Tie2 identifies a hematopoietic lineage of proangiogenic monocytes required for tumor vessel formation and a mesenchymal population of pericyte progenitors

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Summary

Bone marrow-derived cells contribute to tumor angiogenesis. Here, we demonstrate that monocytes expressing the Tie2 receptor (Tie2-expressing monocytes [TEMs]) (1) are a distinct hematopoietic lineage of proangiogenic cells, (2) are selectively recruited to spontaneous and orthotopic tumors, (3) promote angiogenesis in a paracrine manner, and (4) account for most of the proangiogenic activity of myeloid cells in tumors. Remarkably, TEM knockout completely prevented human glioma neovascularization in the mouse brain and induced substantial tumor regression. Besides TEMs and endothelial cells (ECs), Tie2 expression distinguished a rare population of tumor stroma-derived mesenchymal progenitors representing a primary source of tumor pericytes. Therefore, Tie2 expression characterizes three distinct cell types required for tumor neovascularization: ECs, proangiogenic cells of hematopoietic origin, and pericyte precursors of mesenchymal origin.

Introduction

Angiogenesis, the induction and growth of new blood vessels, is a rate-limiting step for tumor growth (Carmeliet and Jain, 2000). In angiogenesis, new blood vessels originate from the preexisting vasculature through the proliferation of endothelial cells (ECs), which maintain a high clonogenic potential throughout adulthood, and the recruitment of vascular mural cells (Jain, 2003). Besides the origin of tumor ECs from preexisting vessels, it has been proposed that endothelial progenitor cells (EPCs) exist in the adult bone marrow (BM), circulate in the peripheral blood (PB), and may incorporate into new blood vessels (Rafii and Lyden, 2003; Urbich and Dimmeler, 2004; Khakoo and Finkel, 2005). The existence of a BM reservoir of EPCs and their selective involvement in neovascularization has attracted interest because these cells may represent a novel target for therapeutic intervention. However, although EPCs were shown to contribute to the blood vessels of certain experimental tumors, the general importance of these cells in tumor angiogenesis remains controversial (Jain and Duda, 2003; Luttun and Carmeliet, 2004). A consensus on the identity and biological activity of EPCs is still lacking. Some studies have defined EPCs as cells that (1) are mobilized from the BM in response to cytokine treatment, ischemia, vascular injury, and tumor growth; (2) express surface markers of both ECs and hematopoietic progenitor cells (HPCs); (3) can be distinguished from mature circulating ECs (CECs) by the expression of the stem cell markers AC133 and c-kit, and by their outgrowth potential in in vitro clonogenic assays; and (4) integrate within the endothelium of blood vessels as bona fide ECs (Rafii and Lyden, 2003). Other studies have reported that EPCs coexpress EC and mature hematopoietic cell (HC) markers, including monocyte and dendritic cell markers, and localize both within the endothelium of blood vessels and extravascularly (Urbich and Dimmeler, 2004). It is not clear whether EPCs originate in the BM from primitive (hemo)angioblasts and are immature

SIGNIFICANCE

The role of BM-derived cells in angiogenesis is the subject of intense scrutiny and controversy. We show that a distinct lineage of proangiogenic monocytes exists in the BM and peripheral blood that express Tie2, an angiopoietin receptor previously supposed to be restricted to ECs and hematopoietic stem cells (HSCs). These proangiogenic cells, and not BM-derived endothelial progenitors (EPCs), had a requisite role in the vascularization and growth of a clinically relevant tumor model. Our findings support the paradigm that BM-derived cells recruited to tumors, rather than providing building blocks for postnatal vasculogenesis, comprise a functionally distinct myeloid lineage of paracrine inducers of angiogenesis. The proangiogenic cells should represent a promising target of novel anticancer therapies.

cells possibly related to hematopoietic stem cells (HSCs), rather than differentiating from lineage-committed HCs, such as monocytes, which can acquire an EC-like phenotype in vitro and in vivo under angiogenic conditions (Schmeisser et al., 2001). Moreover, CECs, believed to originate from sloughing of vessel walls, may substantially contribute to the EPC pool (Lin et al., 2000).

The functional importance of EPCs in tumor vascularization was underscored in a study that used the angiogenesis-defective Id mutant mice. Replacement of Id mutant BM by wild-type BM cells restored angiogenesis and tumor growth in these mice (Lyden et al., 2001). However, the extent to which EPCs contributed to the generation of the tumor endothelium has been highly variable in different studies, from substantial (Lyden et al., 2001), to low and dependent on the tumor type and grade (Ruzinova et al., 2003; Li et al., 2004; Peters et al., 2005), to negligible (De Palma et al., 2003a; Machein et al., 2003; Gothert et al., 2004; Rajantie et al., 2004; Shinde Patil et al., 2005), possibly due to differences in the experimental models and the technical difficulty of distinguishing tightly juxtaposed periendothelial and bona fide ECs in tissues by light microscopy.

Besides EPCs, it has been demonstrated that the HCs recruited to tumors play a crucial role in angiogenesis and tumor progression (Coussens and Werb, 2002; Pollard, 2004). Myeloid lineage HCs, such as tumor-associated macrophages (TAMs), can stimulate angiogenesis by secreting angiogenic factors, or indirectly by producing extracellular matrix-degrading proteases, which in turn release sequestered angiogenic factors. This function was highlighted in a mouse model of skin tumorigenesis, which showed a decreased incidence of invasive cancer upon inactivation of the MMP9 gene. Transplantation of wild-type BM cells restored tumor development in these mice (Coussens et al., 2000). Genetic deletion of the CSF-1 gene (*Csf1*$^{op/op}$) significantly attenuated the development of invasive carcinomas in tumor-prone transgenic mice, a phenotype associated with the failure to recruit macrophages into neoplastic tissues in the absence of CSF-1 (Lin et al., 2001). In one study, CD11b$^+$Gr-1$^+$ myeloid cells obtained from the spleens of tumor-bearing mice were shown to promote angiogenesis when coinjected with tumor cells; interestingly, deletion of MMP9 in these cells abolished their tumor-promoting ability (Yang et al., 2004). Of note, TAMs and CD11b$^+$ myeloid cells are overlapping and functionally heterogeneous cell populations, and it would be desirable to know whether their proangiogenic activity in tumors is associated with a distinct subset of these cells. Thus, whereas all these studies underscored the general importance of BM-derived myeloid cells in tumor angiogenesis, little characterization of the specific activity of the different hematopoietic subsets involved in these processes has been provided.

We recently identified a small subset of CD11b$^+$ myeloid tumor-infiltrating cells characterized by the expression of the Tie2 angiopoietin receptor (Tie2-expressing monocytes [TEMs]; De Palma et al., 2003a). Here, by using a combination of genetic approaches, transplantation strategies, and stringent models of oncogenesis, we investigated the origin, phenotype, functional role, and relevance of TEMs in tumor angiogenesis. Our findings provide novel insight into the cellular mechanisms of tumor vessel formation and have implications for the design of anticancer therapies.

Results

Tie2 expression in the tumor stroma

To identify the cell types that express Tie2 in tumors, we generated transgenic mice expressing GFP [TgN(Tie2-GFP)] under the control of a lentiviral vector (Tie2p/e LV; De Palma et al., 2003b) containing transcription regulatory sequences of the *Tie2/Tek* gene (Jones et al., 2001). We injected the LV into the perivitelline space of fertilized oocytes, as described (Lois et al., 2002 and the Supplemental Experimental Procedures in the Supplemental Data available with this article online) and selected mouse lines carrying multiple vector copies per genome to amplify detection of Tie2 transcriptional activity. GFP immunofluorescence (IF) staining showed robust and EC-specific expression of GFP in all organs of TgN(Tie2-GFP) mice (n = 9), including heart, liver, brain, gut, and spleen (Figure S1).

We then analyzed N202 mouse mammary tumors grown subcutaneously (s.c.) and found that GFP was expressed in the majority of vascular ECs (CD31$^+$ and CD34$^+$; Figure 1A and data not shown) and by a small fraction of tumor-infiltrating leukocytes, identified as TEMs (CD45$^+$; Figure 1A). Fluorescence-activated cell sorting (FACS) of tumors made into single-cell suspensions showed that the Tie2-GFP$^+$ cells were uniformly Tie2$^+$ and Sca-1$^+$ and accounted for 1%–2% of the total cells (Figure 1B). The majority of the tumor-derived Tie2-GFP$^+$ cells were ECs (Tie2$^+$CD31$^+$CD45$^-$), whereas ~5% were identified as Tie2$^+$CD11b$^+$CD45$^+$ TEMs. These TEMs, which expressed Sca-1, were c-kit$^-$. Interestingly, a significant fraction of tumor-derived Tie2-GFP$^+$ cells were Tie2$^+$CD31$^-$CD45$^-$; the identity of these cells will be discussed below. In summary, three distinct cell populations expressed Tie2 in tumor grafts: vascular ECs, hematopoietic TEMs, and a population of stromal cells distinct from ECs and HCs (see Table S1 for a list of markers expressed by these cell populations).

Tie2 expression in the hematopoietic system

We analyzed GFP expression in the BM and PB of TgN(Tie2-GFP) mice (Figures 1C and 1D, Table S1, and Figure S2). Two to five percent of BM cells were Tie2-GFP$^+$ (Figure 1C). FACS analysis showed that these Tie2-GFP$^+$ cells were ECs (<5% CD31$^+$CD45$^-$; data not shown) and HCs (>95% CD45$^+$). The Tie2-GFP$^+$ HCs were enriched in Sca-1$^+$ (~30%) and c-kit$^+$ (~50%) progenitors. Colony-forming cell (CFC) assays of sorted cells showed that the Tie2-GFP$^+$ fraction of c-kit$^+$CD45$^+$ BM cells did not form colonies, in contrast to the Tie2-GFP$^-$ fraction, which gave 10% outgrowth, suggesting that Tie2 expression was associated with the more primitive HPCs/HSCs (data not shown). In agreement with this notion, we showed that Tie2 receptor$^+$ HCs were highly enriched in the BM HSC fraction of wild-type mice (Figure S2). Interestingly, the Tie2-GFP$^+$ HCs of the BM of TgN(Tie2-GFP) mice also contained a population of myeloid lineage (CD45$^+$CD11b$^+$) cells (Figure 1C). These cells were distinct from committed progenitors, because they did not form colonies in CFC assays (data not shown). Thus, in the BM, Tie2 was expressed by ECs and putative HSCs, as shown in previous studies (Yano et al., 1997; Arai et al., 2004), and by a subset of myeloid lineage cells distinct from HPCs.

