Neuronal and glioma-derived stem cell factor induces angiogenesis within the brain

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Summary

Stem cell factor (SCF) is overexpressed by neurons following brain injury as well as by glioma cells; however, its role in gliomagenesis remains unclear. Here, we demonstrate that SCF directly activates brain microvascular endothelial cells (ECs) in vitro and induces a potent angiogenic response in vivo. Primary human gliomas express SCF in a grade-dependent manner and induce normal neurons to express SCF in brain regions infiltrated by glioma cells, areas that colocalize with prominent angiogenesis. Downregulation of SCF inhibits tumor-mediated angiogenesis and glioma growth in vivo, whereas overexpression of SCF is associated with shorter survival in patients with malignant gliomas. Thus, the SCF/c-Kit pathway plays an important role in tumor- and normal host cell-induced angiogenesis within the brain.

Introduction

Malignant gliomas are among the most lethal tumors, with median survivals of less than a year for patients with the most common type of glioma, glioblastoma, despite aggressive surgery, radiation, and chemotherapy. In an attempt to identify novel therapeutic targets, many investigators have focused on the profound angiogenic response associated with malignant gliomas (Plate and Risau, 1995). Tumor-induced angiogenesis is one of the pathological hallmarks of malignant gliomas and has been demonstrated experimentally to be important for glioma progression. Thus, antiangiogenic strategies have great potential as a treatment approach for gliomas (Stratmann et al., 1997; Tanaka et al., 1998; Purow and Fine, 2004). Identification of the most important angiogenic pathways utilized by gliomas is therefore of interest and significance.

It has been shown that many putative angiogenic factors are expressed by glioma cell lines in vitro; however, few of these factors (with the possible exception of vascular endothelial growth factor [VEGF]), have been conclusively demonstrated to have any pathophysiological role in glioma progression in vivo (Goldbrunner et al., 2000; Dunn et al., 2000; Lamszus et al., 2004). This is important, for it is known that tumor angiogenesis involves a complex interplay between tumor cells, endothelial cells (ECs), and other host cells as well as their surrounding extracellular matrix (host stroma) (Liotta et al., 1991; Carmeliet and Jain, 2000; Tlsty and Hein, 2001; Kalluri, 2003). Thus, a putative angiogenic factor may have very different functions in diverse tissue types and vascular beds. Given the unique microenvironment and microvascular architecture within the central nervous system (CNS), it would therefore not be surprising that various cytokines might have very different biological effects on the angiogenic process within the CNS compared to that in systemic capillary beds.

Stem cell factor (SCF) expression has been demonstrated in the past in a number of glioma cell lines, although its significance remains unclear. We and others have recently reported that various forms of brain injury induce neuronal expression of SCF, and that SCF mediates neural stem cell (NSC) migration to the site of cerebral injury (Zhang and Fedoroff, 1999; Jin et al., 2002; Sun

SIGNIFICANCE

Tumor angiogenesis is a hallmark of malignant gliomas and is thought to play an important role in glioma progression. SCF expression in glioma cells has been reported, but its significance remains unclear. We have now demonstrated that SCF expression, both by neurons as well as by infiltrating glioma cells, results in a marked angiogenic response in vivo. Downregulation of SCF expression in vivo results in improved survival in orthotopic mouse glioma models, whereas overexpression of SCF is associated with a worse prognosis in patients with glioblastoma. This work, therefore, shows that SCF, expressed by both tumor and normal host cells, is an important antiangiogenic target in malignant gliomas and possibly other SCF-expressing tumors.

et al., 2004; Erlandsson et al., 2004). Likewise, recent data demonstrate that hepatic injury induces SCF production and that SCF in turn promotes liver regeneration, an angiogenesis-dependent process (Ren et al., 2003; Greene et al., 2003). Consistent with a potential role in angiogenesis, the SCF receptor c-Kit is found on circulating endothelial precursor cells and human umbilical vein EC (HUVEC) (Broudy et al., 1994; Peichev et al., 2000; Matsui et al., 2004). We hypothesized that, as an adoptive response to tumor-induced brain injury, CNS host cell (i.e., neuron)-mediated SCF expression contributes to glioma-derived SCF expression in inducing a proangiogenic environment within the CNS that is conducive to tumor progression.

In this study, we demonstrate that SCF is a potent angiogenic factor in vitro and in vivo. Furthermore, SCF expression is upregulated in gliomas in a grade-dependent manner, and tumor cells with the strongest SCF expression, as well as normal SCF-expressing neurons, are found predominantly within the infiltrating tumor border, an area that colocalizes with prominent angiogenesis. High SCF expression is associated with shorter patient survival, and downregulation of SCF expression in glioma cells suppresses glioma-induced angiogenesis and improves survival of the mice bearing intracranial glioma xenografts. Thus, the SCF/c-Kit pathway plays a prominent role in pathological angiogenesis within the CNS and may provide a promising molecular target for glioma treatment.

Results

Aberrant expression of SCF in gliomas

Under normal conditions, little or no SCF expression is detectable in the normal brain (Zhang and Fedoroff, 1999; Manova et al., 1992). To evaluate whether SCF is associated with the prominent angiogenesis seen in malignant gliomas, we first determined SCF expression levels in human malignant glioma cell lines. Quantitative real-time RT-PCR revealed high levels of both the secreted *SCF* (s*SCF*) and membrane bound *SCF* (*mSCF*) mRNA splicing isoforms in all glioma cells examined (Figure 1A). We further demonstrate by ELISA that high levels of SCF protein are secreted into the conditioned media (CM) by glioma cell lines in contrast to very low levels found in the CM from normal human astrocytes in vitro (Figure 1B). Additionally, lysates of glioma cell lines reveal high levels of SCF protein as detected by immunoblotting (Figure 1C). The three bands seen on the Western blot correspond to the sSCF (33 kDa), cleaved fraction of sSCF (19 kDa), and mSCF (29 kDa).

