Impaired recruitment of bone-marrow–derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth

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The role of bone marrow (BM)-derived precursor cells in tumor angiogenesis is not known. We demonstrate here that tumor angiogenesis is associated with recruitment of hematopoietic and circulating endothelial precursor cells (CEPs). We used the angiogenic defective, tumor resistant Id-mutant mice to show that transplantation of wild-type BM or vascular endothelial growth factor (VEGF)-mobilized stem cells restore tumor angiogenesis and growth. We detected donor-derived CEPs throughout the neovessels of tumors and Matrigel-plugs in an *Id1*$^{+/-}$*Id3*$^{-/-}$ host, which were associated with VEGF-receptor-1–positive (VEGFR1^{+}) myeloid cells. The angiogenic defect in Id-mutant mice was due to impaired VEGF-driven mobilization of VEGFR2^{+} CEPs and impaired proliferation and incorporation of VEGFR1^{+} cells. Although targeting of either VEGFR1 or VEGFR2 alone partially blocks the growth of tumors, inhibition of both VEGFR1 and VEGFR2 was necessary to completely ablate tumor growth. These data demonstrate that recruitment of VEGF-responsive BM-derived precursors is necessary and sufficient for tumor angiogenesis and suggest new clinical strategies to block tumor growth.

In the initial phases of tumor angiogenesis, endothelial cells (ECs) are recruited from the neighboring pre-existing capillaries[1–4]. Although evidence has supported the notion that bone-marrow (BM)-derived endothelial-like cells may contribute to wound healing[5–9], it is not established whether BM-derived precursor cells can contribute to tumor neo-angiogenesis. Moreover, studies evaluating the significance of endothelial precursor cells (CEPs) or hematopoietic cells in tumor angiogenesis have been hampered by the lack of suitable *in vivo* models.

We have taken advantage of the angiogenic defect in Id-mutant mice[10] to determine the functional importance of the recruitment of BM precursor cells in tumor angiogenesis. The Id proteins interact with other helix–loop–helix transcription factors, thereby modulating cellular differentiation in early fetal development[11]. *Id1*$^{+/-}$*Id3*$^{-/-}$ double-mutant mouse embryos display vascular malformations in the forebrain leading to fatal hemorrhage. Adult mice with reduced *Id* gene dosages cannot support neo-angiogenesis when challenged with tumor[10]. Therefore, Id-deficient mice provide a valuable model to examine the role of BM-derived hematopoietic and CEPs in initiating and sustaining a functional tumor vasculature.

Vascular endothelial growth factor (VEGF)[12] mediates the angiogenic switch[2,13–15] by interacting with two tyrosine kinase receptors, VEGF receptor-1 (VEGFR1, Flt-1)[16] and VEGFR2 (Flk-1, KDR)[17,18]. Although VEGFR2 regulates proliferation and survival of ECs, the function of VEGFR1 in the modulation of angiogenesis is not known. VEGFR1-deficient mice have vascular malformations suggesting that VEGFR1 has a role in vascular remodeling[16,19,20]. Nonetheless, the mechanism whereby activation of VEGFR2 or VEGFR1 results in recruitment of endothelial and hematopoietic cells, and their role in tumor angiogenesis is not fully understood.

Here, we demonstrate that transplantation and engraftment of β-galactosidase–positive (β-gal^{+}) wild-type BM or VEGF-mobilized stem cells into lethally irradiated Id-mutant mice is sufficient to reconstitute tumor angiogenesis. Analysis of the tumors demonstrates incorporation of BM-derived VEGFR2^{+} CEPs into vessels surrounded by VEGFR1^{+} myeloid cells. Defective angiogenesis in Id-mutant mice is associated with impaired VEGF-induced mobilization and proliferation of the BM precursor cells. We show that inhibition of both VEGFR1 and VEGFR2 signaling was necessary to block tumor angiogenesis and induce tumor necrosis.

Transplantation of BM restores Id-mutant angiogenic defects

We lethally irradiated *Id1*$^{+/-}$*Id3*$^{-/-}$ mice and reconstituted them

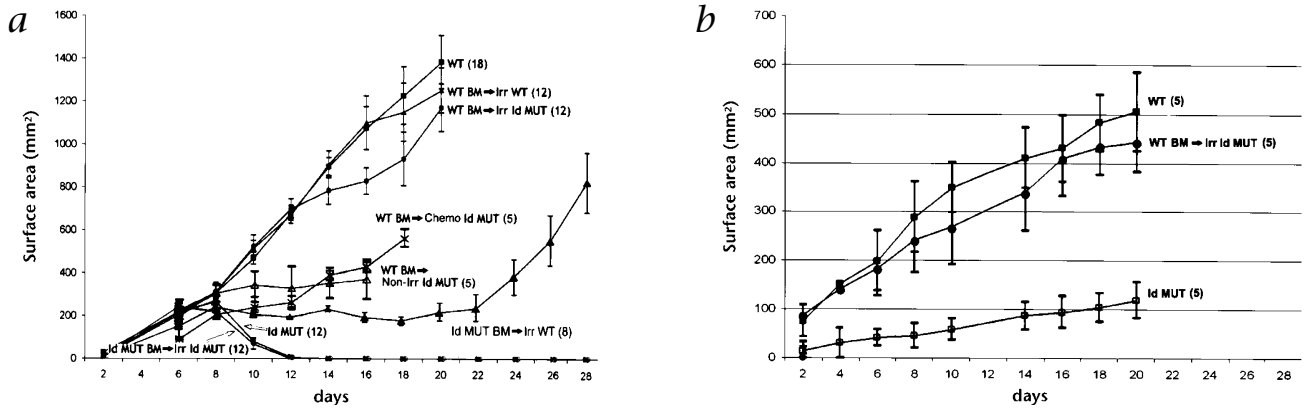


Fig. 1 Transplantation of wild-type (WT) BM rescues tumor growth in Id-mutant mice. ***a*** and ***b***, WT or Id-mutant (Id MUT) BM donor cells were injected via tail veins into lethally-irradiated (Irr) WT or Id-mutant mice. Following reconstitution, mice were injected intradermally with 2×10^7 cells of either B6RV2 lymphoma cells (*a*) or Lewis Lung Carcinoma (LLC) cells (*b*). Irradiated Id-mutant mice rescued with WT BM restores B6RV2 and LLC tumor growth similar to either WT or irradiated WT mice transplanted with the WT BM. This is in contrast to Id-mutant or irradiated Id-mutant mice reconstituted by Id-mutant BM that failed to support tumor growth. Engraftment of Id-mutant BM into irradiated WT host resulted in profound delay in tumor B6RV2 growth. Rosa-26 WT BM micro-transplanted into non-irradiated Id-mutant mice (10% BM chimerism, Non-Irr Id MUT), inoculated with B6RV2 tumor cell, support and maintain tumor growth (*a*). Similarly, 50% BM chimeric 5-FU-treated Id-mutant (Chemo Id MUT) mice support the growth of inoculated B6RV2 tumors. Tumor surface area mean ± s.d. are shown and number of mice is given in parentheses.

with donor wild-type BM cells. Four weeks after hematopoietic engraftment, we intradermally inoculated mice with either B6RV2 lymphoma or Lewis lung carcinoma cells (LLCs) (Fig. 1). In these reconstituted mice, tumor growth paralleled that observed in wild-type animals. Moreover, as in wild-type animals, Id-mutant mice engrafted with wild-type BM cells and inoculated with B6RV2 lymphoma developed widespread mesenteric lymph node metastases and died before day 26. In contrast, we observed rapid tumor regression in non-transplanted Id-mutant mice or irradiated Id-mutant mice receiving Id-mutant BM cells.