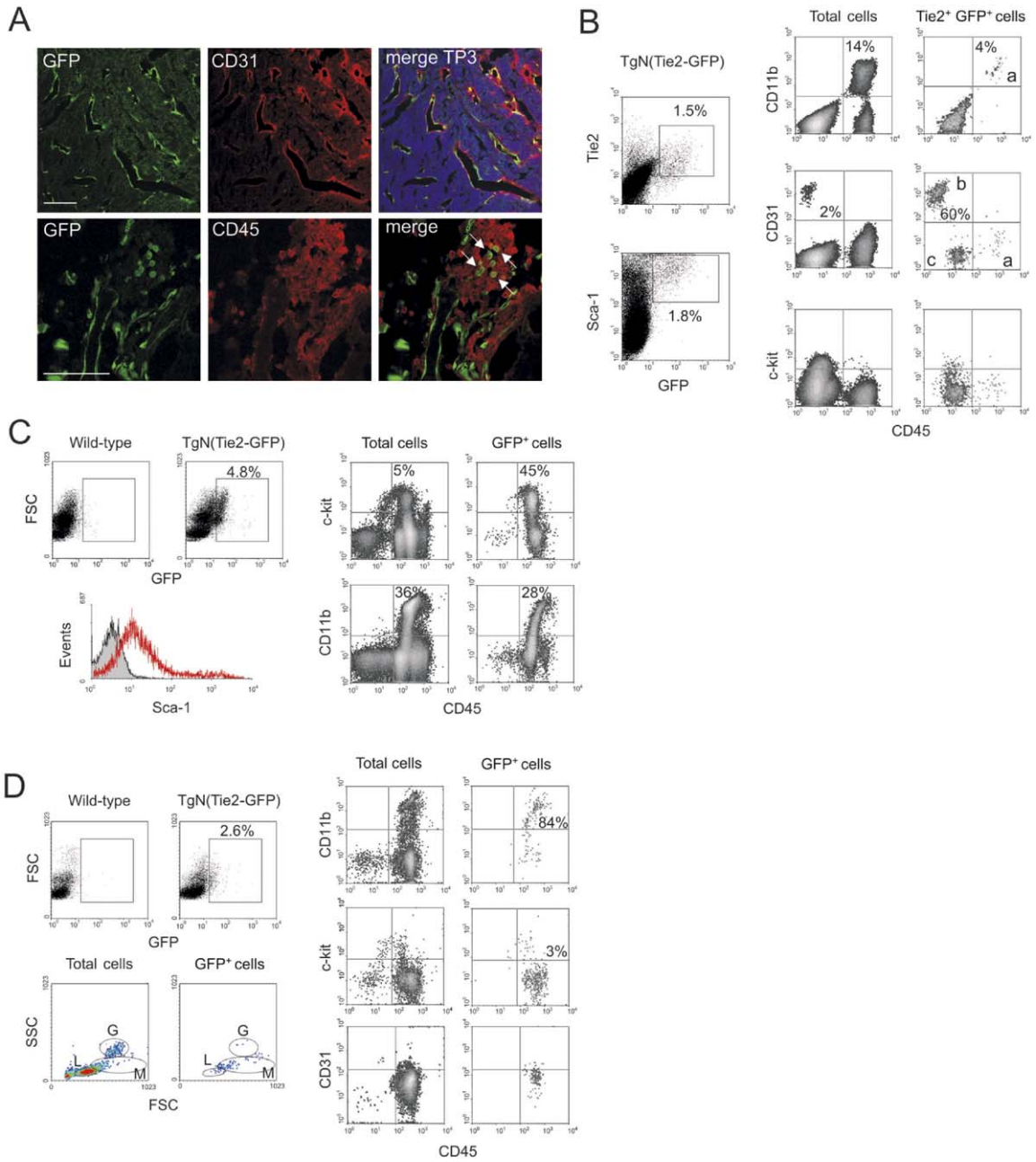

Figure 1. Tie2 expression in tumors and in the hematopoietic system of Tie2-GFP transgenic mice

A: Confocal IF analysis of N202 tumors shows GFP expression (green) in CD31$^+$ ECs (red) and in CD45$^+$ TEMs (arrows; red). Nuclei labeled by TO-PRO-3 (TP3; blue). Scale bar, 120 μm.

B: Tumor FACS analysis. All GFP$^+$ cells expressed Tie2 and Sca-1 (dot plots on the left). The Tie2$^+$GFP$^+$ cells (density plots on the right) were CD45$^+$CD11b$^+$ TEMs (**Ba**), CD45$^-$CD31$^+$ ECs (**Bb**), or CD45$^-$CD31$^-$ cells (**Bc**). The Tie2$^+$GFP$^+$CD45$^+$ TEMs were c-kit$^-$.

C: BM FACS analysis. The GFP$^+$ cells (dot plots on the left) were enriched in Sca-1$^+$ cells (open histogram; filled histogram is the IgG isotype control) and CD45$^+$c-kit$^+$ cells. GFP was also expressed by CD45$^+$CD11b$^+$ myeloid cells (density plots on the right).

D: PB FACS analysis. The GFP$^+$ cells were monocytes (dot plots on the bottom left; L, lymphocytes; M, monocytes; G, granulocytes) and were mostly CD45$^+$CD11b$^+$/CD45$^+$c-kit$^-$/CD45$^+$CD31$^-$ myeloid lineage hematopoietic TEMs (density plots on the right).

In the PB (Figure 1D), a small fraction (~1%–2%) of the leukocytes were identified as TEMs (Tie2-GFP$^+$). These circulating TEMs were 99% CD45$^+$ and >85% CD11b$^+$, accounted for approximately 10% of the total myeloid CD11b$^+$ cells, were distinct from granulocytes according to light scattering features and the expression of Gr-1, and mostly did not express the HSC/HPC markers c-kit (95% c-kit$^-$) and Sca-1 (>70% Sca-1$^-$). TEMs did not express the B cell marker CD19, the T cell marker CD3, or the pan-NK marker CD49b (Figure S2). In addition, TEMs were CD31^{Low} (Figure 1D) and VEGFR-2$^-$ (Figure S2) and

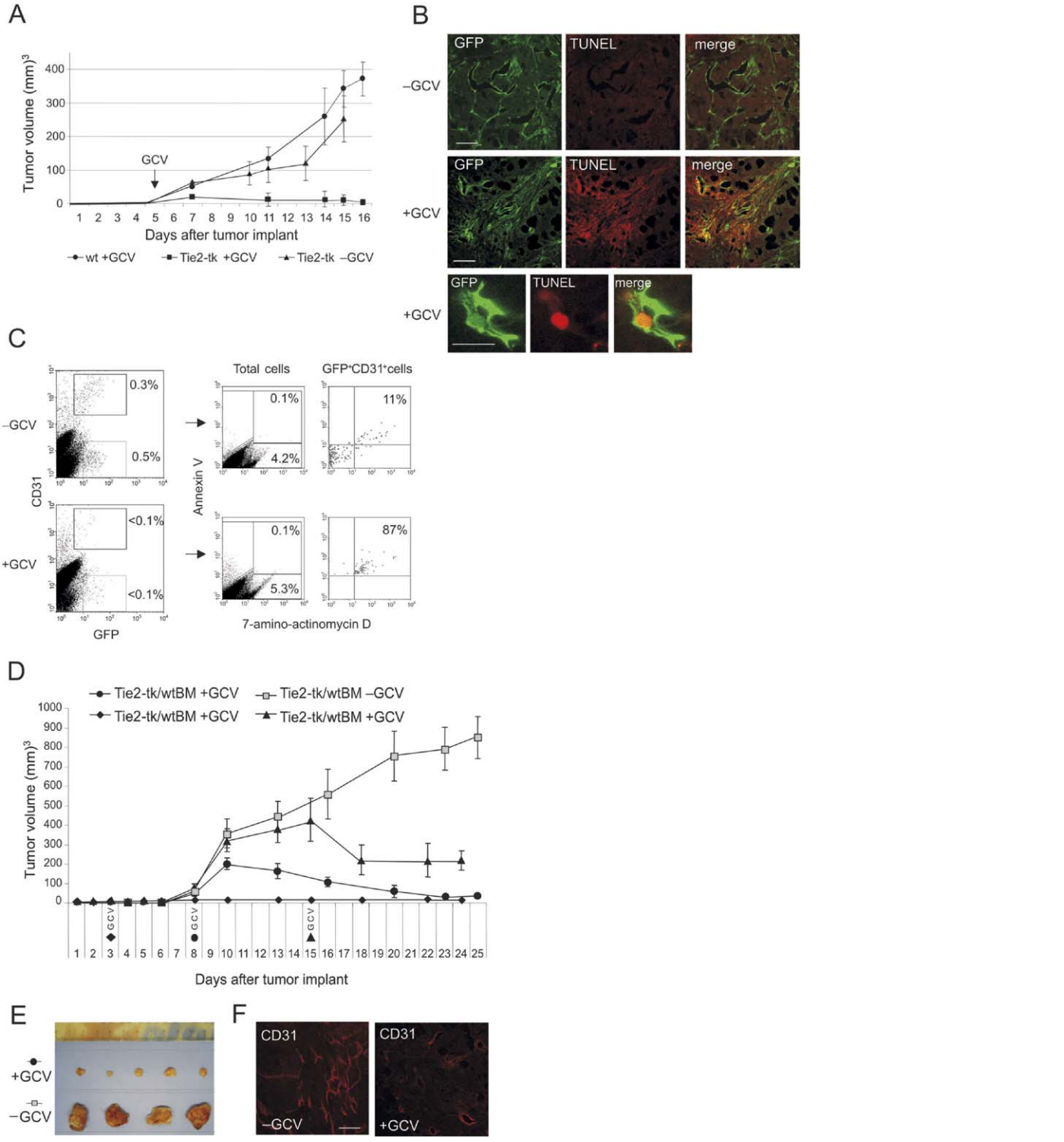

Figure 2. Transplantation of wild-type BM cells does not rescue the angiogenesis-defective phenotype of Tie2-tk transgenic mice

A: N202 tumor growth (average volume ± SEM) in GCV-treated wild-type (n = 5) and Tie2-tk mice (n = 6), and in untreated Tie2-tk mice (n = 6).

B: TUNEL assays of day 5 untreated (−GCV; n = 3) and GCV-treated (+GCV; n = 3) tumors grown in Tie2-tk mice. TUNEL reactivity (red) was scant in tumors from untreated mice, whereas it was marked in the GFP^+ cells (green) of tumors from treated mice. Scale bar, 120 μm. A small blood vessel containing a $TUNEL^+$ EC nucleus is shown in the lower panels (scale bar, 30 μm).

C: Tumor FACS analysis of apoptosis/necrosis by annexin V/7-amino-actinomycin D staining. The GFP^+CD31^+ tumor ECs of untreated mice (n = 3; dot plot on the top left) contained few double apoptotic/necrotic cells (9% ± 2%; density plot on the top right). There were much fewer GFP^+ cells in GCV-treated mice (<0.1% versus >0.3% in untreated mice; n = 3; $p < 0.05$; dot plot on the bottom left), and most of the GFP^+ ECs were double apoptotic/necrotic (78% ± 9%; density plot on the right).

thus were distinct from EPCs and CECs. In summary, the phenotype of circulating TEMs indicates that they represent a distinct subset of monocytes.

Generation of conditional angiogenesis-defective, tumor-resistant transgenic mice

To study the function of the different Tie2$^+$ cell types observed in tumors, we generated transgenic mice expressing the conditionally toxic gene thymidine kinase (tk) under the control of the Tie2p/e LV [TgN(Tie2-tk)]. In these mice, GFP expression was linked to that of tk by an IRES element. The GFP pattern in TgN(Tie2-tk) mice was similar to that observed in TgN(Tie2-GFP) mice, although the expression level was weaker because it was IRES dependent, and labeling with anti-GFP antibodies was required to detect GFP expression (data not shown).