In order to evaluate the level of *SCF* expression in primary human gliomas, rather than in just cell lines, we performed mRNA expression profiling of 157 primary human gliomas and 23 nontumor human brain samples using the Affymetrix Human Genome U-133 plus 2.0 GeneChip. A total of four probe sets corresponding to the *SCF* gene are present, and Figure 1D demonstrates the data from a typical probe set showing a significant elevation of *SCF* message in gliomas in comparison to nontumor brain ($p < 0.001$). High-grade gliomas differ significantly from low-grade gliomas both biologically and genetically. Thus, we investigated the levels of *SCF* expression in gliomas of different grades. The results revealed a statistically significant higher level of *SCF* expression in high-grade gliomas (grade III or anaplastic astrocytoma, n = 31; grade IV astrocytoma or glioblastoma, n = 81) compared to low-grade gliomas (grade II astrocytoma, n = 45) or normal brain (n = 23) ($p < 0.001$) (Figure 1D). The microarray expression data were confirmed by quantitative real-time RT-PCR demonstrating a significant elevation of *SCF* in grade IV gliomas compared to low-grade gliomas or nontumor brain ($p < 0.05$) (Figure 1E). Since high-grade gliomas are characterized by a much greater amount of tumor-associated angiogenesis compared to low-grade gliomas, the positive correlation of SCF expression with increasing glioma grade is consistent with a potential role for SCF in glioma-associated angiogenesis.

Effects of SCF on brain microvascular EC

Even though the SCF receptor c-Kit is often expressed on glioma cells in vitro (Natali et al., 1992; Tada et al., 1994; Stanulla et al., 1995), even high concentrations of SCF failed to induce proliferation in our glioma cells in vitro, consistent with previous observations (data not shown) (Berdel et al., 1992; Stanulla et al., 1995). Given the lack of effect of SCF on glioma cell growth in vitro, yet the positive correlation between glioma grade and higher levels of SCF expression, we reasoned that SCF might be involved in glioma progression in vivo through some indirect or paracrine mechanism. To explore the possibility that this indirect effect was a proangiogenic one, we first set out to determine whether c-Kit was expressed on primary ECs in vitro. All ECs examined exhibited c-Kit protein on their cell surfaces by FACS analysis, including bovine brain microvascular EC (BMVEC-b), HUVEC, and human dermal microvascular EC (HMVEC-d) (Figure 2A). By contrast, all of seven glioma cell lines demonstrated the presence of intracytoplasmic but little membrane bound c-Kit (Figures S1A and S1B in the Supplemental Data available with this article online; Table S1).

We next exposed BMVEC-b, HUVEC, and HMVEC-d in basal medium to SCF and determined the rate of thymidine incorporation at 48 hr or 72 hr. SCF stimulated proliferation in all three primary ECs in a dose-dependent manner even at low concentrations (i.e., BMVEC-b proliferation increased approximately 6-fold and 7-fold following exposure to 1 ng or 10 ng of SCF per ml for 72 hr, respectively) (Figure 2B). Additionally, we exposed human brain microvascular EC (HBMEC) to various concentrations of SCF (from 0.1 to 10 ng per ml) and demonstrated a similar dose-dependent pro-proliferative response to that seen with the BMVEC-b (Figure 2C). It is particularly noteworthy that SCF alone, without the addition of other cytokines such as VEGF, was sufficient to sustain human brain microvascular EC and other EC cell growth and survival.

We next used a wound healing assay and Matrigel tube formation assay to further characterize the effects of SCF on BMVEC-b migration and differentiation in vitro. SCF induced a 2- to 3-fold increase in BMVEC-b migration compared to control in a dose-dependent manner (Figures 2D and 2E). Additionally, SCF induced BMVEC-b and HBMEC to migrate in Matrigel within 4 hr of exposure and to form mature appearing capillary-like structures by 18 hr. By contrast, these primary human and bovine brain ECs exposed to the vehicle control neither migrated nor formed capillary-like structures (Figure 2F). In order to establish whether glioma cells could be exerting a proangiogenic effect on neighboring ECs through SCF and other angiogenic proteins, we evaluated the effects of glioma cell CM on EC proliferation. EC proliferation increased 5-fold following exposure to glioma CM compared to basal media. Blocking antibodies to both SCF and VEGF were able to abolish the mitogenic effects of the glioma CM on EC proliferation (Figure 2G). These data