To determine if BM-derived precursor cells are necessary for tumor growth, we transplanted BM from Id-mutant mice into lethally irradiated wild-type mice and inoculated them with B6RV2 cells (Fig. 1*a*). Tumor growth was substantially retarded for three weeks, demonstrating that BM precursor cells are required at least for the early phases of tumor angiogenesis. Residual wild-type BM may account for the later growth of the tumors.

Given the four-week recovery time after irradiation and before tumor challenge, it is unlikely that irradiation facilitated BM recruitment by compromising the host ECs ability to contribute to tumor vascularization. Nonetheless, to test this we generated chimeric Id-mutant mice either by micro-transplanting BM from Rosa-26 mice into naive Id-mutant, non-irradiated recipients or Id-mutant mice that were treated with a single dose of 5-flurouracil (5-FU) (Fig. 1*a*). This results in the generation of a chimeric Id-mutant BM with 10% (micro-transplantation) and 50% (5-FU treatment) donor-derived cells. Remarkably, growth of B6RV2 cells was restored in all chimeric Id-mutant mice. As compared with irradiated Id-mutant mice engrafted with wild-type BM, chimeric Id-mutant mice displayed slower tumor growth (Fig. 1). However, the degree of tumor growth correlated with the level of engraftment of wild-type BM into the Id-mutant host. These data suggest that tumor growth in Id-mutant mice transplanted with wild-type BM was not due to vascular alterations induced by irradiation, but rather to the direct contribution of wild-type BM-derived cells.

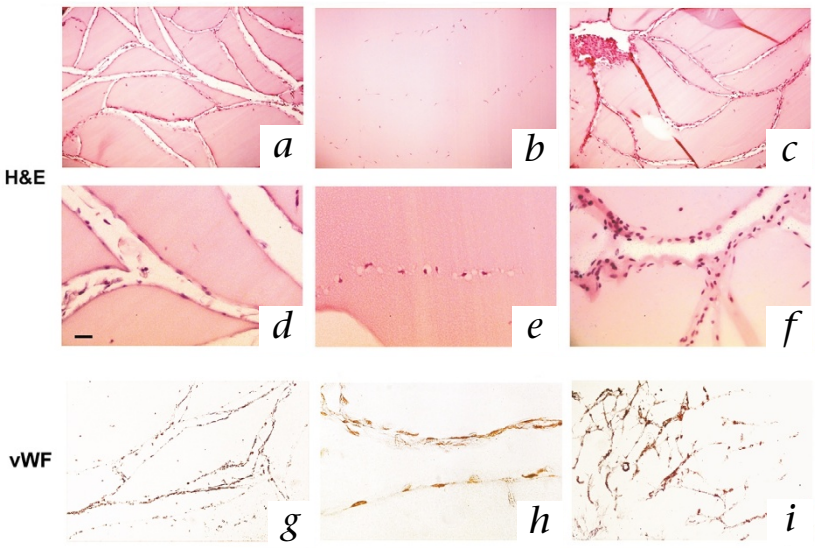


Fig. 2 Transplantation of WT BM restores vascular channel formation. ***a–f***, In the Matrigel/VEGF assay on day 10 after implantation, channel formation is observed in (*a* and *d*) WT ($n = 12$) (*b* and *e*) absent in Id-mutant ($n = 10$), and rescued in H&E-stained plugs of (*c* and *f*) irradiated Id-mutant mice engrafted with WT BM ($n = 12$). ***g–i***, vWF staining identifies endothelial cells lining the vessels on day 10 (*g* and *h*) and sprouting mature vessels on day 21 (*i*) of Matrigel plugs implanted into lethally irradiated $Id1^{+/-}Id3^{-/-}$ mutant recipient mice reconstituted with WT BM. Scale bar, 100 μm (*a–c*); 25 μm (*d–f*); 100 μm (*g*); 25 μm (*h*); 50 μm (*i*).

To determine whether the contribution of wild-type donor-BM in Id-mutant mice to neovascularization was restricted to tumor vasculature, we examined vascular channel formation in VEGF-loaded Matrigel plugs (Fig. 2). Histological analysis of the plugs removed after either 10 or 21 days after implantation showed