Five to twelve days after N202 tumor challenge, we treated TgN(Tie2-tk) mice with GCV daily for 12 days (2.5 mg/mouse) or left them untreated, and monitored tumor growth for up to 60 days (Figure 2A and data not shown). GCV completely prevented tumor growth in TgN(Tie2-tk) mice (n = 15). Tumor growth rates were similar in wild-type FVB (n = 5) and untreated TgN(Tie2-tk) mice (n = 15), indicating that GCV treatment or tk expression alone had no effect on tumor growth. Interestingly, the 12 day GCV treatment completely prevented the tumors from relapsing during the 6–8 week observation time that followed the end of the treatment, indicating eradication of the tumor challenge (data not shown). In some of these experiments, GCV treatment was started as the tumors had reached a large size (day 12 postinjection). Even in these conditions, all tumors rapidly regressed (data not shown and Figure 2D).

TUNEL assays performed on tumor sections (Figure 2B) and FACS of tumors made into single-cell suspensions and analyzed for cell apoptosis/necrosis by annexin V/7-amino-actinomycin D double staining (Figure 2C) revealed substantial EC apoptosis/necrosis and effective reduction of GFP$^+$ cells in tumors from GCV-treated TgN(Tie2-tk) mice (5 day treatment, started at day 12 after tumor challenge; n = 3), as compared to untreated mice (n = 3). These data indicated that tumor resistance in GCV-treated TgN(Tie2-tk) mice was a direct consequence of impaired angiogenesis mediated, at least in part, by EC death.

Elimination of TEMs without myelosuppression and without loss of long-term repopulating HSCs

Although Tie2 is likely expressed by HSCs (see above), GCV-treated TgN(Tie2-tk) mice had normal hematopoiesis for up to 8 weeks after the end of the treatment, the longest time point analyzed. GCV-treated mice, however, had substantially reduced frequency of Tie2-GFP$^+$ cells in the BM and almost no circulating TEMs in PB. Despite the depletion of the majority of Tie2-expressing cells, BM cells exposed to GCV in vivo remained capable of radioprotecting lethally irradiated mice in a BM transplantation (BMT) model (Figure S3). Eight weeks after the transplant, all recipients were surviving and had normal hematopoiesis, indicating long-term engraftment of Tie2-tk BM cells exposed to GCV. These results strongly suggested that HSCs, even if they expressed Tie2, were resistant to GCV. Furthermore, because elimination of the majority of the BM Tie2-GFP$^+$ cells did not cause obvious myelotoxicity in GCV-treated mice, the GCV-sensitive Tie2-GFP$^+$ cells likely represented a specific lineage of HCs, rather than multipotent HPCs.

Transplantation of wild-type BM cells does not rescue angiogenesis in angiogenesis-defective transgenic mice

Transplantation of wild-type BM in Tie2-tk transgenic mice might provide a source of EPCs capable of rescuing the genetically hampered supply of ECs. To explore this scenario, we generated Tie2-tk/wtBM chimeras by transplanting wild-type BM cells into lethally irradiated TgN(Tie2-tk) mice. Engraftment of long-term repopulating wild-type HSCs was assessed by CFC assays and PCR for vector sequences 10–12 weeks after the transplant, showing that ~80% (n = 60 CFCs from seven mice) of the CFCs were of donor origin in the transplanted mice. Six to eight weeks after the transplant, we injected N202 tumor cells and found that GCV completely prevented tumor growth in Tie2-tk/wtBM mice (n = 5; Figure 2D). As observed in nontransplanted TgN(Tie2-tk) mice, the 12 day GCV treatment of Tie2-tk/wtBM mice prevented the tumors from relapsing during the 4–6 week observation time that followed the treatment (data not shown). To rule out the possibility that BM-derived wild-type EPCs required an established tumor to be efficiently mobilized, or a preformed tumor vasculature to contribute to tumor angiogenesis, we also started GCV treatment 8 (n = 5) or 15 (n = 5) days after tumor challenge, when tumors had reached a large size. GCV induced a rapid regression of the tumor mass in all treated mice (Figure 2E). The regressing tumors displayed marked necrosis (data not shown) and drastically reduced vascular density (Figure 2F), as compared to those growing in untreated mice. Interestingly, the few blood vessels observed in regressing tumors had large diameter and were not branched. Overall, these results indicate that putative BM-derived EPCs were not capable of rescuing tumor angiogenesis in GCV-treated Tie2-tk/wtBM mice. Of note, the experimental conditions that were used were biased toward the mobilization and incorporation of putative donor BM-derived EPCs into tumor blood vessels by eliminating the competition from endogenous, host-derived ECs.

A possible caveat of the experiments described above was that, in GCV-treated mice, tk-expressing ECs might spread toxicity to neighboring tk-negative cells ("bystander effect"), possibly affecting the incorporation and survival of putative BM-derived EPCs into tk-expressing tumor vessels in our transplantation model. To address this issue, we coinjected wild-type tumor-derived ECs together with tumor cells in Tie2-tk transgenic mice and found that the exogenous ECs integrated within the growing tumor vasculature in the presence of GCV (Figure S4). Interestingly, despite the relatively low amounts of exogenous ECs present in the tumors of treated mice at the

D: N202 tumor growth (average volume ± SEM) in Tie2-tk mice transplanted with wild-type BM cells and treated with GCV starting at the indicated time after tumor challenge, or left untreated (n = 5 for each group).
E: Photos of tumors grown in mice treated from day 8 as in **D**, or left untreated, and excised at day 25.
F: CD31 IF staining (red) of representative tumors from **E**. Scale bar, 120 μm.

time of analysis, these cells rescued tumor angiogenesis and growth, in contrast with BM-derived cells that failed to do so in the BMT experiments described above (Figure S4). Overall, these data argue against the possibility that tk-mediated bystander effect was responsible for the lack of incorporation of BM-derived EPCs into the vasculature of tk-transgenic mice.

Tumor TEMs promote tumor angiogenesis

To directly assay the proangiogenic activity of tumor TEMs, we isolated GFP$^+$CD11b$^+$ cells from N202 tumors grown in TgN(Tie2-GFP) mice by FACS and injected these cells (>95% purity; Figure S5) together with N202 tumor cells (1:20 ratio; 2.5×10^4 TEMs) in nude mice. As controls, we injected N202 cells alone, and N202 cells with GFP$^-$CD11b$^+$ tumor-derived myeloid cells, which included all myeloid lineage cells, except for TEMs. We studied tumor vascularization 6 days postinjection, when tumors were at an early stage of growth and had a diameter of 2–3 mm (Figure 3A). We observed scant vascularization in tumors (n = 3) derived from the injection of N202 cells alone. In these tumors, CD31$^+$ or CD34$^+$ (Figure 3B) blood vessels were few, had a small diameter, and were little branched. On the contrary, tumor challenges coinjected with TEMs (n = 3) were much more vascularized, and the blood vessels were larger and irregularly shaped and had a more developed branching pattern. Computer-assisted digital image analysis showed that the overall vascular area was 4-fold higher in tumors coinjected with TEMs than in control tumors. Interestingly, tumor angiogenesis was not enhanced by the coinjection of N202 cells and CD11b$^+$ myeloid cells depleted of TEMs (n = 3). These results indicated that TEMs have a superior proangiogenic activity among tumor-infiltrating myeloid cells. We did not detect GFP$^+$ cells in the tumors 6 days after injection of the cells, suggesting a rapid turnover of TEMs in growing tumors, a circumstance that argues against the possibility that these cells are immature HPCs. Interestingly, all tumors displayed abundant infiltration of both CD45$^+$ hematopoietic cells and NG2$^+$ pericytes/myofibroblasts (Figure 3B), suggesting that these cells colonize tumors during the early stages of development, possibly before a functional vasculature has been established, or in concomitance with the angiogenic switch.

Circulating TEMs promote angiogenesis and are the likely precursors of tumor TEMs

To explore the relationship between circulating and tumor-homing TEMs, and to investigate whether commitment to a proangiogenic function already occurs in circulating cells rather than being locally induced within the tumor stroma, we purified TEMs from the PB of TgN(Tie2-GFP) mice by FACS (purity >95%; Figure S5) and tested their proangiogenic activity in an in vivo matrigel plug assay. We injected matrigel alone (n = 3), or matrigel containing freshly isolated TEMs (7×10^4 cells; n = 5), or an equal number of peripheral blood mononuclear cells (PBMCs) depleted of TEMs (n = 5), or an excess of total PBMCs (2.5×10^5 cells; n = 2), s.c. in nude mice. We excised matrigel plugs 8 days later and found that, while matrigel alone contained few cells, TEMs induced a robust capillary network, as shown by CD34 immunostaining of matrigel sections (Figure 3C). These CD34$^+$ vascular structures were GFP negative, indicating that they did not originate from the injected cells; however, a few GFP$^+$CD45$^+$ TEMs, often associated with the developing vascular network, were still present in the matrigel 8 days postinjection (data not shown). Remarkably, PBMCs depleted of TEMs did not induce significant angiogenesis in matrigel ($p < 0.01$ versus TEMs), and an excess of total PBMCs was no more effective than purified TEMs. Thus, PB TEMs had superior capacity to trigger an early angiogenic response among PBMCs in the experimental conditions used.

The proangiogenic activity of PB TEMs suggested that these cells were the likely precursors of tumor TEMs. To further investigate this relationship, we transplanted wild-type mice with TgN(Tie2-tk) transgenic BM cells, and 8 weeks later we challenged them with N202 tumors and administered GCV before the tumors became visible, in order to eliminate TEMs before they reached the tumor site. GCV-treated mice (n = 5) showed a drastically reduced tumor growth as compared with untreated mice (n = 5), indicating that elimination of circulating TEMs was sufficient to inhibit tumor growth (Figure 3D). Interestingly, when we stopped GCV treatment, the tumors remained dormant for more than 2 weeks before resuming their growth (data not shown), suggesting that reconstitution of the TEM lineage was required before the tumors could turn on angiogenesis and resume their growth. Taken together, these findings imply that circulating TEMs represent a distinct lineage of proangiogenic monocytes required to promote vascular growth in vivo.

Recruitment of TEMs to spontaneously arising mouse pancreatic tumors and orthotopic xenografts of human glioblastomas

In order to extend the aforementioned findings to clinically relevant models of oncogenesis, we studied the recruitment of TEMs to both spontaneously arising pancreatic and orthotopically growing brain tumors.

In RIP-Tag2 transgenic mice, a fraction of the pancreatic islets spontaneously develops into adenomas/invasive adenocarcinomas (Hanahan, 1985). We transplanted newborn RIP-Tag2 mice with BM lin$^-$ cells transduced with either the Tie2p/e-GFP or the ubiquitously expressed PGKp-GFP LV, to obtain Tie2-BMT (n = 11) and PGK-BMT (n = 4) RIP-Tag2 mice, respectively. Orthotopically growing brain tumors were induced by injecting human glioma cells (isolated from glioblastoma multiforme; Galli et al., 2004) into the right striatum of Tie2-BMT (n = 8) and PGK-BMT (n = 3) nude mice, which had been previously transplanted with transduced lin$^-$ cells.