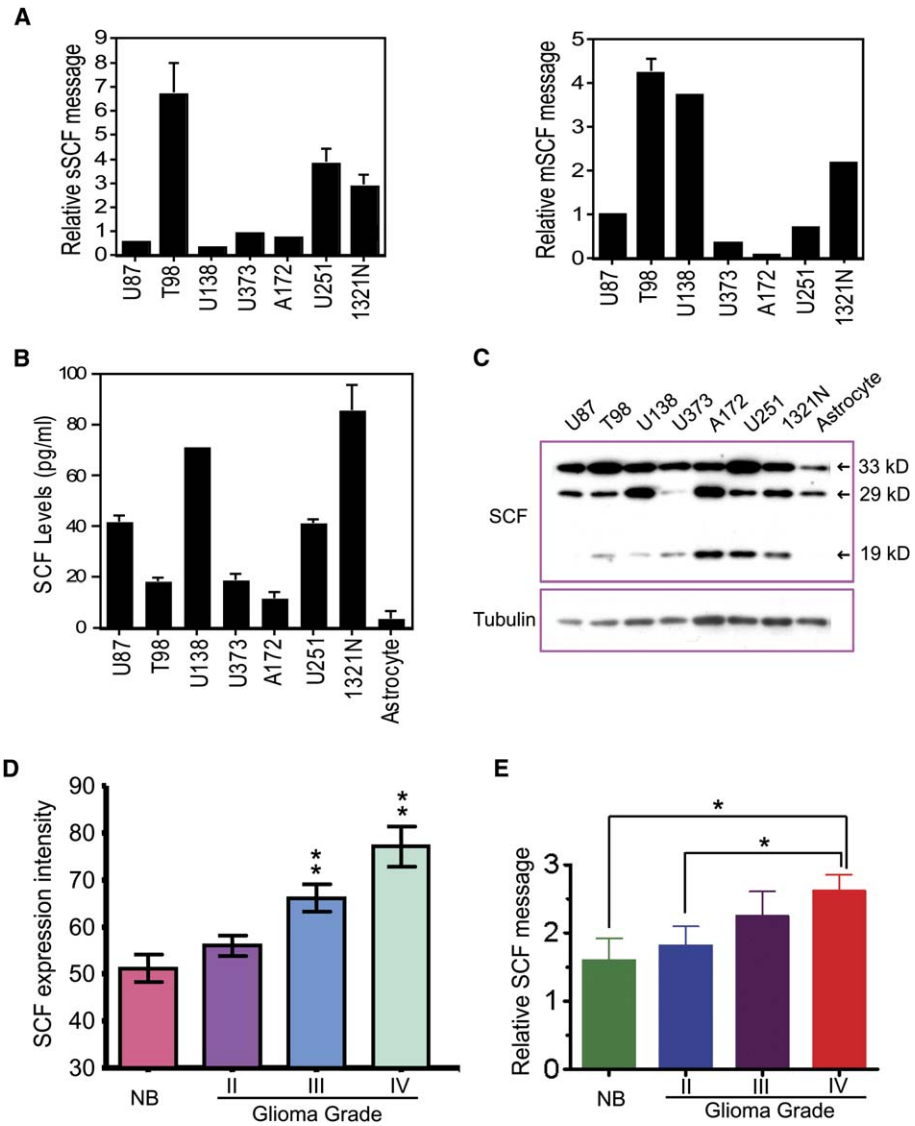

Figure 1. Aberrant expression of SCF in gliomas

A–C: Expression of the two splicing isoforms of SCF in human glioma cell lines. **A:** *sSCF* and *mSCF* were determined by real-time PCR with isoform-specific probe sets, respectively. **B:** Secreted SCF in cell conditioned media was measured using ELISA and normalized by total protein. **C:** Western blot analysis of SCF expression. Tubulin was used as a loading control. Note that both *SCF* isoforms are expressed at higher levels by glioma cells than by normal astrocytes.

D and E: *SCF* mRNA is overexpressed in primary human high-grade glioma specimens. **D:** cDNA microarray analysis of differential *SCF* expression in gliomas according to WHO grades. Note the positive correlation of *SCF* expression with increasing grade (grade III and IV gliomas compared to nontumor brain [NB]; $^{**}p < 0.001$). **E:** Increased *SCF* mRNA in high-grade gliomas was confirmed by real-time RT-PCR. Grade II oligodendroglioma (n = 9) and astrocytoma (n = 9); grade III, anaplastic oligodentrocytoma (n = 8), anaplastic astrocytoma (n = 9); grade IV (n = 28); NB (n = 9). ($^{*}p < 0.05$). Relative message indicates the ratio of *SCF* to *β-actin*.

Columns and bars show the mean and SEM, respectively.

demonstrate the ability of SCF to induce proliferation, migration, and differentiation of primary brain microvascular ECs in vitro.

SCF/c-Kit activates signal transduction in EC

SCF-mediated c-Kit signaling has been shown to induce activation of the mitogen-activated protein kinase (MAPKs) and phosphoinositide 3-kinase (PI3K)/Akt pathways in known SCF target cells (Jin, 2005). A recent report suggests that SCF stimulates a HUVEC angiogenic response through activation of these pathways (Matsui et al., 2004). We investigated whether SCF could induce these two pathways specifically in primary brain ECs rather than just in HUVEC. Confluent BMVEC-b and HUVEC cells were treated with SCF after serum starvation. SCF rapidly induced c-Kit auto-tyrosine-phosphorylation, which peaked at 10–15 min and lasted for 30 min following exposure (Figure 3A). Additionally, phosphorylation of MEK1/2, as well as its downstream p44/42 MAPK, was dramatically increased. SAPK/JNK was similarly activated 10–15 min following SCF exposure, whereas p38 MAPK exhibited a prolonged activation for over 30 min.

Like the MAPK pathway, the PI3K pathway was activated following BMVEC-b and HUVEC exposure to SCF. Phosphorylation of Akt was significantly enhanced by SCF treatment, which in turn resulted in phosphorylation of mTOR. Activation of mTOR increased phosphorylation of 4E-BP1 consistent with a possible diminished inhibition of 4E-BP1 on eIF4E, which could result in diminished cap-dependent translation. Thus, SCF activates the MAPK and PI3K pathways in BMVEC-b in a manner similar to its effects on HUVEC.

Additionally, SCF (50 ng/ml) induced immediate phosphorylation of the c-Kit, p44/42 MAPK, and Akt proteins in HBMECs (Figure 3B). Thus, SCF-induced activation of the MAPK and PI3K pathways appears to occur in ECs from both systemic capillary beds as well as from the brain. In contrast to BMVEC-b, SCF could not induce phosphorylation of p38 MAPK and SAPK/JNK in HBMEC (data not shown). Whether this represents a species-specific difference in SCF-mediated signaling or reflects different subtypes of ECs from the brain remains to be determined.

