < 0.05 was considered significant. Results are expressed as mean value ± SEM.

SUPPLEMENTAL DATA
The Supplemental Data include five figures, Supplemental Experimental Procedures, one table, and two movies and can be found with this article online at http://www.cell.com/cell-stem-cell/supplemental/S1934-5909(09)00009-5.

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