RIP-Tag2 mice were euthanized at 12–13 weeks of age, when the pancreata of all mice contained macroscopic vascularized lesions. Total body irradiation and BMT prolonged the average mouse survival by 1 to 2 weeks but did not affect the incidence or progression of adenomas to invasive tumors (data not shown). IF staining of pancreatic sections obtained from PGK-BMT mice (hematopoietic chimerism was 81% ± 7%, as measured by frequency of GFP$^+$ cells in the PB) showed abundant GFP$^+$ cells both throughout the exocrine pancreatic tissue (Figure 4A) and in the tumors (Figure 4B). In the tumors, virtually all GFP$^+$ cells were hematopoietic (CD45$^+$; data not shown) and were mostly CD11b$^+$ or F4/80$^+$ (data not shown) tumor-infiltrating macrophages. Both the CD31$^+$ or CD34$^+$ ECs and the α-smooth muscle actin (α-SMA)$^+$ pericytes/smooth muscle cells (data not shown) were GFP negative, indicating that the blood vessels of the pancreatic tumors were not BM derived. In brain tumors grown for 6 weeks in PGK-BMT mice (hematopoietic chimerism was 83% ± 12%), more than 85% of the F4/

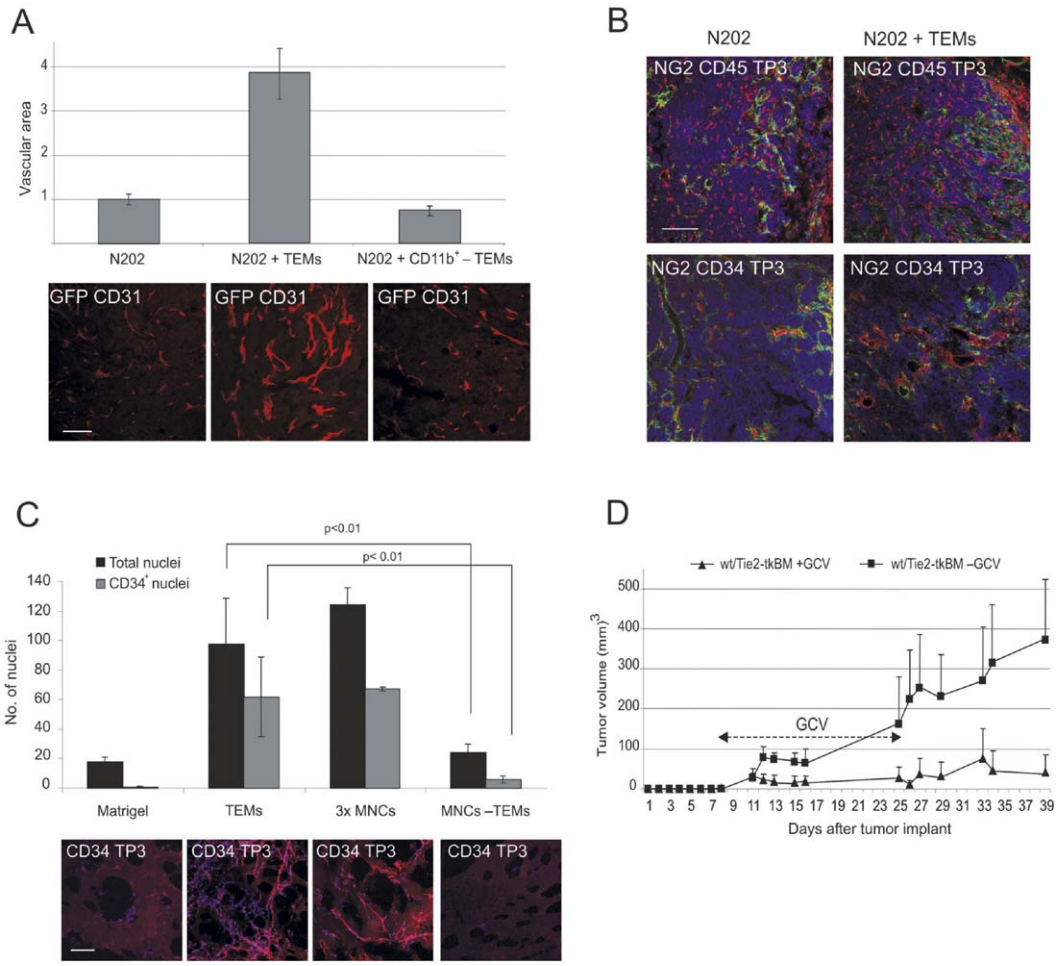

Figure 3. Proangiogenic activity of tumor and circulating TEMs

A: Vascular area (histograms showing average fold increase over reference value ± SEM) in tumors originated from the injection of N202 cells (n = 3; reference value), N202 cells and TEMs (n = 3), or N202 cells and CD11b$^+$ myeloid cells depleted of TEMs (n = 3), and grown for 5 days in nude mice. Representative pictures of tumor sections immunostained for CD31 (red) and GFP (green) are shown on the bottom. Scale bar, 120 μm.

B: Confocal IF analysis for NG2 (green), CD45 or CD34 (red), and TP3 (blue) staining of tumors originated by the injection of N202 cells (left) or N202 cells and TEMs (right), as in **A**. Scale bar, 120 μm.

C: Average number ± SEM of cell nuclei (black bars) and of nuclei belonging to CD34$^+$ vascular structures (gray bars) per ×200 microscope field in matrigel sections. Matrigel alone (n = 3), matrigel containing TEMs (n = 5), matrigel containing an excess of PBMCs (n = 2), or matrigel containing PBMCs depleted of TEMs (n = 5) were injected 8 days earlier s.c. in nude mice. Representative pictures of sections immunostained for CD34 (red) and stained with TP3 (blue) are shown on the bottom. Scale bar, 120 μm.

D: N202 tumor growth (average volume ± SEM) in wild-type FVB mice transplanted with transgenic Tie2-tk BM cells 8 weeks earlier, and either treated with GCV for the indicated time (n = 5) or left untreated (n = 5).

80$^+$ monocyte/macrophage lineage cells were GFP$^+$ (>1000 F4/80$^+$ cells analyzed), whereas in the brain parenchyma only a small fraction of these cells were GFP$^+$, consistent with the expected turnover of microglia in noninjured brains (Biffi et al., 2004) and with an enhanced infiltration of the tumors by macrophages (Figure 4C). In the tumors, all GFP$^+$ cells were hematopoietic (CD45$^+$ or F4/80$^+$) and did not colocalize with CD31$^+$ or CD34$^+$ ECs, indicating that the tumor blood vessels were not BM derived. In addition, the GFP$^+$ cells were α-SMA$^-$, and virtually all were NG2$^-$ (>98%; >250 NG2$^+$ cells analyzed), indicating that the occurrence, if any, of BM-derived pericytes/myofibroblasts in brain tumors is extremely rare. Overall, these data show that transplanted BM cells and their progeny maintained hematopoietic lineage fidelity in both spontaneous and orthotopic tumors and did not adopt vascular cell fates with significant frequency.

We then studied the recruitment of TEMs to these tumors. In Tie2-BMT RIP-Tag2 mice, Tie2-GFP$^+$CD11b$^+$ TEMs were selectively present in the pancreatic tumors, and not in the surrounding exocrine tissue or normal islets (Figures 5A and 5B). These TEMs often had a perivascular location but were CD31$^-$ and CD34$^-$ and distinct from vascular ECs (Figure 5B and data not shown). In the brain of Tie2-BMT nude mice, TEMs were present within the tumor (Figure 5C) but were never found in the noninfiltrated brain parenchyma. TEMs were an important fraction of the total CD45$^+$ HCs found within brain tumors

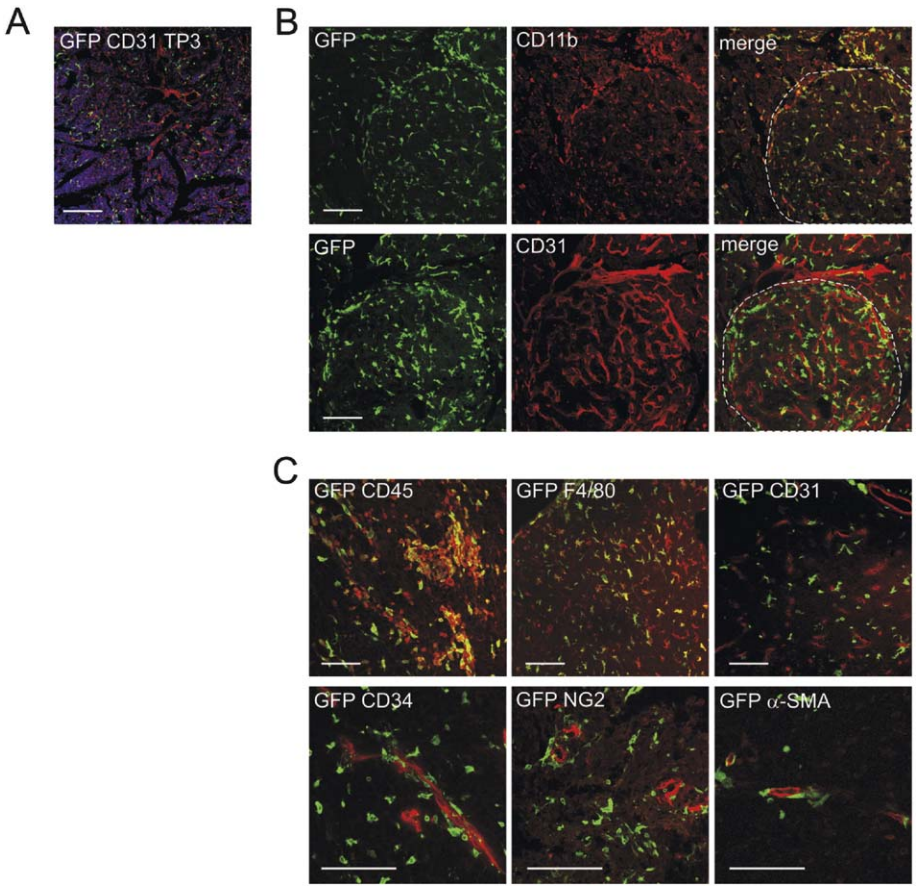

Figure 4. Contribution of BM-derived cells to spontaneous pancreatic and orthotopic brain tumors

A and B: GFP (green) and CD11b or CD31 (red) IF analysis and TP3 (blue) staining of the exocrine pancreatic tissue (**A**) and pancreatic islet tumors (**B**) from PGK-BMT RIP-Tag2 mice. The dashed line indicates the tumor perimeter. Scale bars, 120 μm.

C: GFP (green) and CD45, F4/80, CD31, CD34, NG2, or α-SMA (red) IF analysis of human orthotopic glioblastomas from PGK-BMT nude mice. Scale bars, 120 μm.