SCF promotes angiogenesis in vivo

We next evaluated whether SCF has proangiogenic activity in vivo by subcutaneously implanting Matrigel impregnated

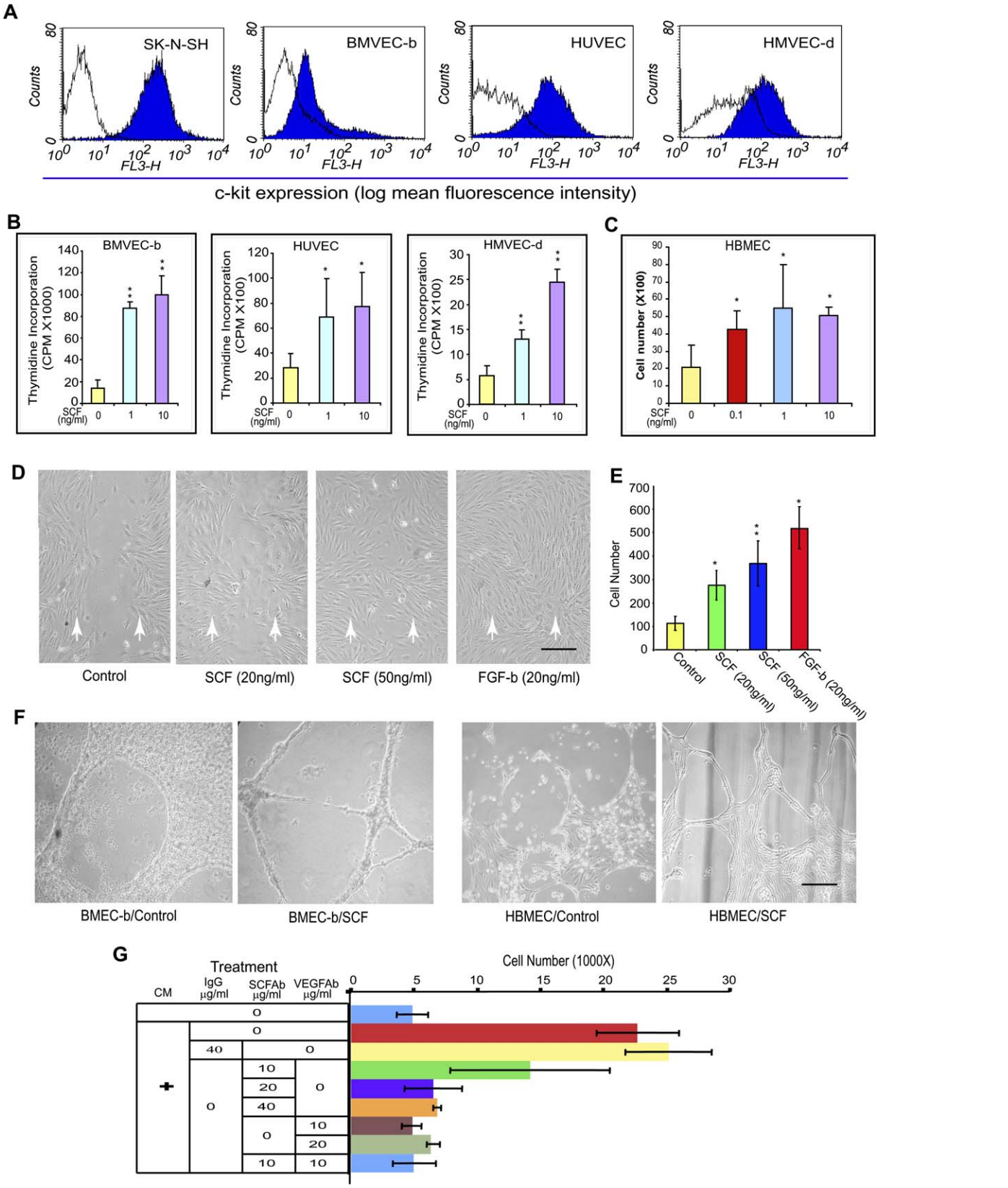

Figure 2. SCF promotes angiogenesis in vitro

A: c-Kit expression in ECs was determined by FACS in BMVEC-b, HUVEC, and HMVEC-d cells. The blue shaded histograms represent the fluorescence intensity of c-Kit, and the black line indicates the control IgG isotype staining. SK-N-SH and NIH3T3 cells were used as positive and negative controls, respectively (NIH3T3 not shown).

with SCF, β-FGF (positive control), or vehicle alone into the adult SCID mice. Newly formed blood vessels in the Matrigel plugs were visualized by hematoxylin/eosin (H&E) staining and confirmed by immunofluorescent staining for factor VIII-related antigen (vWF)-positive ECs. In addition, Tie2 protein levels in the Matrigel plugs were determined. Fourteen days after injection, vehicle control-impregnated Matrigel plugs demonstrated few vWF-positive vessels and had essentially no detectable Tie2 protein expression (Figure 4). By contrast, SCF (as well as the positive control β-FGF) induced substantial angiogenesis, as demonstrated by abundant intact blood vessels as well as high Tie2 expression within the SCF-containing Matrigel plug. These data demonstrate that SCF can promote angiogenesis in vivo.

Silencing SCF attenuates glioma angiogenesis

To evaluate the effect of SCF on glioma angiogenesis and progression, we engineered glioma cells expressing low levels of SCF by retrovirally transducing an antisense *SCF* construct or a *SCF* shRNA into wild-type U373, U87, and U251 glioma cells expressing high levels of SCF. The resulting U373 cell line (U373/as-SCF), U87 cell line (U87/as-SCF), and U251 cell line (U251/shRNA SCF) were expanded, and then stable low level-expressing clones were selected by antibiotics and verified by Western blot (Figure 5A). To avoid the confounding variable of concurrent VEGF expression, we verified by Western blots that suppression of SCF expression in our cloned glioma cell lines had no effects on VEGF expression (Figure 5A). Next, we implanted U373/as-SCF or U373/vector cell lines together with Matrigel subcutaneously into SCID mice in order to assess glioma-induced angiogenesis in vivo with and without SCF stimulation. Within 2 weeks, a profound angiogenic response was seen in the Matrigel containing the control U373/vector cells with multiple vWF-positive perfused vessels and abundant Tie2 protein in the Matrigel plug homogenates. By contrast, Matrigel containing the U373/as-SCF cells demonstrated far fewer capillaries with vWF staining and significantly less Tie2 protein in the plug homogenates (Figures 5B–5D). Thus, suppression of SCF in glioma cells results in significant inhibition of glioma-induced angiogenesis in vivo. Consistent with these observations, U251/as-SCF did not form palpable subcutaneous tumors several weeks after implantation, in contrast to their matched U251/vector cells, which formed tumors so large that animals needed to be euthanized (Figure S2). Likewise, systemically administered bevacizumab (Genentech, San Francisco, CA) inhibited U251/vector produced VEGF resulting in profound tumor growth suppression. We could not, however, demonstrate an additive or synergistic effect of SCF knockdown and VEGF inhibition, since SCF knockdown alone resulted in complete tumor growth inhibition (Figure S2).