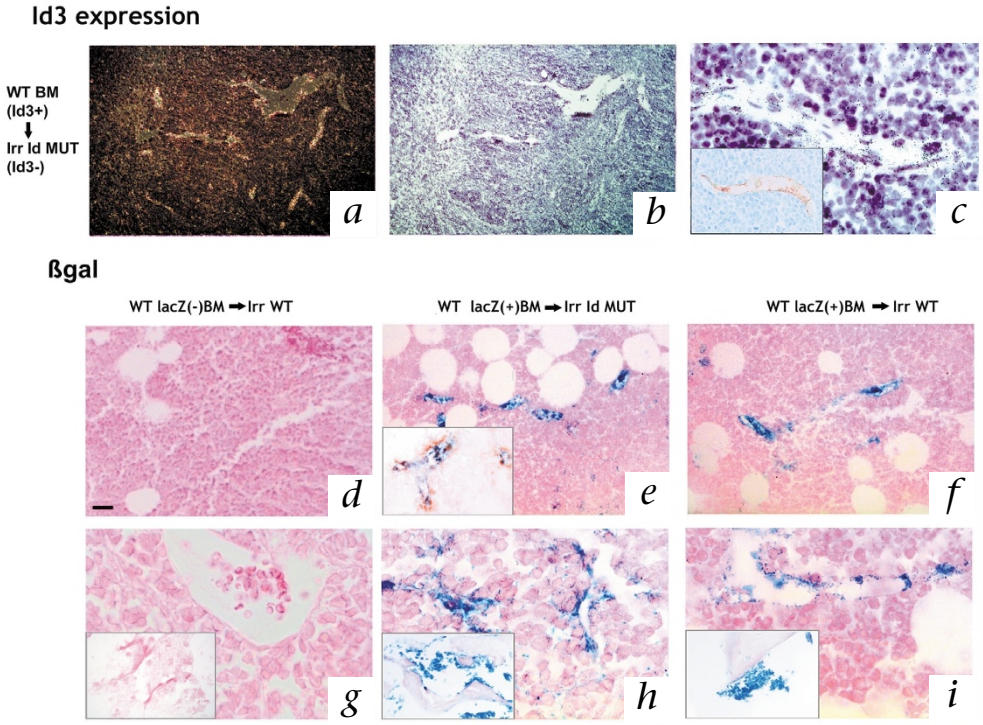


Fig. 3 BM-derived cells reconstitute the angiogenic defect in $Id1^{+/-}Id3^{-/-}$ mutant mice. ***a–i***, Irradiated Id3$^-$ mutant mice transplanted and engrafted with Id3$^+$ WT BM were stained for H&E in bright field (*b* and *c*) and vWF (*c*, inset) that correspond to: *Id3* gene expression in dark field (*a*) of a blood vessel in a day 14 B6RV2 tumor (n = 12). As control experiments, transplanted β-gal$^-$ WT BM into irradiated WT hosts failed to stain for LacZ (*d* and *g*), D14 B6RV2 tumors, blood vessels and BM (*g*, inset; n = 8). Nearly all vessels were LacZ$^+$ in D14 B6RV2 tumors growing in both irradiated Id-mutant (*e* and *h*; n = 14) and WT (*f* and *i*; n = 12) recipients, engrafted with Rosa 26 BM, with LacZ$^+$ cells also detected in *h* and *i* (insets) BM cells (eosin for bone). vWF stains β-gal$^+$ blood vessels (*e*, inset). Scale bars, 25 μm (*b–c* and *g–i*); 50 μm (*a* and *d–f*); 100 μm (insets).

no vascular channel formation in Id mutants (n = 10) (Fig. 2*b* and *e*), whereas vessel sprouting can be seen in Matrigel plugs of the wild-type mice (n = 12) (Fig. 2*a* and *d*). Notably, reconstitution of the Id-mutant mice (n = 12) with wild-type BM cells restored channel formation in the Matrigel plugs (Fig. 2*c* and *f*). By day 21 of implantation, von Willebrand Factor-postive (vWF$^+$) vessels contained blood cells that formed numerous vWF$^+$ sprouting vessels (Fig. 2*i*). These data demonstrate that wild-type BM contributes to the restoration of vascular channels in Id-mutant host mice in a non-tumor setting.

Incorporation of BM-derived CEPs into neovessels

To assess whether BM-derived cells were recruited to the neo-angiogenic site in B6RV2 tumors, we performed RNA *in situ* hybridization for Id3 (Fig. 3). Id3 expression was detected in tumor vasculature in $Id1^{+/-}Id3^{-/-}$ mice (n = 12) transplanted with donor $Id1^{+/+}Id3^{+/+}$ wild-type BM (Fig. 3*a* and *b*). vWF co-expressed with Id3 established the presence of dilated vessels typical of tumor vasculature (Fig. 3*c*). To confirm the contribution of BM-derived ECs to the tumor vasculature, we implanted B6RV2 tumors into lethally irradiated Id-mutant mice engrafted with β-gal$^+$ BM (Rosa-26 mice, n = 14). Because Rosa-26 mice express the β-gal transgene in all tissues, LacZ staining of the tumor can reveal donor-derived BM cells readily. We detected LacZ$^+$ vessels in tumors implanted for 14 days. (Fig. 3*e* and *h*). Approximately 90% of the vessels expressing vWF were characterized as LacZ$^+$ (Fig. 3*e*, inset, and data not shown) demonstrating that wild-type BM-derived cells are incorporated into vessels associated with the B6RV2 tumors grown in Id-mutant mice.

The contribution of donor cells to the tumor vasculature may reflect the inability of the neighboring Id-deficient ECs to be recruited to the vascular bed, thereby forcing the recruitment of donor-BM cells. To examine the relative contribution of pre-existing and BM-derived CEPs in a more physiological setting, BM from Rosa-26 mice was transplanted into lethally irradiated wild-type mice (n = 12) and then challenged with tumor. As in tumor vessels of BM-reconstituted Id-mutant mice, we detected LacZ-stained blood vessels throughout the vasculature of the tumor grafts in the wild-type host mouse (Fig. 3*f* and *i*). In addition, LacZ staining was detected in all BM cells verifying a complete engraftment of the host BM, whereas the bone itself showed only eosinophilic staining (Fig. 3*h* and *i*, insets). No LacZ staining was seen in either the BM or tumor tissue when BM cells of wild-type β-gal$^-$ mice were transplanted (n = 8) (Fig. 3*d* and *g*, inset). Collectively, these results underscore the capacity of BM-derived cells to be mobilized and incorporated into the tumor vasculature.

VEGFR1$^+$ cells are co-mobilized with VEGFR2$^+$ CEPs to tumors

VEGF not only induces mobilization of CEPs but also hematopoietic stem and progenitor cells[22]. Therefore, transplantation of VEGF-mobilized cells into lethally irradiated Id-mutant mice should rescue hematopoiesis, replenish CEPs and allow cells to reconstitute angiogenesis in Id-mutant mice. We injected Rosa-26 mice with adenoviral vectors carrying $VEGF_{165}$ transgene ($AdVEGF_{165}$), and mobilized cells collected from the peripheral circulation were transplanted into lethally irradiated Id-mutant mice. Similar to the reconstitution with BM-derived cells, transplantation of VEGF-mobilized cells resulted in restoration of angiogenesis and tumor growth of the implanted B6RV2 cells in Id-mutant mice. On day 2 after tumor implantation, LacZ-stained cells could be detected in the blood vessels of the B6RV2 tumors (Fig. 4*a* and *b*), demonstrating that VEGF-mobilized CEPs are capable of contributing to neo-angiogenesis. Analysis of tumors demonstrated the presence of vWF$^+$LacZ$^+$ vessels (Fig. 4*c*), decorated with VEGFR1$^+$LacZ$^+$ mononuclear cells (Fig. 4*d*). The VEGFR1$^+$ cells were of myelomonocytic origin since they co-stained for myeloid-specific markers including monocyte macrophage (MOMA) (Fig. 4*e*) and CD11b (Mac1). Also, day-2 tumors were stained with the endothelial specific marker, VE-Cadherin. VEGFR1$^+$ cells, which are composed mostly of LacZ$^+$MOMA$^+$ myelomonocytic cells (Fig. 4*e*), were found in asso-