(average 9% ± 3%; >1000 $CD45^+$ cells analyzed; Figure 5C), sometimes had a branched morphology, and uniformly expressed CD11b (data not shown). As observed in pancreatic tumors, TEMs were $CD31^-$ and $CD34^-$ and were not integrated within the EC layers of blood vessels. Many TEMs had a perivascular location similar to that of pericytes; however, TEMs did not express NG2 (Figure 5C) and α-SMA (data not shown), indicating that TEMs are bona fide HCs distinct from pericytes and smooth muscle cells. Interestingly, both TEMs and $NG2^+$ pericytes had an intriguingly similar perivascular location, but in $NG2^+$ ("coated") vessels TEMs had an adluminal position with respect to the pericyte layer.

TEMs trigger the angiogenic phase in human glioma orthotopic xenografts

IF staining of brain tumors showed that the wide majority of TEMs expressed basic fibroblast growth factor (bFGF), a potent proangiogenic molecule (Figure 5D), suggesting that TEMs might promote angiogenesis via the release of bFGF, and possibly other proangiogenic factors. We then asked whether TEMs play a critical role during the angiogenic phase of glioma development. We transplanted human glioma cells (U87 cells; Winkler et al., 2004) in the striatum of nude mice, which were previously transplanted with Tie2-tk transgenic BM-derived lin^- cells. One day after the injection of the tumor cells, we randomly sorted the mice into two groups and left them untreated (n = 7) or treated them with GCV (2.5 mg/mouse) daily for 12 days (n = 7) in order to eliminate TEMs. Eighteen days later, we analyzed the tumors by magnetic resonance imaging (MRI), histology, and IF analysis (Figure 6 and Figure S6). GCV treatment and TEM elimination (see below) did not prevent tumor take, consistent with the ability of gliomas to initially grow by coopting the preexisting vasculature. MRI, however, showed that the treated tumors were on average smaller than the untreated tumors and displayed a markedly reduced contrast (gadolinium) uptake, with mixed hyperintense and hypointense signal, indicating impaired perfusion and widespread cell loss (Figure 6A). MRI volumetry indicated that tumor necrosis was dramatically increased in GCV-treated as compared to untreated mice (>10-fold; $p < 0.02$; n = 5; Figure S6). Consistent with MRI, hematoxylin/eosin staining of brain sections showed that GCV-treated gliomas were mostly made of nonviable tissue (Figure 6B). Extensive necrosis was also prevalent at the tumor margins, a finding in sharp contrast with the untreated tumors. Moreover, the proliferation index of the GCV-treated tumors was extremely low, as indicated by the paucity of $Ki67^+$ nuclei both in the inner tumor mass and at the tumor margins (Figure 6C). Taken together, these data strongly suggested that gliomas of GCV-treated mice were regressing at the time of analysis.

CD31 IF staining showed that the tumors of GCV-treated mice were almost avascular, whereas those of untreated mice were highly angiogenic (Figure 6D). Computer-assisted digital image analysis indicated that, at the tumor periphery, the total

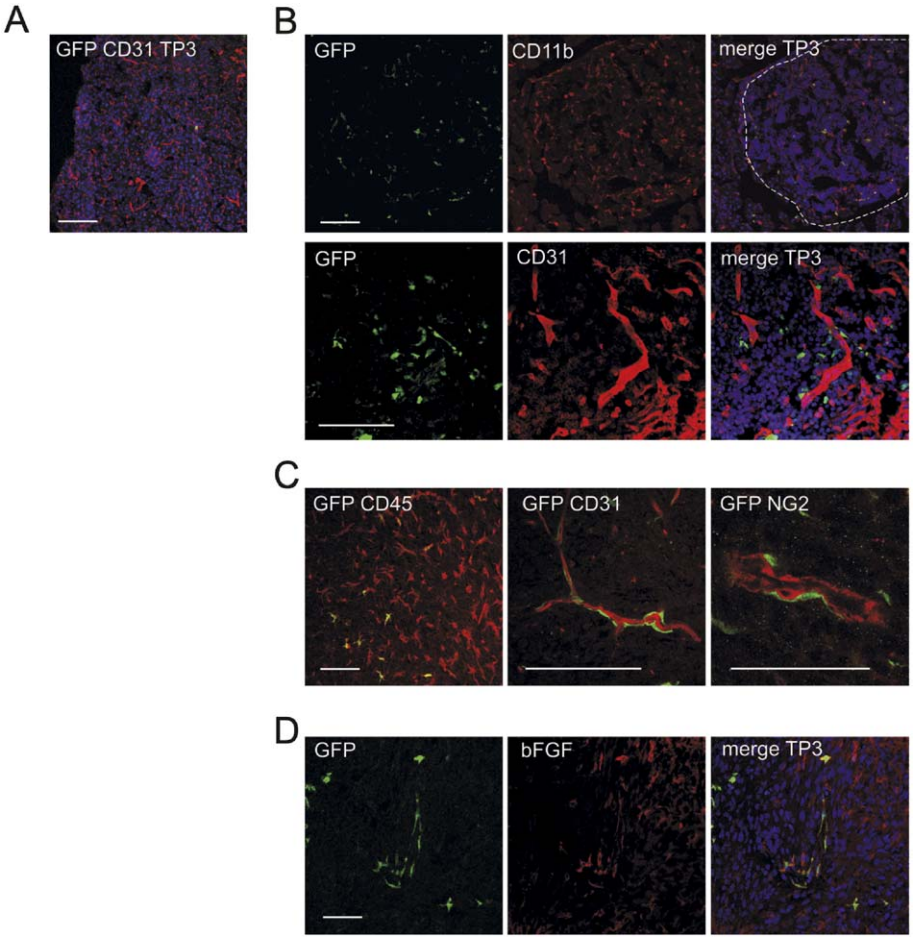

Figure 5. Contribution of TEMs to spontaneous pancreatic and orthotopic brain tumors

A and B: GFP (green) and CD11b or CD31 (red) IF analysis and TP3 (blue) staining of the exocrine pancreatic tissue (**A**) and pancreatic islet tumors (**B**) from Tie2-BMT RIP-Tag2 mice. The dashed line indicates tumor perimeter. Scale bars, 120 μm.

C and D: GFP (green) and CD45, CD31, NG2, or bFGF (red) IF analysis of human orthotopic glioblastomas from Tie2-BMT nude mice. Scale bars, 120 μm.

vascular area was >20-fold lower in the treated mice than in the untreated ($p < 0.01$; $n = 4$) and that the vessel density was also greatly reduced (34 ± 7 and 141 ± 12 vessels/mm^2 in treated and untreated mice, respectively; $p < 0.01$; $n = 4$). Interestingly, the rare blood vessels observed in GCV-treated tumors had regular shape and small diameter, were covered by NG2$^+$ or α-SMA$^+$ (data not shown) pericytes, and thus likely represented residual coopted vessels. By contrast, the blood vessels of control tumors were abnormally enlarged and tortuous, contained many Ki67$^+$ EC nuclei, and had loosely associated pericytes, all typical features of angiogenesis (Figure 6D). Thus, GCV treatment severely impaired the angiogenic phase in orthotopic gliomas.

GCV effectively cleared GFP$^+$ TEMs from tumors but did not inhibit the recruitment of CD11b$^+$ macrophages and Gr-1$^+$ granulocytes (Figure 6E). Moreover, the large necrotic areas of treated tumors were heavily infiltrated by these scavenging cells. Taken together, these results proved the selective action of the tk/GCV system in our transplantation model and indicated that tumor-infiltrating macrophages and granulocytes were not able to locally reconstitute the TEM cell pool or surrogate their function, supporting the notion that TEMs are a distinct proangiogenic lineage of HCs required for tumor vascularization.

Mesenchymal progenitors that express Tie2 in tumors are the likely precursors of vascular pericytes

In addition to ECs and TEMs, a third distinct cell population expressed Tie2 in tumors. These Tie2$^+$ cells only expressed the membrane-associated aminopeptidase N (CD13) and Sca-1 among a panel of "mesoderm lineage" markers and accounted for a sizeable fraction of all tumor Tie2-GFP$^+$ cells (~30%; Figure 7A). The surface phenotype of these cells (Tie2$^+$CD13$^+$Sca-1$^+$CD31$^-$CD45$^-$) was reminiscent of that of certain types of mesenchymal progenitor cells (MPCs), including mesoangioblasts (Sampaolesi et al., 2003). In tumor sections (Figure 7B), the Tie2-GFP$^+$CD13$^+$CD31$^-$CD45$^-$ cells had an elongated spindle shape and were mostly found clustered near blood vessels but did not express the pericyte NG2 and α-SMA (data not shown) markers.

To investigate the nature of these cells, we isolated Tie2-GFP$^+$CD13$^+$CD31$^-$CD45$^-$ cells from N202 tumors grown in TgN(Tie2-GFP) mice by cell sorting (purity >98%; Figure S5). The cells efficiently differentiated into pericyte/smooth muscle-like, osteoprogenitor-like and, to a lesser extent, adipocyte-like cells when cultured in vitro under specific growth conditions (Figure 7C and Supplemental Experimental Procedures). Although the demonstration of multipotency would require clonal analyses, our results indicate that this cell population is highly enriched in MPCs.

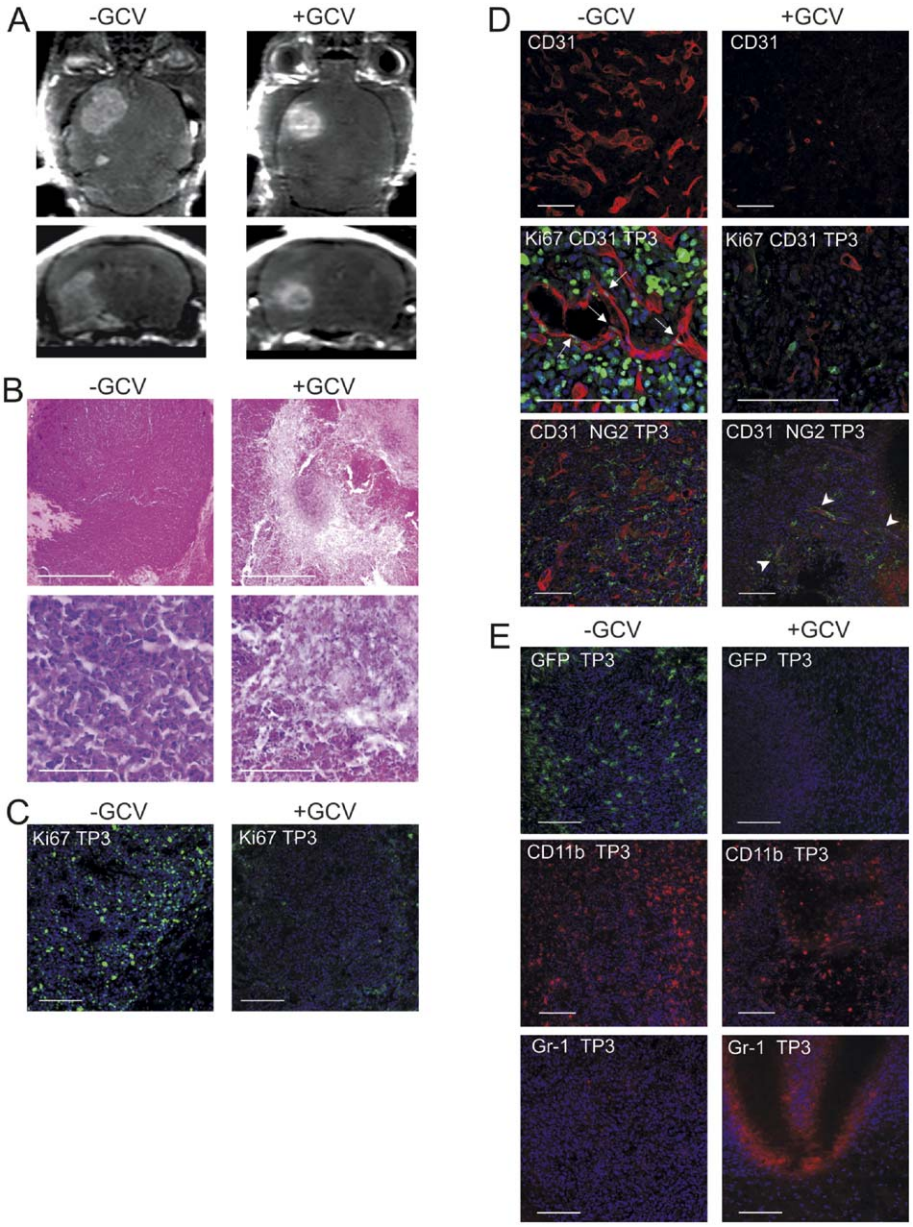

Figure 6. TEMs trigger the angiogenic phase in human glioma orthotopic xenografts