SCF effects on survival in intracranial tumor model

We next evaluated whether suppression of SCF would affect the survival of animals with intracranial gliomas. U373/as-SCF or U373/vector cells, U87/as-SCF or U87/vector cells, and U251/shRNA SCF or U251/vector cells were stereotactically implanted into the cerebral subcortex of adult athymic *nu/nu* mice. Log-rank analysis of the Kaplan-Meier survival curves demonstrated a significant survival advantage for the SCF low-expression glioma-bearing mice compared to their matched SCF high-expression parental vector-infected glioma-bearing animals ($p < 0.05$) (Figure 6A). Examination of 4-week-old U373/vector intracranial tumors demonstrated significant SCF expression within the tumor cells and a robust angiogenic response. By contrast, there was significantly lower SCF expression within the tumor cells of the intracranial U373/as-SCF tumors with an attenuated angiogenic response within the tumor (Figure 6B).

Immunohistological staining demonstrated that, although SCF is generally expressed in most tumor cells within the central U373/vector tumor mass, SCF expression was most marked in tumor cells within the tumor border and in the few infiltrating glioma cells at the invasion front. Increased SCF expression was also observed in the normal brain tissue adjacent to the tumor mass (Figure 6C). In situ hybridization for *SCF* mRNA revealed that it was the normal host neurons within the cerebral cortex, adjacent to the invading tumor mass, that accounted for the non-tumor cell-associated high-level *SCF* expression (Figure 6D). Additionally, a similar pattern and level of normal neuronal overexpression of SCF was seen in cortex immediately adjacent to U373/as-SCF tumors despite the fact that the actual tumor cells made very little SCF (in contrast to the U373/vector cells). Normal neurons at increasingly farther distances from the tumor site, however, demonstrated progressively less SCF expression, such that SCF expression was barely detectable in neurons within the cortex contralateral to the growing tumor mass (Figure 6E). Together, these results support a role for tumor cell and normal host neuron-mediated SCF-induced tumor angiogenesis in glioma progression.

Biological and clinical significance of SCF in glioma-infiltrated human cerebral cortex

To evaluate whether the patterns of glioma-associated SCF expression in the intracranial mouse xenograft model reflected patterns seen in human brains with malignant gliomas, we performed extensive immunohistochemical analysis of surgical specimens from patients with glioblastoma. We observed generally four important features of the heterogeneous SCF expression in the brains of patients with glioblastoma. In some areas, close to the center of tumor mass, very little or no SCF was detected (Figure 7A). SCF was generally expressed at high levels by glioma cells infiltrating into the cerebral white matter (Figure 7B). In the glioma-infiltrated cerebral cortex abundant SCF expression was seen not only in the tumor cells, but also in normal host neurons (Figure 7C). Thus, there was profound expression of SCF in cerebral cortex infiltrated by glioma cells secondary to both tumor- and normal host neuron-associated SCF expression (Figure 7D). In summary, SCF expression appears to

B: SCF alone sustained EC proliferation and survival as determined by thymidine incorporation.
C: HBMEC also showed dose-dependent growth in the presence of SCF.
D–F: SCF activates brain microvascular ECs. **D:** In the presence of SCF, BMVEC-b migrated into the denuded zone 18 hr after scraping. Arrows indicate the wound edge. **E:** Total cells in denuded zone were counted from three separate fields in each well from triplicates. **F:** BMVEC-b formed tubular structures on Matrigel in the presence of SCF but not in controls.
G: The U251-derived CM significantly increased HUVEC proliferation in culture, an effect nearly completely blocked by neutralizing antibodies to either SCF or VEGF. Scale bar, 200 μM.
Columns and bars show the mean and SEM, respectively.