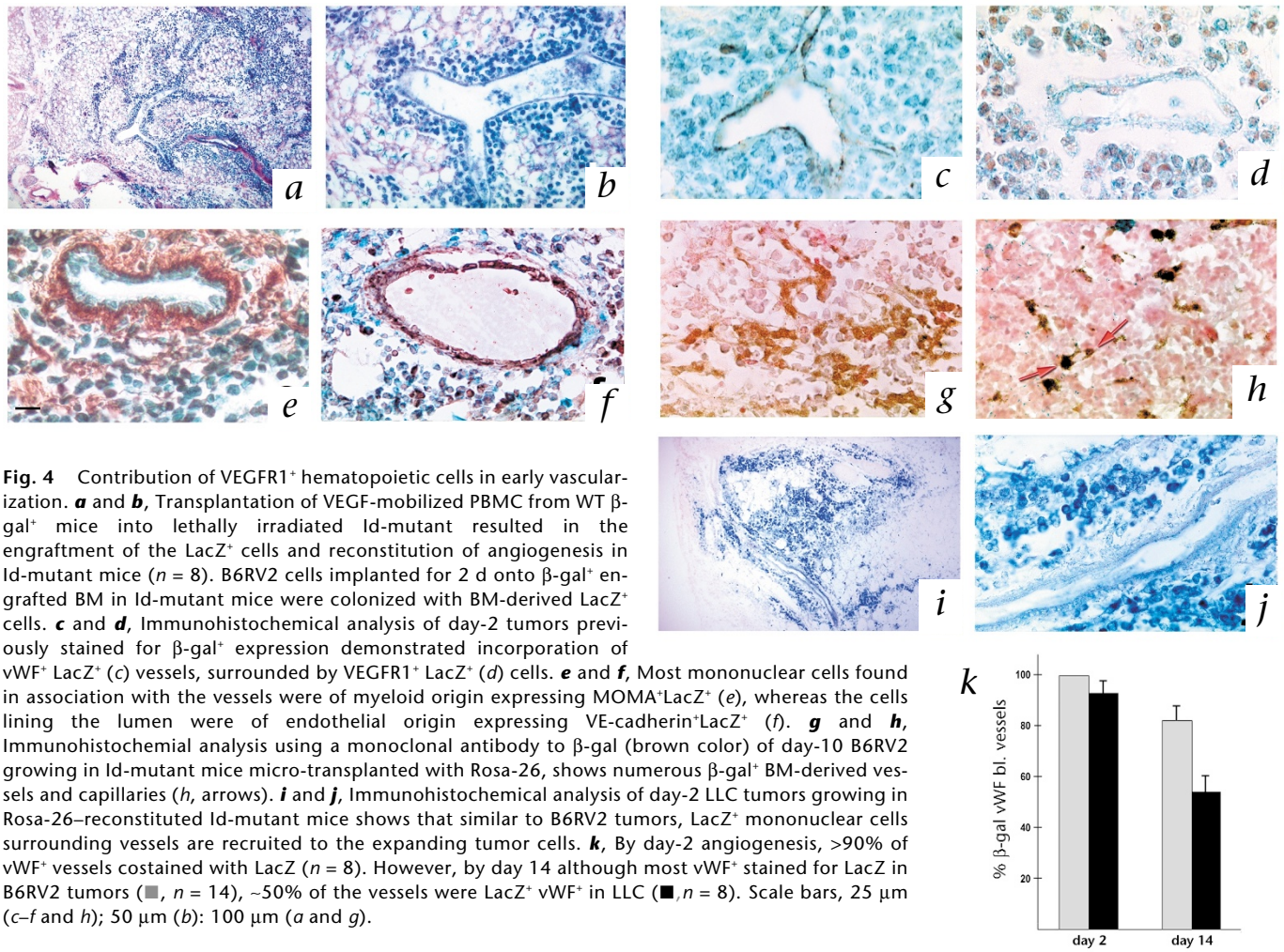


Fig. 4 Contribution of VEGFR1$^+$ hematopoietic cells in early vascularization. ***a*** and ***b***, Transplantation of VEGF-mobilized PBMC from WT β-gal$^+$ mice into lethally irradiated Id-mutant resulted in the engraftment of the LacZ$^+$ cells and reconstitution of angiogenesis in Id-mutant mice (n = 8). B6RV2 cells implanted for 2 d onto β-gal$^+$ engrafted BM in Id-mutant mice were colonized with BM-derived LacZ$^+$ cells. ***c*** and ***d***, Immunohistochemical analysis of day-2 tumors previously stained for β-gal$^+$ expression demonstrated incorporation of vWF$^+$ LacZ$^+$ (*c*) vessels, surrounded by VEGFR1$^+$ LacZ$^+$ (*d*) cells. ***e*** and ***f***, Most mononuclear cells found in association with the vessels were of myeloid origin expressing MOMA$^+$LacZ$^+$ (*e*), whereas the cells lining the lumen were of endothelial origin expressing VE-cadherin$^+$LacZ$^+$ (*f*). ***g*** and ***h***, Immunohistochemial analysis using a monoclonal antibody to β-gal (brown color) of day-10 B6RV2 growing in Id-mutant mice micro-transplanted with Rosa-26, shows numerous β-gal$^+$ BM-derived vessels and capillaries (*h*, arrows). ***i*** and ***j***, Immunohistochemical analysis of day-2 LLC tumors growing in Rosa-26–reconstituted Id-mutant mice shows that similar to B6RV2 tumors, LacZ$^+$ mononuclear cells surrounding vessels are recruited to the expanding tumor cells. ***k***, By day-2 angiogenesis, >90% of vWF$^+$ vessels costained with LacZ (n = 8). However, by day 14 although most vWF$^+$ stained for LacZ in B6RV2 tumors (■, n = 14), ~50% of the vessels were LacZ$^+$ vWF$^+$ in LLC (■, n = 8). Scale bars, 25 μm (*c*–*f* and *h*); 50 μm (*b*): 100 μm (*a* and *g*).

ciation with LacZ$^+$VE-Cadherin$^+$ (Fig. 4*f*) vessels. β-gal$^+$ vessels could also be identified in tumors growing in the Id-mutant mice micro-transplanted with Rosa-26 wild-type BM (10% wild-type BM chimerism) (Fig. 4*g* and *h*). Irradiated Id-mutant mice transplanted with wild-type Rosa-26 BM were also inoculated with LLCs. Similar to early tumor angiogenesis seen in B6RV2 tumors, we detected LacZ$^+$ mononuclear cells surrounding newly formed LacZ$^+$ vessels in LLC tumors (Fig. 4*i* and *j*).