A: Contrast-enhanced T1-weighted axial (upper panels) and coronal (lower panels) MRI analyses show that gadolinium uptake is homogeneous in the untreated tumor (−GCV; n = 5), whereas it appears highly inhomogeneous in the treated one (+GCV; n = 5), indicating the presence of large areas of tumor cell loss. Note that the untreated tumor displays widespread striatal infiltration extending toward the frontal lobe and leptomeningeal spreading.

B: Hematoxylin/eosin staining of representative tumor sections from untreated (−GCV; n = 7) and treated (+GCV; n = 7) mice shows widespread necrosis and massive cell loss in the treated tumor. Scale bars, 1200 μm (upper panels) and 120 μm (bottom panels).

C–E: Ki67, NG2, or GFP (green) and CD31, CD11b, or Gr-1 (red) IF analysis and TP3 (blue) staining of representative tumor sections from untreated (−GCV; n = 5–7) and treated (+GCV; n = 5–7) mice. Arrows indicate Ki67$^+$ EC nuclei; arrowheads indicate small blood vessels covered by NG2$^+$ pericytes. Tumor areas with scant nuclear staining (TP3; blue) reflect tissue necrosis. Scale bars, 120 μm.

We next injected freshly isolated Tie2-GFP$^+$CD13$^+$CD31$^-$CD45$^-$ MPCs together with N202 tumor cells (1:20 ratio; 2.5 × 10^4 MPCs) s.c. in nude mice (Figure 7D). We analyzed the tumors 8 days later and found abundant Tie2-GFP$^+$ cells in the tumor stroma, suggesting that these cells proliferated in vivo while keeping Tie2 expression on. Although the majority of the GFP$^+$ cells were associated with blood vessels, they were CD31$^-$ and did not differentiate into ECs. Of note, control populations of tumor-derived Tie2-GFP$^+$CD31$^+$CD13$^-$CD45$^-$ ECs efficiently integrated within the endothelium of blood vessels. Furthermore, the wide majority of the Tie2-GFP$^+$ cells did not express the pericyte markers NG2 and α-SMA (data not shown), suggesting that these cells maintained an undifferentiated phenotype in vivo at 8 days postinjection. Alternatively, it is possible that Tie2 (and GFP) expression was shut off upon differentiation of the cells in vivo. To rigorously track the fate of Tie2$^+$CD13$^+$ cells in tumors, we crossed Tie2-GFP transgenic with Rosa26 mice, which express the reporter gene β-galactosidase (β-gal) ubiquitously, and obtained double transgenic TgN(lacZ/Tie2-GFP) mice (see the Supplemental Experimental Procedures and Figure S1). MPCs isolated from tumors grown in these mice could be expanded in culture for >20 cell doublings and were phenotypically characterized by FACS and RT-PCR analysis of cell surface molecules, growth factors, and receptors (Figure 8A). In vitro, the cells uniformly expressed β-gal and maintained an undifferentiated MPC phenotype. Indeed, the cultured cells continued to express high levels of Sca-1 and low levels of Tie2 and CD13. Furthermore, RT-PCR analysis showed that the cells only expressed VEGF-A, bFGF, HGF, and FGFR-1 among a wide panel of growth factors and

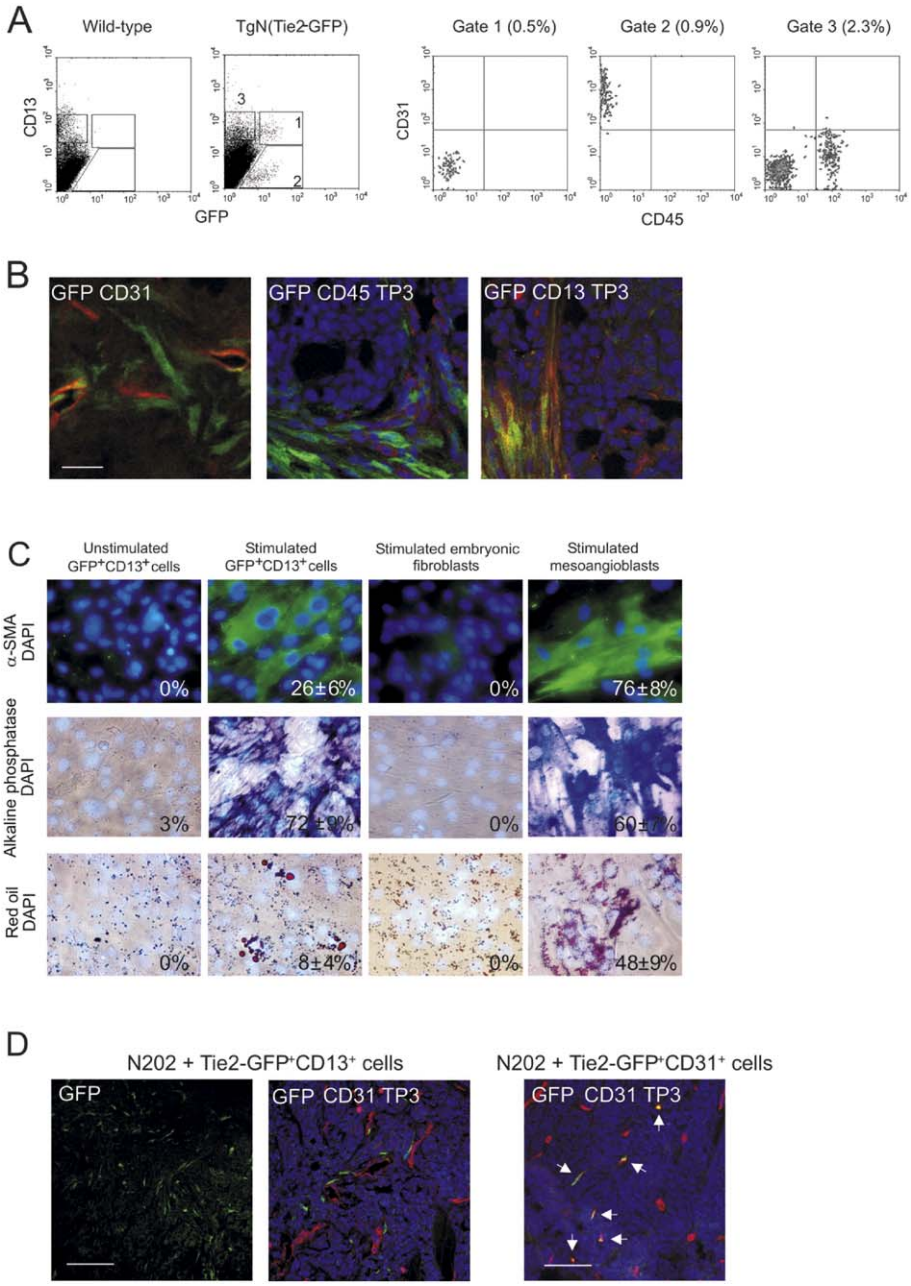

Figure 7. Isolation of Tie2$^+$ mesenchymal progenitors from tumors

A: FACS analysis of tumors grown in wild-type and Tie2-GFP transgenic mice (dot plots on the left). The GFP$^+$CD13$^+$ cells (gate 1) were CD45$^-$CD31$^-$ cells; the GFP$^+$CD13$^-$ cells (gate 2) were CD45$^-$CD31$^+$ ECs; the GFP$^-$CD13$^+$ cells (gate 3) contained CD45$^+$CD31$^-$ HCs (density plots on the right).

B: Confocal IF analysis for GFP (green) and CD31, CD45, or CD13 (red) and TP3 (blue) staining of N202 tumors grown in Tie2-GFP transgenic mice shows the presence of CD31$^-$ (left), CD45$^-$ (middle), and CD13$^+$ (right) GFP$^+$ cells. Scale bar, 30 μm.

C: Photos of cell cultures derived from purified GFP$^+$CD13$^+$CD45$^-$CD31$^-$ cells (MPCs). Cells were stimulated to differentiate into α-smooth muscle actin-positive smooth muscle-like cells, alkaline phosphatase-positive osteoprogenitor-like cells, and red oil-positive adipocyte-like cells, or left unstimulated. The frequency of marker-positive cells is indicated (average of two experiments), as assessed by DAPI nuclear staining (light blue). Control cell populations were unstimulated MPCs and stimulated embryonic fibroblasts and mesoangioblasts.

D: Confocal IF analysis for GFP (green), CD31 (red), and TP3 (blue) staining of tumors originated by the injection of N202 cells together with freshly isolated MPCs (left panels) or ECs (right panel) in nude mice and analyzed 8 days later. Note that Tie2-GFP$^+$CD31$^+$ ECs, but not Tie2-GFP$^+$CD13$^+$ MPCs, integrated within tumor blood vessels (arrows). Both MPCs and ECs were isolated from tumors grown for 2 weeks in TgN(Tie2-GFP) mice. Scale bar, 120 μm.

receptors tested. Remarkably, when we coinjected these cells together with N202 tumor cells (1:10 ratio; 5 × 10^4 MPCs) s.c. in nude mice, we found that they gave rise to a large proportion of the tumor α-SMA$^+$ cells (45% ± 6% β-gal$^+$; >550 α-SMA$^+$ cells analyzed from four tumors) 2 weeks later (Figure 8B). No β-gal$^+$ cells were observed in tumors grown from the injection of tumor cells alone (n = 3). The β-gal$^+$ cells did not express GFP at this time point of analysis, suggesting that the injected cells shut off Tie2 expression while differentiating. We found that the β-gal$^+$ cells did not colocalize with CD31$^+$ ECs, although they often loosely surrounded small blood vessels, in agreement with the expected arrangement of pericytes in tumors (Morikawa et al., 2002). We obtained similar results by using MPCs transduced ex vivo with a LV constitutively expressing GFP from a PGK-GFP expression cassette (Figure 8C). Taken together, these results strongly suggest that a rare population of MPCs, characterized by the expression of Tie2 and CD13 and found within the tumor stroma, may represent the major source of vascular mural cells in tumors.