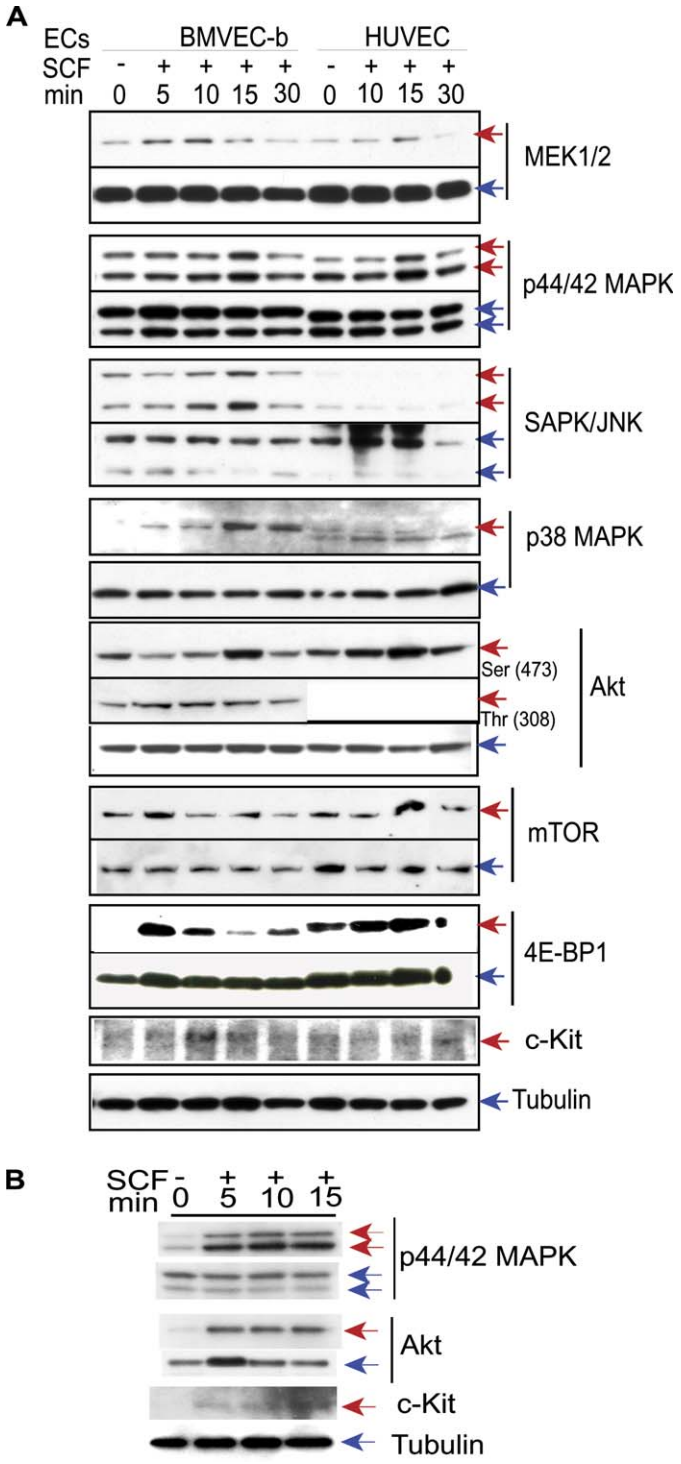

Figure 3. SCF/c-Kit activates MAPKs and Akt pathways in brain microvascular ECs

Cell lysates were collected at the indicated times after SCF stimulation of BMVEC-b and HUVEC (**A**) as well as HBMEC (**B**) and analyzed by Western blot with the indicated anti-phosphorylated protein antibodies as well as anti-total protein antibodies. Arrows: red, phosphorylated protein; blue, total protein. Tubulin was used as an equal loading control.

reside most prominently in the invasive front of the infiltrating glioma, suggesting its roles in tumor progression. Most notably, this area of high SCF expression colocalizes with areas of dense microvasculature sprouting and branching, consistent with a role for SCF in glioma- and normal host neuron-mediated angiogenesis (Figure 7E). In addition, abundant c-Kit expression was also detected on the glioblastoma-associated ECs but little on the actual tumor cells, consistent with our in vitro data (Figure 7F). The clinical significance of these findings is reflected by the fact that patients with glioblastoma (grade IV; n = 64), otherwise matched for known prognostic factors (i.e., age, treatment), who had tumors that expressed high levels of *SCF* experienced significantly shorter survival than patients with tumors expressing relatively low levels of *SCF* ($p = 0.0004$ by log-rank analysis; Figure 7G). By contrast, *VEGF* expression levels did not correlate with survival in patients with glioblastoma ($p = 0.24$).

Discussion

The work reported here describes a role for SCF in tumor-associated angiogenesis and specifically in glioma progression. SCF has been long recognized as an important growth factor for a number of cell types, including hematopoietic stem cell, mast cells, melanocytes, and germ cells (Zsebo et al., 1990; Galli et al., 1992; Mackenzie et al., 1997). In these divergent cell types, SCF has been shown to have a number of different biological properties. A role for SCF as a potential angiogenic factor, however, has not been previously entertained until a recent report demonstrated its mitogenic effects on HUVEC cells in vitro (Matsui et al., 2004). We have now expanded on those observations by demonstrating that SCF has potent mitogen, differentiation, and chemotactic properties on a variety of primary EC lines in vitro, and furthermore can induce a robust angiogenic response and contribute to tumor-associated angiogenesis in vivo.

It has been previously reported that glioma cell lines can express SCF and c-Kit, implicating SCF as an autocrine growth factor for gliomas (Hamel and Westphal, 2000). Previous observations, however, could not demonstrate a mitogenic effect of SCF on glioma cells. Thus, the role for SCF in glioma pathogenesis remained unclear until now. Our data now confirm that SCF expression is not a direct glioma mitogen but rather a potent glioma-associated angiogenic factor. Although SCF has been previously implicated in angiogenesis through its ability to regulate mast cell migration with subsequent release of VEGF (Zhang et al., 2000), no direct role for SCF in angiogenesis had been previous described except for a recent report of its ability to induce HUVEC proliferation and migration in vitro (Matsui et al., 2004). We have extended those observations by showing that not only HUVECs, but also primary ECs (including human brain microvascular ECs) express c-Kit and that SCF-mediated c-Kit signaling in ECs results in activation of the MAPK and AKT pathway, leading to enhanced proliferation, survival, and migration of ECs even in the absence of VEGF, β-FGF, or other, thought to be obligate, EC growth factors.