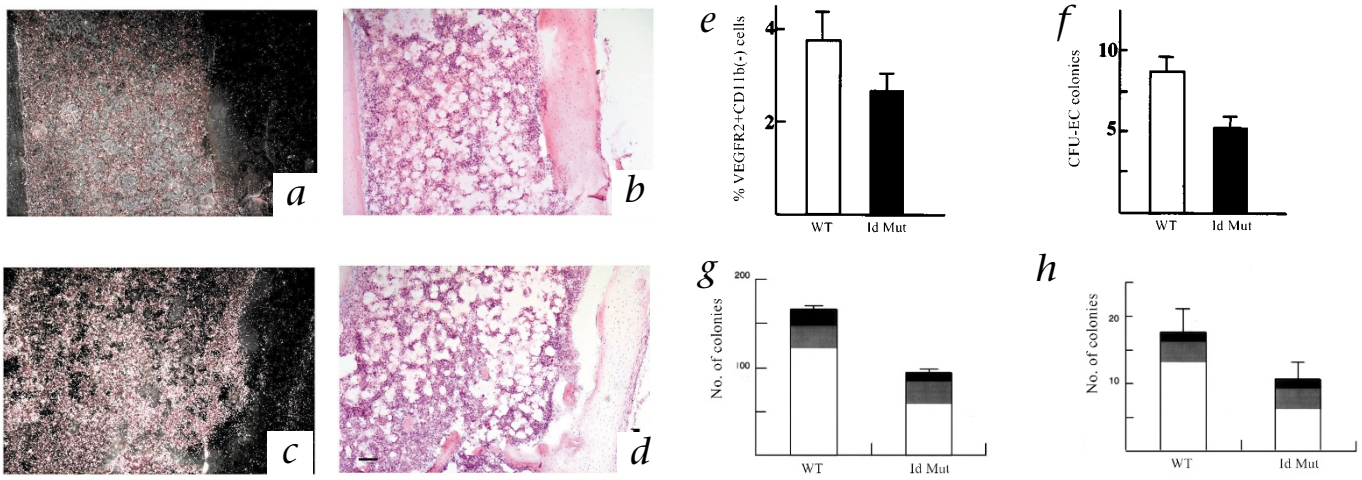
Quantification of the vWF$^+$LacZ$^+$ vessels demonstrated that by day 2 most of the neo-vessels were vWF$^+$LacZ$^+$ in both B6RV2 and LLC tumors (Fig. 4*k*), emphasizing the predominant contribution of BM-derived precursor cells to tumor growth. However, by day 14 although more than 90% of vessels within the B6RV2 tumors were still vWF$^+$LacZ$^+$, only 50% of the vessels within the LLC tumors were vWF$^+$LacZ$^+$ (Fig. 4*k*), suggesting that the persistence of BM-derived precursors in tumor angiogenesis is dependent to some extent on the tumor type.

Inhibition of VEGFR2 and *Id* impairs CEP motility

VEGF is critical for the survival, differentiation and chemokinesis of CEPs (refs. 21,22). *Id* genes are not detected by *in situ* hybridization in BM cells in adult wild-type mice (Fig. 5*a* and *b*). However, elevation of $VEGF_{165}$ plasma levels in wild-type mice resulted in the upregulation of *Id1* and *Id3* expression in BM (Fig. 5*c* and *d*). Although there are no differences in the blood counts of wild-type and Id-mutant mice, there was a 20% decrease in the total number of VEGFR1$^+$CD11b$^+$ myeloid cells and colony forming units (CFU-ECs) (Fig 5 *e* and *f*), and 30% reduction of hematopoietic progenitors (CFU-Cs), in the BM of Id-mutant mice (Fig. 5*g* and *h*). However, given that VEGF-induced angiogenesis is severely blocked in the Id-mutant mice, it seemed likely that the impaired recruitment rather than the absolute decrease in the number of the BM precursors that was responsible for tumor resistance in Id-mutant mice.

To test this hypothesis, *Id1*$^{+/-}$*Id3*$^{-/-}$ mutant and wild-type mice were injected with $AdVEGF_{165}$, which allows release of the VEGF (average plasma level of 750 pg/ml) into the circulation. Elevation of plasma $VEGF_{165}$ levels in wild-type mice (n = 6) induced mobilization of VEGFR2$^+$ CEPs that lacked the myelomonocytic marker (CD11b, Mac1) (11% on day 3, 5% on day 5 and 2% on day 10) (Fig. 5*i*). Notably, $VEGF_{165}$ failed to recruit any VEGFR2$^+$CD11b$^-$ CEPs to the circulation (0.3% on day 3, 0.2% on day 5 and 0.2% on day 10) (Fig. 5*i*) in Id-mutant mice (n = 6). The VEGFR2$^+$ cells were BM-derived CEPs, rather than mature ECs, given that they formed VEGFR2$^+$ late-outgrowth endothelial colonies (CFU-EC) in *in vitro* cultures (Fig. 5*j*).

We demonstrate that inoculation of B6RV2 cells into wild-type mice, but not the Id-mutant mice, also results in mobilization of VEGFR2$^+$ CEPs (Fig. 5*k*). Injection of a neutralizing antibody against VEGFR2 following the inoculation of B6RV2