Discussion

A distinct lineage of Tie2$^+$ monocytes promotes tumor vascularization

Our data provide evidence for the existence of a distinct monocyte lineage, which we termed Tie2-expressing monocytes

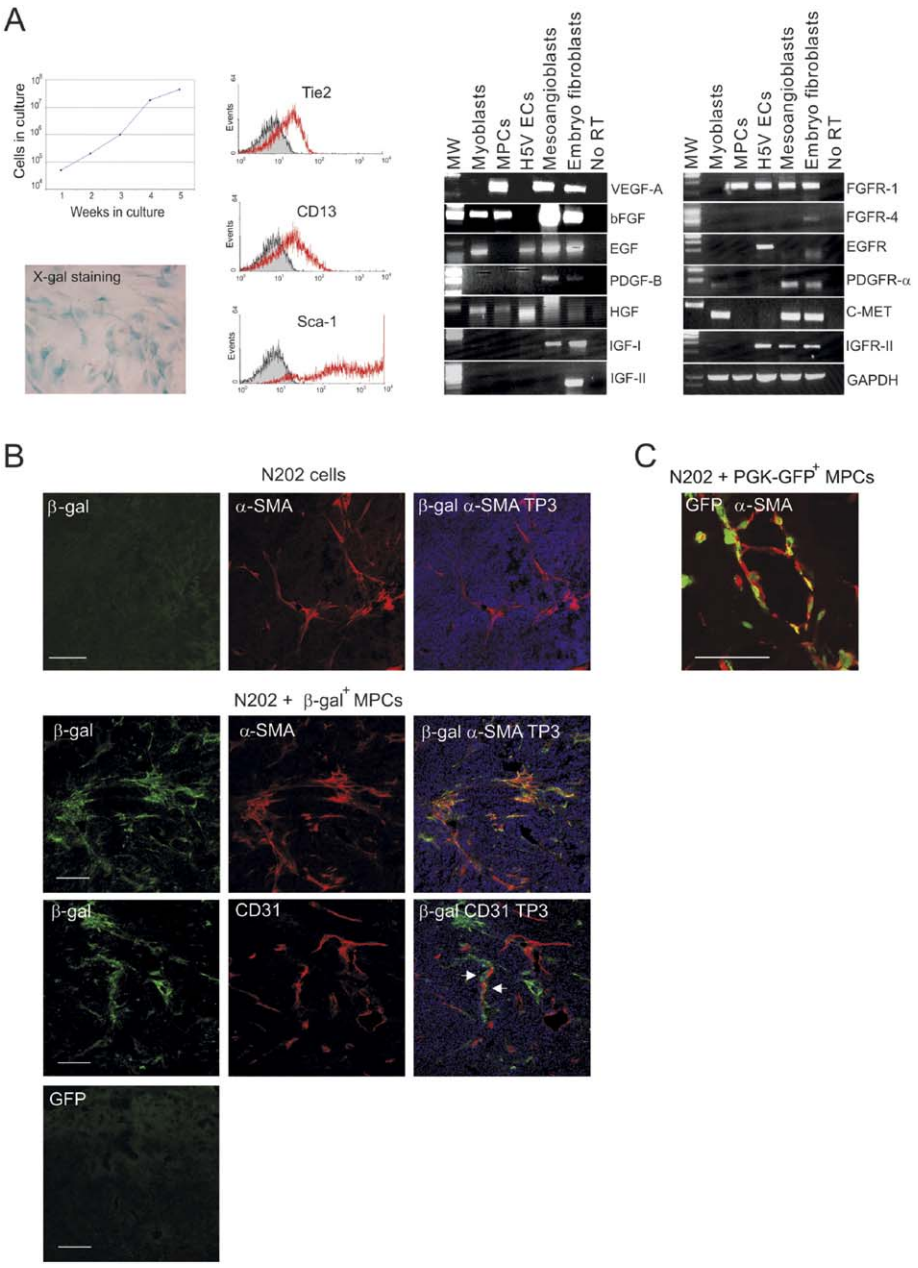

Figure 8. Tie2$^+$ mesenchymal progenitors generate tumor vessel pericytes

A: In vitro culture and characterization of MPCs, isolated as in Figure 7 from tumors grown for 2 weeks in double transgenic TgN(lacZ/Tie2-GFP) mice. Cells were counted at each passage (top left graph). MPCs cultured for ten population doublings were stained with X-gal to detect β-galactosidase (β-gal) activity (bottom left panel), or labeled with PE-conjugated antibodies against Tie2, CD13, and Sca-1, for FACS analysis (histogram plots in the middle; filled histogram is the IgG isotype control), or lysed for RNA extraction and RT-PCR analysis of VEGF-A, bFGF, EGF, PDGF-B, HGF, IGF-I, IGF-II, FGFR-1, FGFR-4, EGFR, PDGFR-α, c-Met, IGFR-II, and GAPDH transcripts (right panel). RNA from the other indicated cell cultures was included for comparison in the RT-PCR analysis.

B: Confocal IF analysis of β-gal, NG2, or GFP (green), α-SMA or CD31 (red), and TP3 (blue) staining of tumors originated by the injection of N202 cells alone or N202 cells together with MPCs (10:1 ratio) in nude mice and analyzed 2 weeks later. The injected MPCs differentiated into β-gal$^+$α-SMA$^+$ pericytes, which often surrounded CD31$^+$ blood vessels (arrows). Scale bar, 120 μm.

C: Confocal IF analysis for GFP (green) and α-SMA (red) of a tumor originated by the injection of N202 cells together with MPCs (10:1 ratio), isolated as in **A** and transduced by a PGK-GFP LV, in nude mice and analyzed 2 weeks later. Scale bar, 120 μm.

(TEMs), endowed with proangiogenic activity and required to promote tumor vascularization. These cells were identified in tumors and in the PB as a fraction of CD11b$^+$ myeloid cells specifically expressing Tie2, a receptor tyrosine kinase previously known to be restricted to ECs. Although the functional role of Tie2 expression has been elucidated in HSCs (Yano et al., 1997; Arai et al., 2004), its occurrence in a distinct subset of monocytes has only been recently described (De Palma et al., 2003a).

We identified TEMs using a sensitive reporter system based on LV-mediated transduction and transgenesis of transcription regulatory sequences from the *Tie2/Tek* gene driving GFP expression. We observed consistent frequency of TEMs in the PB and BM of several transgenic lines and in mice transplanted with ex vivo-transduced HSCs and verified Tie2 receptor expression in the GFP-expressing cells, indicating that the expression pattern of the Tie2-GFP transgene faithfully reproduced that of the endogenous locus with little influence of the vector integration site.

Evidence supporting the existence of a distinct TEM lineage was obtained from GCV-treated Tie2-tk mice and transplant recipients of GCV-treated Tie2-tk BM. In these studies, the majority of the Tie2$^+$ HCs were eliminated from the BM without obvious effects on hematopoiesis, strongly suggesting that the GCV-sensitive Tie2$^+$ HCs were distinct from multipotent HPCs and HSCs. Because GCV only kills proliferating tk-expressing cells, these findings also imply that primitive Tie2$^+$ HSCs must be quiescent or selectively GCV resistant and that they must

turn off Tie2 expression in their clonogenic progeny. This scenario is in agreement with a recent study that showed that 5-FU, an agent that selectively kills proliferating cells, preserved the Tie2$^+$ HSCs in the BM stem cell niche (Arai et al., 2004). On the other hand, the authors observed that 5-FU treatment eliminated a population of Tie2$^+$ HCs in the BM vascular zone. Since the specific elimination of Tie2$^+$ HCs did not detrimentally affect hematopoiesis in our experimental models, it is tempting to speculate that these 5-FU-sensitive Tie2$^+$ HCs represented the BM reservoir of committed TEM precursors.

Additional evidence for the existence of a distinct lineage of TEMs was provided by the delayed recovery of tumor growth observed after halting GCV treatment of Tie2-tk BMT mice. This finding suggested that the GCV-resistant HCs, including circulating and tumor-infiltrating monocyte/macrophages, were not capable of acquiring a TEM phenotype in the tumors when counterselection by GCV was relieved and that the TEM cell pool had to be reconstituted in the BM from putative TEM cell progenitors.

Our data also indicate that circulating TEMs are already committed to a proangiogenic function. Since TEMs constitutively circulate in PB but are not found in quiescent tissues, they may respond to specific cues associated with tissue remodeling and angiogenesis to extravasate, possibly acquire a specialized phenotype, and eventually exert their function. Interestingly, TEMs are a small fraction of the hematopoietic infiltrate found at sites of angiogenesis at steady state. It thus appears that TEMs are short-lived effectors that are selectively retrieved from a pool of circulating cells and are quickly exhausted at angiogenesis sites. However, further studies are needed to conclusively establish the developmental relationship between circulating and tissue-homing TEMs.

Remarkably, TEM activity accounted for most of the proangiogenic activity of PB and tumor-infiltrating myeloid cells in our experimental models. It is well established that TAMs exert proangiogenic activity in tumors, although functional heterogeneity within this cell population has been documented (Pollard, 2004). Whereas this heterogeneity may reflect modulation of the monocyte/macrophage function between growth-promoting and growth-inhibitory activities by the local environment, our data indicate the occurrence of a subset of these cells specifically committed to a proangiogenic function. We cannot rule out that the TEM population, which we have operationally defined and isolated according to the expression of a combination of surface markers, may have some degree of functional heterogeneity, which is commonly observed in other HC subsets. Although the molecular bases of the proangiogenic activity of TEMs need to be elucidated, the selective involvement of these cells in angiogenesis makes them promising antitumor target candidates.

Proangiogenic TEMs, but not BM-derived EPCs, are recruited to spontaneous and orthotopic tumors and are required for tumor neovascularization

The specific recruitment and association of TEMs with the neovasculature of spontaneous pancreatic adenocarcinomas and human brain tumors grown orthotopically in mice highlighted the general relevance of these cells in tumor angiogenesis. By using gene marking approaches based on BMT, we did not find evidence of BM-derived ECs and pericytes in these tumors, in agreement with our previous findings in s.c. tumor grafts (De Palma et al., 2003a). Nevertheless, ad hoc mouse and tumor models may enhance the recruitment of the elusive EPCs from the BM. To force the mobilization of putative EPCs from the BM and their incorporation into tumor vessels, we generated transgenic mice in which EC proliferation and angiogenesis could be specifically blocked by GCV administration. These mice had a remarkable tumor-resistant phenotype, which was not rescued by the transplantation of wild-type BM cells, indicating that putative BM-derived EPCs could not restore angiogenesis when the local supply of preexisting ECs was hampered.