Our data clearly demonstrate the ability of SCF to induce a potent angiogenic response both subdermally and within the brain. Furthermore, we show using several different cell lines and using several different methodologies that downregulation of SCF expression in an orthotopic glioma model results in diminished angiogenesis and prolongation of animal survival. We believe that these observations have clinical significance based on our demonstration that the level of *SCF* expression in primary human gliomas directly correlates with the grade of the tumor

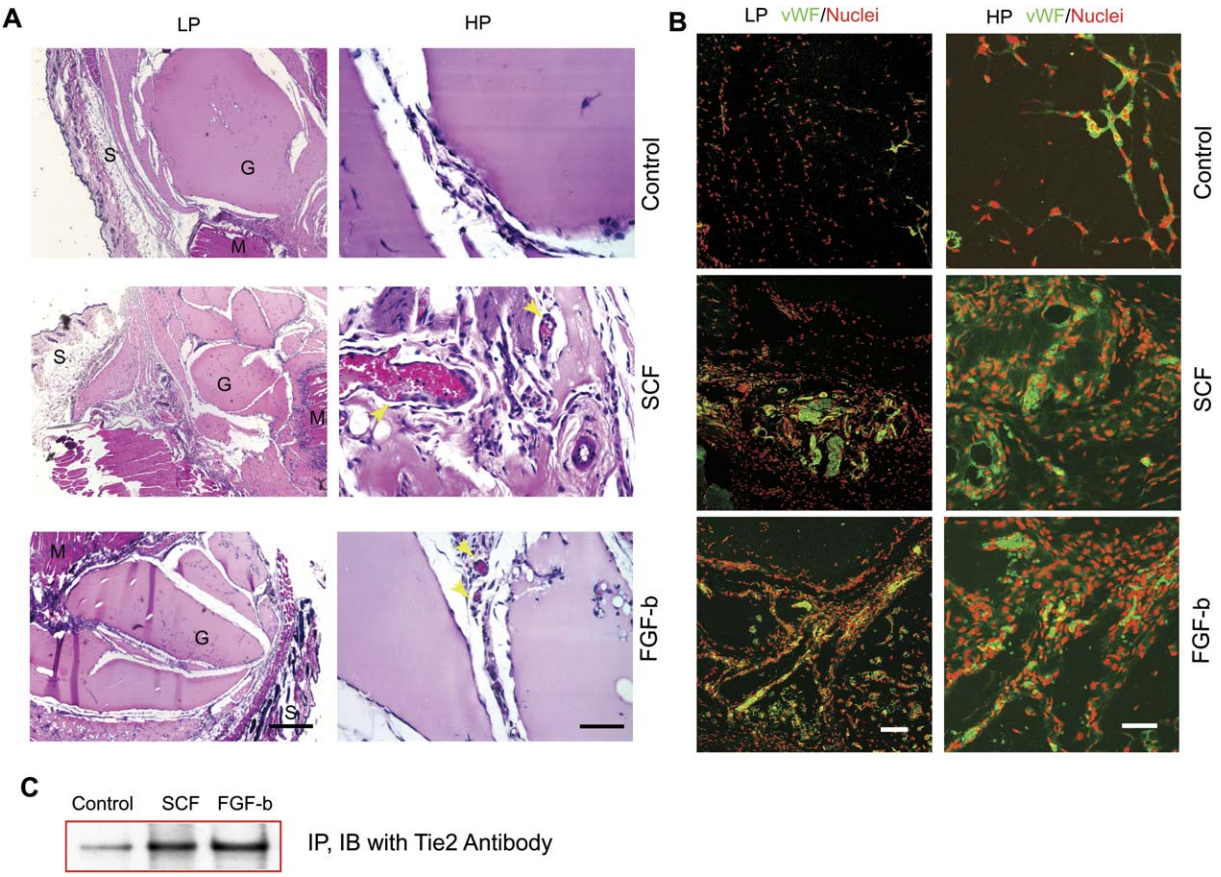

Figure 4. A high density of blood vessels was induced in SCF- or β-FGF-supplemented Matrigel plugs

A: H&E staining. Scale bars, 500 μM (LP), 50 μM (HP).

B: Immunofluorescence staining for vWF. Scale bars, 100 μM (LP), 50 μM (HP).

C: Western blot for Tie2 in homogenates of Matrigel plugs. Arrows: neovasculature containing red blood cells. LP, low power; HP, high power; M, muscle; S, skin; G, gel.

and patient survival. These data demonstrating that high-grade gliomas express significantly more *SCF* than low-grade gliomas or nontumor human brain tissue are fully consistent with the fact that high-grade gliomas are significantly more angiogenic than low-grade gliomas. In fact, endothelial proliferation is one of the pathological criteria for grading a glioma high grade (Kleihues and Cavenee, 2000). Additionally, our demonstration of increased angiogenic activity colocalizing with increased SCF expression in surgical sections of human brains with malignant gliomas further supports the relevance of SCF-induced angiogenesis in the pathogenesis of malignant gliomas.

It is becoming increasingly clear that tumorigenesis in vivo involves not just the biology of the tumor cell itself, but rather a complex interplay between tumor cells and the deregulation of normal host physiological functions that ultimately aid in tumor progression (Tlsty and Hein, 2001; Liotta and Kohn, 2001). Others and we have previously demonstrated that SCF expression is induced in normal neurons in vivo following various types of brain trauma (Zhang and Fedoroff, 1999; Sun et al., 2004). Gliomas cause significant damage to normal brain parenchyma through numerous mechanisms including disruption of the blood-brain barrier with resultant increased cerebral edema and increased intracranial pressure, glioma-mediated degradation of the extracellular matrix through overexpression of metalloproteases, and release of neurotoxic excitatory molecules such as glutamate (Forsyth et al., 1999; Takano et al., 2001; VanMeter et al., 2001; Davies, 2002; Chantrain et al., 2004). Thus, we wondered whether, along with the direct release of SCF from glioma cells, tumor growth could be eliciting expression of SCF from normal brain tissue in a manner analogous to that seen in our brain injury models. Data from both our orthotopic model and the surgical sections from human brains with malignant gliomas demonstrate that normal host neurons within the area of cerebral cortex, adjacent to the tumor mass and within the invading front of glioma cells, do express SCF to very high levels, whereas neurons in areas of cortex away from the growing tumor do not. These areas of neuronal and glioma SCF overexpression overlap precisely with areas of sprouting and branching angiogenesis. Thus, normal host cells within the brain appear to have been coopted to induce pathological angiogenesis in support of the infiltrating tumor cells. The expression of SCF by normal host tissue may also help explain why downregulation of direct glioma SCF expression through our antisense and siRNA constructs in our orthotopic models decreased glioma-mediated angiogenesis but did not abolish it and only led to moderately prolonged animal survival. Full angiogenesis inhibition will likely depend on inhibition of all SCF expression (or c-Kit inhibition), including both tumor- and normal host neuron-mediated expression, possibly in conjunction with inhibition of other angiogenic pathways.