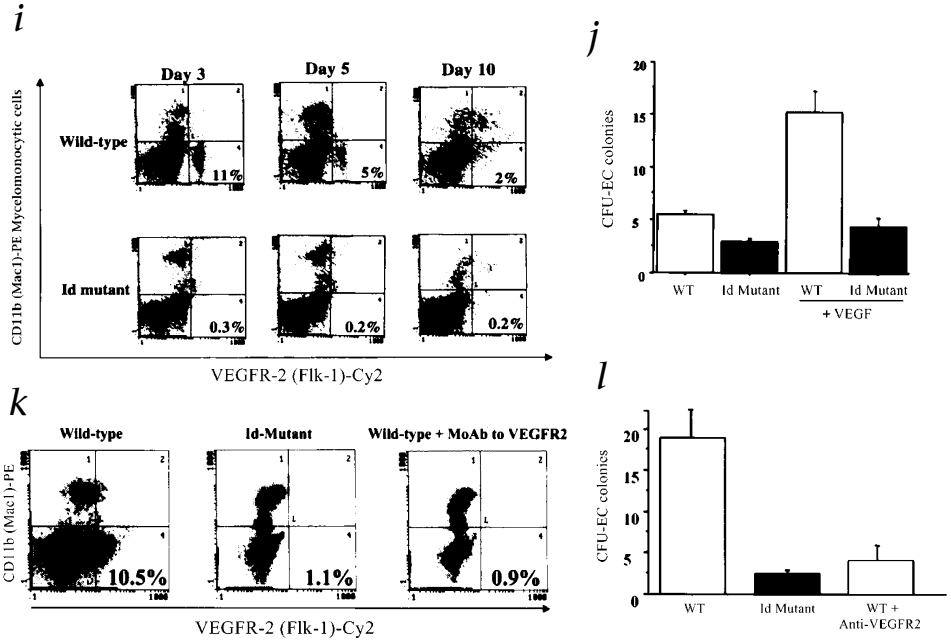


Fig. 5 Loss of the *Id* genes and neutralizing antibody against VEGFR2 impairs mobilization of CEPs. ***a*–*d***, Id-mutant mice or WT mice were injected i.v. with Ad-null and $AdVEGF_{165}$. As compared to untreated WT mice ($n = 6$) (*a* and *b*), VEGF induced *Id3* expression in the BM ($n = 6$) (*c* and *d*). Left, dark field; right, bright field. Scale bar, 100 µm. ***e*** and ***f***, BM from Id-mutant mice (■) and WT control (□) were collected and the number of $VEGFR2^+CD11b^-$ cells were determined by flow cytometry ($n = 5$) (*e*). The number of BM-derived CEPs was quantified by CFU-EC (*f*). As compared with WT BM, the number of CFU-ECs and $VEGFR2^+CD11b^-$ cells are decreased by 25% in Id-mutant BM ($n = 6$, $P < 0.05$). ***g*** and ***h***, The number of BM (*g*) or splenic (*h*) hematopoietic progenitors were determined by CFU-C progenitor assay. ■, CFU-GEMM; ■, BFU-E; □, CFU-GM. The total number of CFU-Cs was decreased by 30% both in the spleen and the BM of the Id-mutant mice ($n = 4$, $P < 0.05$). Mobilized peripheral blood cells were isolated from $AdVEGF_{165}$ treated mice and identified as either $VEGFR2^+$ or $CD11b^+$ cells. The $VEGFR2^+CD11b^-$ cells were mobilized early on in the WT, but were undetectable in the Id-mutant mice ($n = 8$) (*h*). ***i*** and ***j***, VEGF-mobilized peripheral blood of WT mice contained significant numbers of CFU-ECs, whereas in Id-mutant mice less colonies were detected ($n = 8$, $P < 0.05$) (*i*). WT (□, $n = 7$) and Id-mutant mice (■, $n = 7$) were inoculated with B6RV2 cells. A group of WT mice inoculated with B6RV2 was also injected i.p. with 400 µg/ml antibody to VEGFR2 (*j*). ***k*** and ***l***, After 5 d of tumor growth the number of mobilized $VEGFR2^+CD11b^-$ cells (*k*) and CFU-ECs (*l*) were determined in the peripheral blood. Compared with untreated WT (□) mice, Id-mutant (■) and anti-VEGFR2–treated WT (■) mice showed an impairment of the mobilization of $VEGFR2^+$ cells (*k*) and CFU-ECs (*l*). ***m***, Failure of expansion of VEGFR1+CD11b+ cells derived from the BM of $Idb1^{+/-}Idb3^{-/-}$ mice in $VEGF_{165}$-supplemented cultures were evaluated in *in vitro* cultures. Values in the table represent the total number of $VEGFR1^+$ or $VEGFR1^+CD11b^+$ cells undergoing expansion in the presence of $VEGF_{165}$.

m

		VEGFR1+ cells ($\times 10^3$)	VEGFR1+/CD11b+ cells ($\times 10^3$)
Wild-Type	*Baseline*	5±2	4.5±3.0
	Day 3	50±10	50±20
	Day 6	160±40	130±50
$Id1^{+/-}Id3^{-/-}$	*Baseline*	3.5±1.0	3.5±1.0
	Day 3	0.5±0.2	0.2±0.1
	Day 6	0.2±0.1	0.2±0.1

into the wild-type mice also inhibited mobilization of $VEGFR2^+$ cells similar to Id-mutant mice (Fig. 5*k*). Most $VEGFR2^+$ cells mobilized by B6RV2 inoculation are BM-derived CEPs with capacity of forming CFU-ECs (Fig. 5*l*). Neutralizing antibodies against VEGFR2 also inhibited tumor-induced mobilization of CFU-ECs (Fig. 5*l*). However, there was complete abrogation of CFU-EC mobilization in Id-mutant mice that were inoculated with B6RV2 tumors (Fig. 5*l*). These data support the notion that defective angiogenesis observed in Id-mutant mice is only partly a consequence of impaired VEGF-induced mobilization of $VEGFR2^+$ CEPs.

Inhibition of VEGFR signaling blocks tumor growth

We demonstrate that in Id-mutant mice the total number of

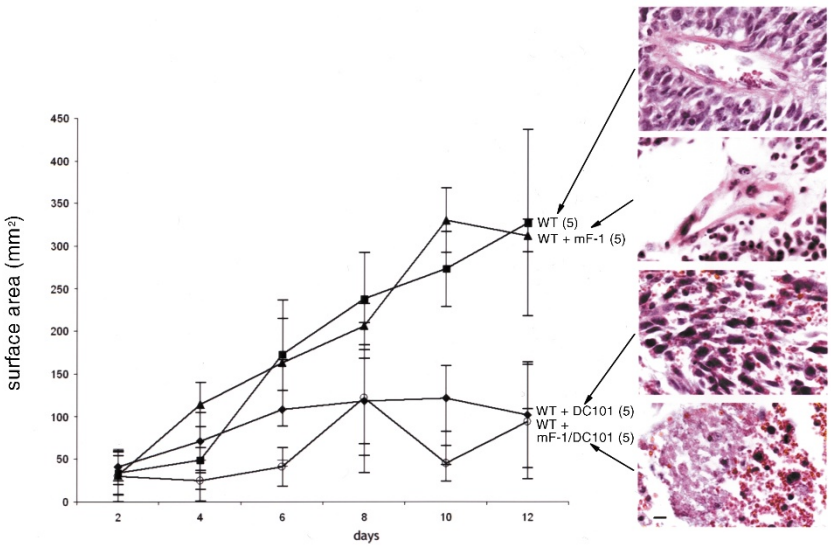


Fig. 6 Neutralizing antibody against both VEGFR1 and VEGFR2 is essential to completely block tumor angiogenesis and growth. Wild-type C57Bl/6 mice inoculated with LLCs and injected i.p. with neutralizing antibody to murine VEGFR1 (mF-1, $n = 5$; 400 μg//injection) and VEGFR2 (DC101, $n = 5$; 800 μg per injection) or in combination with both antibodies or control (saline injection) on days 2, 4, 6 and 8 after tumor injection. Inhibition of VEGFR2 results in disruption of all vessels, whereas VEGFR1 inhibition results in generation of vessels without significant perivascular mononuclear infiltrates. Treatment of mice with both antibodies results in the widespread tumor necrosis and complete absence of viable tumor cells and blood vessels. Histological sections were done on day 12. Scale bar, 25 μm.

VEGFR1⁺CD11b⁺ cells was only modestly decreased in the BM (Fig. 5*m*). However, VEGFR1⁺CD11b⁺ cells failed to expand to VEGF in long-term BM cultures (Fig. 5*m*). This suggests that in early phases of neo-angiogenesis, corecruitment and proliferation of BM-derived cells, composed of VEGFR2⁺ CEPs and VEGFR1⁺ myeloid precursors, may be impaired in Id-mutant mice, which may explain the lack of tumor growth in these animals.