Our results and their interpretation differ from those of Lyden et al., who showed that wild-type BMT rescued the angiogenic defect of *Id1*$^{+/-}$*Id3*$^{-/-}$ mice (Lyden et al., 2001). The authors suggested that mobilization and recruitment of EPCs and HPCs from the BM was impaired in Id mutant mice and that this defect was responsible for the tumor-resistant Id mutant phenotype. Id mutant mice display both deficiencies in EC response to angiogenic stimuli and defective HC mobilization from the BM (Ruzinova et al., 2003; Volpert et al., 2002; Benezra et al., 2001). In one study, *Id1*$^{-/-}$ mice were paradoxically more susceptible to skin carcinogenesis than wild-type mice, and this effect was linked to the impaired recruitment of γδ T cells to the tumors (Sikder et al., 2003). It is difficult to establish whether the defective angiogenic phenotype of *Id1*$^{+/-}$*Id3*$^{-/-}$ mice is primarily due to cell-autonomous deficiencies in ECs, rather than to the defective recruitment of HCs at sites of angiogenesis, or both. Because Id gene loss only impairs the growth of some tumor types, Id-defective ECs may not be inherently incapable of undertaking angiogenesis. Indeed, in tumor-prone transgenic mice that are affected by Id gene loss, reduced tumor viability, rather than decreased tumor incidence or improved survival have been observed (Ruzinova et al., 2003; Li et al., 2004). Although BMT rescued tumor viability in these mice, the contribution of BM-derived EPCs was estimated to be low in these tumors, suggesting that tumor-infiltrating BM-derived cells promoted the formation of functional vessels mostly in a paracrine manner. Wild-type tumor-infiltrating myeloid cells indeed express mitogens and matrix remodeling factors, such as MMPs, which are downregulated by Id gene disruption in ECs (Ruzinova et al., 2003). Based on these data, one can speculate that Id mutant HCs may have altered tumor-homing and/or proangiogenic capacity, and that these defects largely contribute to the angiogenesis-defective and tumor-resistant Id mutant phenotype.

These observations and our results support the emerging paradigm that BM-derived cells recruited to tumors and other sites of angiogenesis, rather than providing a source of building blocks for "postnatal vasculogenesis," comprise distinct HC subsets that promote angiogenesis by secreting or mobilizing growth factors, or by physically cooperating with ECs in making new blood vessels, for instance, by drilling "tunnels" in the extracellular matrix and supporting the migration of vascular cells. Because TEMs share some markers with ECs, closely associate with them in newly formed vessels, and exert a proangiogenic activity, they may have been referred to as "EPCs" in some previous studies. However, if the term "EPC" indicates a cell type capable of robust differentiation into bona fide ECs and effective incorporation within the vascular endothelium, this term should not be used to denote cell types that maintain a hematopoietic phenotype throughout their life span and exert

their function perivascularly, possibly by providing a temporary scaffold or paracrine support for endothelialization. Intriguingly, CD11b$^+$ myeloid cells were recently reported to infiltrate avascular tumors (Okamoto et al., 2005), suggesting that HCs engage in the angiogenic process before the occurrence of the angiogenic switch. Mature HCs likely represent the preeminent cells in the body endowed with the ability to migrate within tissues—even in hypoxic conditions—and with the capacity to modify the extracellular matrix and amplify paracrine signals.

It has been established that orthotopic gliomas initially grow by exploiting the preexistent vasculature in a process known as "vessel cooption." The coopted blood vessels eventually regress, leading to a secondarily avascular tumor characterized by massive tumor cell loss. Tumor viability and growth are belatedly rescued by de novo vessel formation through an "angiogenic phase," a process primarily occurring at the tumor periphery (Holash et al., 1999). Remarkably, elimination of TEMs completely prevented the angiogenic phase of human gliomas grafted in the mouse brain and caused widespread tumor necrosis and regression. Consistent with these findings, purified TEMs, and not other tumor-derived myeloid cells, triggered the early phases of tumor vascularization in s.c. models. Thus, TEMs may be pivotal effectors of the angiogenic switch, and their recruitment from the circulation appears to be a rate-limiting factor for tumor growth.

Tumor Tie2$^+$ mesenchymal progenitors generate tumor pericytes

Besides ECs and TEMs, we found that a rare population of CD13$^+$ cells in the tumors expressed Tie2. This cell population was highly enriched in MPCs capable of long-term self-renewal and differentiation into different mesodermal lineages in vitro. Remarkably, these cells generated a large fraction of the tumor pericytes in transplantation experiments in vivo. In vitro, these MPCs expressed VEGF-A and bFGF, suggesting that they may also modulate the activity of ECs in the angiogenic process. In agreement with this hypothesis, it is known that tumor perivascular cells produce VEGF, which can serve as a survival factor for ECs (Jain, 2003).

The tissue origin of tumor pericytes is obscure. It has been proposed that mesenchymal cells at the tumor-host interface are triggered by the tumor microenvironment to differentiate first into myofibroblasts, and then into pericyte-like cells (Jain, 2003). Some recent reports have proposed a BM origin of tumor pericytes and myofibroblasts in the adult (Rajantie et al., 2004; Direkze et al., 2004). Our gene marking and BMT studies seem, however, to indicate that the vast majority of pericyte progenitors are recruited to tumors from the local environment, and not from the BM.

The Tie2$^+$CD13$^+$ MPCs shut off expression of Tie2 upon acquisition of a "mature" pericyte phenotype in vivo. Although one recent study reported expression of Tie2 by a subset of mural cell progenitors in the rat aorta (Iurlaro et al., 2003), a role of Tie2-expressing pericyte progenitors in tumor angiogenesis has never been described. Consistent with our findings, it has been shown that angiopoietin-1 (Ang-1) stimulated the migration of Tie2$^+$ mesenchymal cells in matrigel plugs in the presence of VEGF (Metheny-Barlow et al., 2004). Furthermore, brain tumors overexpressing Ang-1 exhibited abnormal infiltration of mesenchymal cells and pericytes (Winkler et al., 2004). Remarkably, either the pharmacological blockade of Ang-1/Tie2 signaling by using a Tie2-blocking antibody or the downregulation of Ang-1 expression in tumor cells by RNA interference reverted this phenotype. Together, these observations suggest that Ang-1 may promote the recruitment of Tie2-expressing pericyte precursors to newly forming vessels, although future studies will need to address whether Ang-1 directly acts on these precursors to exert this activity. It is tempting to speculate that a similar mechanism may also regulate the recruitment of TEMs to sites of angiogenesis.

Tie2 expression by proangiogenic cells of diverse origin: Implications for anticancer therapies

In conclusion, expression of Tie2 in tumors identifies an integrated system of different cell types: ECs, TEMs, and vascular mural cell progenitors, which are all specifically involved in angiogenesis. The combined targeting of these three cell types by a suicide gene may well explain the dramatic tumor resistance observed in the Tie2-tk transgenic mice in our studies. Although future work is needed to establish whether the angiopoietin/Tie2 signaling pathway plays a critical role in each of these cell types, or even integrates their activities in the angiogenic process, our observations, together with the results obtained in preclinical testing of anticancer drugs that interfere with angiopoietin/Tie2 signaling (Oliner et al., 2004), validate the Tie2 receptor as a prime target of antiangiogenic therapies. Since also quiescent vessels express Tie2, the design of therapeutic strategies aimed at targeting this receptor should be carefully evaluated. Nevertheless, this concern is alleviated by the observation that Tie2 expression is upregulated at sites of de novo vessel formation (Peters et al., 2004). The combined targeting of the Tie2-expressing cells in tumors—TEMs of hematopoietic origin, pericyte progenitors of mesenchymal origin, and ECs—should dramatically improve the efficacy of cancer therapies.

Experimental procedures

Lentiviral vector constructs

The Tie2p/e-GFP, Tie2p/e-tk.IRES.GFP, and PGKp-GFP lentiviral transfer vectors were previously described; concentrated VSV.G-pseudotyped LV stocks were produced and titered as described (De Palma et al., 2003a, 2003b).

Mice

C57BL/6, FVB, nude (*nu/nu*), and SCID mice were purchased from Charles River Laboratory (Calco, Milan, Italy); RIP-Tag2 mice were obtained from the NCI-Frederick Mouse Repository (Frederick, MD). All procedures were performed according to protocols approved by the Animal Care and Use Committee of the Fondazione San Raffaele del Monte Tabor and communicated to the Ministry of Health and local authorities according to the Italian law.

Generation of transgenic mice by lentiviral vectors

Transgenic mice were generated using LVs as described by Lois et al. (2002). All transgenic lines were generated in the FVB background. Methods are described as Supplemental Experimental Procedures.

Tumor studies and in vivo matrigel assays

N202.1A cells (5–10 × 10^5) were injected s.c. in syngenic FVB mice, and tumors were grown for 3–8 weeks. Tumor size was determined by caliper measurements, and tumor volume was calculated by a rational ellipse formula ($m_1 \times m_1 \times m_2 \times 0.5236$, where m_1 is the shorter axis and m_2 is the longer axis). To obtain tumor cell suspensions, tumors grown s.c. in mice for at least 2 weeks were processed mechanically and by collagenase digestion for 2 hr at 37°, then filtered and prepared for FACS analysis. To

induce orthotopic brain tumors in nude BMT mice, 2×10^5 human glioblastoma multiforme-derived neural stem cells (line 0627) or human glioma U87 cells were delivered into the right striatum (0.2 μl/min) of deeply anesthetized (Avertin) adult female mice by stereotactic injection (Galli et al., 2004; Winkler et al., 2004). Tumor growth was monitored by MRI (see Figure S6). For matrigel plug assays, we used matrigel matrix with reduced growth factor composition (Becton-Dickinson). Matrigel plugs were obtained by mixing 250 μl of matrigel with 250 μl of IMDM medium containing $0.7–2.5 \times 10^5$ cells and by injecting the resultant cell suspension s.c. in male nude mice. To quantify angiogenesis, three to five sections of each of two to seven tumors or matrigel plugs per group were immunostained for CD31 or CD34 and scanned at ×200 magnification by a confocal microscope to identify regions of high vascular density. We then measured the vascular area by computer-assisted digital image analysis, as described (Wild et al., 2000), or counted individual EC marker-positive vessels (or TO-PRO-3$^+$ nuclei belonging to vessels) in at least five ×200 fields from each section. Counts were averaged to determine the vascular area or the vessel density. In all studies, values are expressed as mean ± SEM. Differences were considered statistically significant at $p < 0.05$ (unpaired Student's t test).

FACS, IF staining, and confocal microscopy

Methods are available as Supplemental Experimental Procedures.

BMT

Six-week-old C57BL/6, FVB, TgN(Tie2-tk), or nude mice were killed with CO_2, and their BM was harvested by flushing the femurs and the tibias. Lin$^-$ cells (10^6) (purified using a kit from StemCell Technologies) or 10^7 total BM cells were infused into the tail vein of lethally irradiated (975 cGy) 6- to 8-week-old mice or injected into the peritoneum of lethally irradiated (625 cGy) 8-day-old RIP-Tag2 mice. Lin$^-$ cells were transduced by LVs as described (De Palma et al., 2003a). Full hematopoietic reconstitution of transplanted mice was assessed by FACS analysis of PB, and CFC assays and PCR of the GFP sequence on BM cells 6–12 weeks after the transplant, as described (De Palma et al., 2003a).

Isolation, characterization, expansion, and differentiation of MPCs

Methods are available as Supplemental Experimental Procedures.

Supplemental data

The Supplemental Data include Supplemental Experimental Procedures, one table, and six figures and can be found with this article online at http://www.cancercell.org/cgi/content/full/8/3/211/DC1/.

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