To test this hypothesis, wild-type mice harboring either LLC or B6RV2 tumors were treated with neutralizing antibodies against either VEGFR1 (mF-1), VEGFR2 (DC101) or a combination of the two antibodies (Fig. 6). Neutralizing antibodies against VEGFR1 had minimal effect on the tumor growth as compared with blocking VEGFR2 or a combination of VEGFR1 and VEGFR2. Histological examination demonstrated that in mice treated with antibodies against VEGFR2, there was a decrease in the vessel density, whereas in mice treated with antibodies against VEGFR1 there was no change in the number of vessels formed. However, in anti-VEGFR1–treated mice there were few perivascular cells (Fig. 6). In mice treated with both neutralizing antibodies against VEGFR1 and VEGFR2, there was extensive necrosis with no evidence of viable capillaries or tumor tissue. Similar experiments performed with B6RV2 cells produced identical results to those observed with LLCs (data not shown). Collectively, these data suggest that inhibition of both VEGFR1 and VEGFR2 signaling blocks early phases of tumor growth by blocking the recruitment of VEGF-responsive BM precursors.

Discussion

Despite recent identification of signaling pathways that regulate tumor angiogenesis, the origin of ECs contributing to tumor growth is not well defined[2,13]. Several lines of evidence suggest that recruitment of neighboring ECs is essential for vascularization of tumor tissue[1,2,5]. We demonstrate here that BM-derived cells, including VEGFR2⁺ CEPs and VEGFR1⁺ hematopoietic precursor cells, contribute to rapid neovascularization and can functionally restore tumor angiogenesis in Id-mutant tumor-resistant mice, thus offering new potential targets for the development of anti-angiogenesis therapies. LacZ staining revealed that in the early phases of tumor growth, most neo-vessels within the tumor mass were derived from transplanted wild-type BM; the majority of the vessels on day 2 post-implantation in both LLC and B6RV2 tumors were LacZ⁺vWF⁺. However, by day 14 only 50% of the neo-vessels in LLC tumors were LacZ⁺vWF⁺, whereas most vessels in B6RV2 tumors remained positive. Therefore, long-term dependence of certain tumors on BM precursors may be dictated by the extracellular matrix constitution and chemocytokine repertoire of a particular tumor. Transplantation of either wild-type BM- or VEGF-mobilized stem cells into wild-type mice also resulted in rapid mobilization and incorporation of donor cells into functional vessels in tumor tissue as well as Matrigel plugs; thus, our results support the notion that BM precursor cells provide a sufficient source of ECs for the initial phases of neovascularization.

To demonstrate that incorporation of BM cells was not due to effects of radiation, chimeric non-irradiated, Id-mutant mouse were generated in which approximately 10–50% of the BM cells were engrafted with wild-type Rosa-26 BM cells. Chimeric Id-deficient mice also supported the growth of B6RV2 cells. The degree of tumor growth correlated with the level of engraftment of wild-type BM into Id-mutant BM. The significance of the degree of BM engraftment is underscored in the study where transplantation of Id-mutant BM into wild-type mice resulted in a significant delay in tumor growth. These results suggest that failure of Id-mutant BM-derived cells to mobilize into the tumor vasculature, in an otherwise angio-competent host, is the primary defect in retarded tumor angiogenesis.

Transplantation of BM cells results in the engraftment of both mature ECs and CEPs. Therefore, the vWF⁺Id3⁺ cells incorporated into the vessel wall could have derived from either mobilized CEPs or mature ECs. However, plasma elevation of VEGF results in the mobilization of cells with CEP potential capable of forming late outgrowth endothelial colonies (CFU-EC)[21,22]. Importantly, these mobilized cells by themselves can functionally rescue tumor vasculature in the Id-mutant mice indicating that circulating mature ECs are most likely not required for this initial phase of tumor angiogenesis.

We as well as others have shown that plasma elevation of VEGF by tumor cells promotes mobilization of CEPs, hematopoietic stem cells and progenitors[22]. It is remarkable that transplantation of VEGF-mobilized wild-type CEPs and hematopoietic stem cells was sufficient to not only reconstitute hematopoiesis, but also restore tumor growth in Id-mutant mice. Moreover, although *Id1* and *Id3* are not expressed in adult BM, VEGF upregu-

lated *Id* expression in BM cells, suggesting that VEGF-activated *Id1* and *Id3* expression is essential for the mobilization and recruitement of CEPs and VEGFR1$^+$ hematopoietic cells.

The physiological significance of myeloid cells is underscored in metalloproteinase-9 (MMP9)-deficient mice in which there is a defect in tumor growth[24]. Transplantation of wild-type MMP9-deficient BM resulted in enhanced tumor growth in MMP9-deficient mice by delivery of MMP9-producing hematopoietic cells to the tumor vasculature. Activated VEGFR1$^+$ myeloid cells have also been shown to release other angiogenic factors such as VEGF, platelet-derived growth factor and brain-derived neurotrophic factor (BDNF), which enhances vessel formation and stability[25,26]. Therefore, VEGFR1$^+$ myeloid cells that are comobilized with VEGFR2$^+$ CEPs contribute to the newly formed vessels. Indeed, here we demonstrate that in both LLC and B6RV2 tumors, inhibition of either VEGFR1 or VEGFR2 signaling is insufficient to induce tumor regression and necrosis. Inhibition of VEGFR2 signaling results in decreased vessel density and diffuse hemorrhage[23], whereas inhibition of VEGFR1 diminishes vascular investment with perivascular cells, consistent with mutually supporting roles for these two cell types. Importantly, a combination of neutralizing antibodies against both VEGFR2 and VEGFR1 was essential to completely block tumor growth and induce tumor necrosis. These data support the notion that incorporation of BM-derived CEPs into rapidly expanding tumors requires corecruitment of VEGFR1$^+$ hematopoietic cells to confer stability to the neovessels. Although VEGFR1 is expressed on ECs as well as hematopoietic cells, the exact function of VEGFR1 on the regulation of tumor angiogenesis is not yet known[20]. Based on data presented here, we propose that one mechanism by which blocking VEGFR1 signaling may exert its anti-angiogenic effect is by interfering with the recruitment of VEGFR1$^+$ hematopoietic cells to the tumor vasculature. Whether VEGFR1 expression on CEPs or tumor ECs also plays a role in promotion of tumor angiogenesis is not yet known.

Here we demonstrate the functional role of BM-derived VEGF-responsive precursor cells in the regulation of angiogenesis and lay the foundation for novel therapies targeting *Id1*$^{+/+}$*Id3*$^{+/+}$ VEGFR2$^+$ CEPs and VEGFR1$^+$ hematopoietic precursor cells in order to inhibit tumor angiogenesis in certain human malignancies.

Methods

BM transplantation for tumor and Matrigel plug assays. Mice were genotyped by PCR of tail DNA as described[10]. Id-mutant (*Idb1*$^{+/-}$*Idb3*$^{-/-}$) and wild-type C57Bl/6/Sv129 mice were lethally irradiated (950 rads). Approximately, 1×10^6 β-gal$^-$ or β-gal$^+$ (Rosa-26 mice) BM cells were injected into tail veins of irradiated, non-irradiated naive or 5-FU–treated recipient mice. After 4 wk, mice were injected intradermally with either 2×10^7 B6RV2 lymphoma cells, LLC (ATCC) or 1 ml of iced Matrigel (Becton-Dickinson, San Jose, California) and admixed with VEGF (Peprotech, 10 μg/ml, Rocky Hill, New Jersey) and heparin (Sigma, 100 μg per ml) into the right lower abdomen. For the tumors, surface area was scored by 3 independent observers (Dial Caliper, Science Ware, Pequannock, New Jersey).

Histological analysis, immunohistochemistry and *in situ* hybridization. Tumor tissue and Matrigel plugs were fixed in 4% paraformaldehyde for 4 h. Paraffin embedding was performed by dehydrating samples through ethanol and Histoclear (National Diagnostics, Atlanta, Georgia) and immersed in paraplast (Fisher Scientific, Pittsburgh, Pennsylvania). Sections (8 μm) were stained with H&E and antibodies to vWF (combined primary and biotinylated secondary antibody, DAKO, Via Real, California), and VEGFR1 (Flt-1, biotinylated mAb, clone mF-1, ImClone Systems, New York, New York), MOMA-1 (Bachem, San Carlos, California), CD11b (Pharmingen, San Diego, California), VE-cadherin (clone E4G10, ImClone Systems) were used. For *in situ* hybridization, sections were hybridized to [α-^{33}P]UTP labeled antisense RNA probes as described[10]. vWF, CD11b, MOMA, VE-cadherin and VEGFR1 staining was performed on LacZ-stained tissues.

Generation of chimeric Id-mutant mice. 1×10^6 BM mononuclear cells (BMMNC) from Rosa-26 BM were micro-transplanted every 3 d for a total of 3 injections into non-irradiated Id-mutant mice. After 1 wk, B6RV2 or LLC cells were implanted. At the time of implantation, 10% of the BM cells were β-gal$^+$ donor-derived. For higher levels of BM chimerism, the Id-mutant mice received 5-FU (150 mg/kg) by a single i.v. administration on day 0. After 2 d, the mice were transplanted with 1×10^7 Rosa-26 BMMNCs. Subsequently, mice were implanted with 2×10^7 B6RV2 or LLC cells.

β-galactosidase and LacZ staining. Tumor tissue and femoral bones were fixed in 4% paraformaldehyde for 2 h. The samples were washed in PBS and PBS containing washing buffer solution (2 mM $MgCl_2$, 5mM EDTA, 0.01% sodium deoxycholate and 0.02% NP-40) and stained in fresh X-gal solution at 37 °C overnight as described[27]. The X-gal–stained tumors and BM then were embedded in paraffin, sectioned and counter-stained with eosin to visualize LacZ-negative tissue. β-gal was confirmed by immunohistochemistry using antibody against β-gal (Clone GAL13, Santa-Cruz Biotechnology, Santa Cruz, California).

VEGF-induced mobilization. Id-mutant or wild-type mice were injected i.v. with 1.5×10^8 multiplicity of infection (MOI) of AdVEGF and Ad-null as described[22]. Mobilized cells were collected by orbital bleeding and stained with FITC-conjugated anti-VEGFR2 (clone DC101) mAb and Phycoerythrin-CD11b (Mac1). Stained cells (1×10^4) were analyzed on a Coulter Elite (Miami, Florida) flow cytometer to determine the percentages of positive populations. For quantification of CEPs with early and late outgrowth potential, 5×10^4 mobilized cells obtained from day 0 to day 21 and plated in modified endothelial growth medium (EGM), X-vivo-20 serum-free medium with VEGF (10 ng/ml), basic-FGF (5 ng/ml), heparin and endothelial growth supplement on collagen/fibronectin0coated plastic dishes as described[9,21]. Endothelial colonies (CFU-EC) formed within 3 d (early outgrowth) or by 14 d (late outgrowth) were quantified by co-staining with DiI-Ac-LDL metabolic labeling and vWF immunostaining[28,29] (mean ± s.e.m.). Transplantation of VEGF-mobilized cells from Rosa-26 mice into lethally irradiated Id-mutant was performed as described above. 5×10^6 VEGF-mobilized cells from day 3 and 5 were collected, layered over a Ficoll gradient and transplanted by tail-vein injections into lethally irradiated hosts.

***In vitro* expansion of BM precursor cells.** Wild-type or *Id1*$^{+/-}$*Id3*$^{-/-}$ femurs were flushed with serum-free medium. BM was passed through a 21-gauge syringe and filtered through strainer (20 μm). The cells were plated at 1×10^6 per ml in a M199-medium supplemented with 10% FCS, VEGF (10 ng per ml) and heparin (10 U per ml). Starting at day 1 and every 3 d thereafter, cells were removed, quantified and the number of VEGFR1 (FITC-labeled mF1 clone, ImClone Systems), and hematopoietic cells (CD11b-PE) were analyzed by two-color flow cytometry.

Hematopoietic colony assay. Spleen or BMMNCs were obtained from wild-type and Id-mutant mice and centrifuged over a discontinuous gradient using Lympholyte-M (Cederlane). BM or spleen cells (1×10^4 cells) were plated in triplicate in 1 ml of 0.8 % methylcellulose containing 30% FCS, 1% L-glutamine, 2.5% hemin, 0.05 mM 5-ME, 50 ng per ml IL-3, 20 ng per ml c-kit ligand and 2 U per ml erythropoietin. Scoring of colonies was performed for 3 CFU types CFU-Cs (CFU-GM, BFU-E and CFU-Mix) with an inverted microscope.

***In vivo* tumor inhibition studies.** C57BL/6 mice were inoculated with 2×10^7 of either B6RV2 or LLC cells. After 2 d, cohorts of mice ($n = 5$ in each group) were injected i.p. with 400 μg of rat anti-mouse neutralizing anitbody to either VEGFR1 (clone mF1, IgG1, ImClone Systems) or 800 μg VEGFR2 (clone DC101, IgG1, ImClone Systems), or a combination of VEGFR1 and VEGFR2 anotbodies. The control group was injected with saline. Tumor size was quantified on a daily basis. Mice were treated every 48 h starting at day 2 for a total of 4 separate injections. No injection was

delivered after day 8, since anti-rat neutralizing antibody may have blocked the effect of the injected antibodies. The mice were killed on day 12 and the tissues were processed for histological analysis.

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