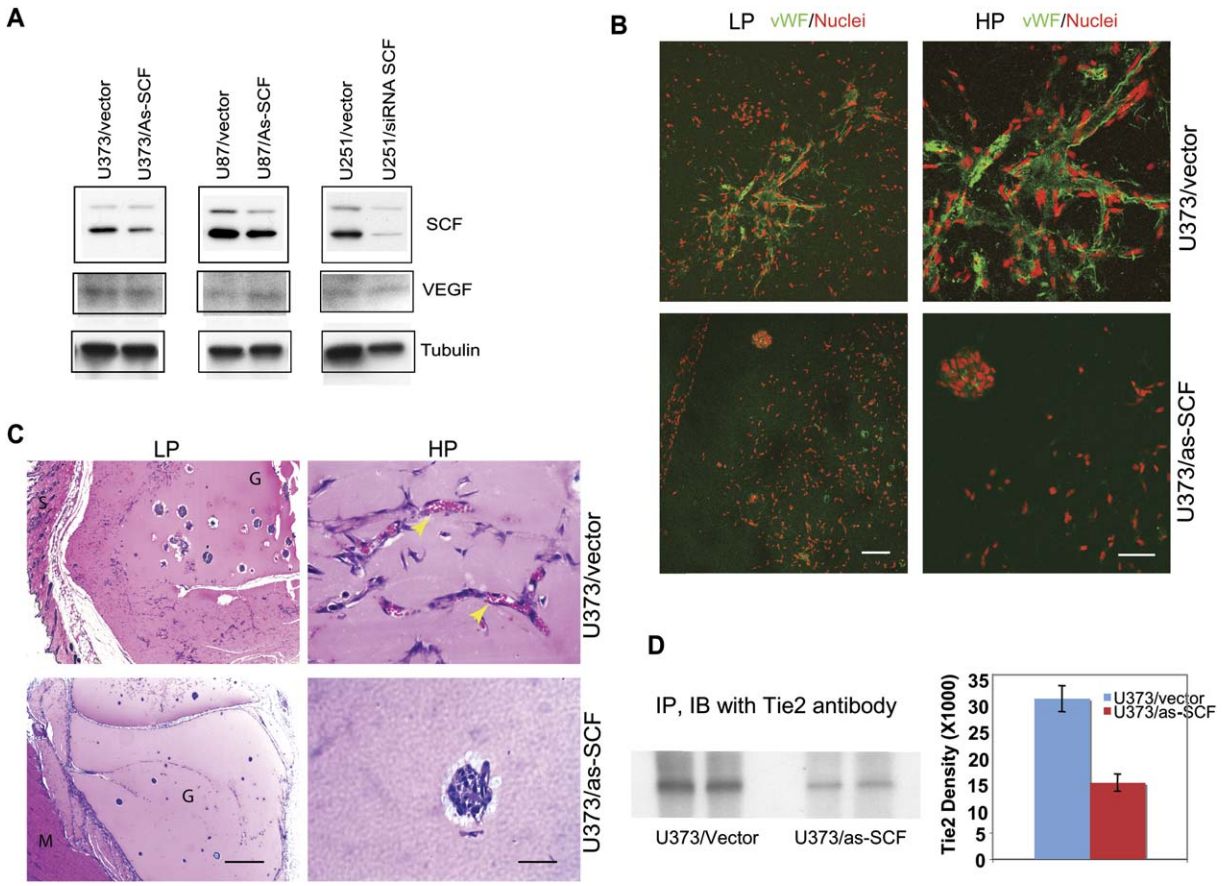

Figure 5. Suppression of SCF in U373 glioma cells attenuates U373-mediated angiogenesis

A: Downregulation of SCF in U373/as-SCF, U87/as-SCF, and U251/shRNA SCF was verified by Western blot. Downregulation of SCF resulted in no effects on VEGF expression from the cells.

B and C: Immunofluorescence staining for vWF (**B**) and H&E staining (**C**) in an in vivo tumor-angiogenesis assay using U373/as-SCF and U373/vector glioma cells. Scale bars, 100 μM (LP in **B**), 500 μM (LP in **C**), 50 μM (HP).

D: Tie2 protein in homogenates of Matrigel plugs (40 mg total protein in each sample) was immunoprecipitated with Tie2 antibody and then detected by Western blot in duplicate. The comparative quantitative intensities of the amount of Tie2 protein were measured by densitometry. Arrows: neovasculature containing red blood cells. LP, low power; HP, high power; M, muscle; S, skin; G, gel. Columns and bars show the mean and SEM, respectively.

These data strongly implicate SCF as an important angiogenic factor in pathogenesis of malignant gliomas. Among the relatively large number of factors that have been implicated in glioma-mediated angiogenesis, the most commonly cited are β-FGF, PDGF, and VEGF (Dunn et al., 2000; Lamszus et al., 2004). Although gliomas clearly express β-FGF in a grade-dependent manner, various studies have failed to find β-FGF receptors on glioma-associated endothelium, leading some to speculate that β-FGF is acting more as a glioma autocrine growth factor rather than an angiogenic factor (Zagzag et al., 1990; Morrison et al., 1994). The various heterodimer PDGF receptors have been demonstrated on glioma-associated endothelium; however, PDGF overexpression tends to occur in low-grade gliomas and higher-grade gliomas that have transformed from low-grade gliomas rather than in the more common primary glioblastomas (Westermark et al., 1995; Ribom et al., 2002; Dai et al., 2001). Thus, PDGF may also be playing a largely mitogenic autocrine role in glioma growth and may not have a major angiogenic function in the majority of high-grade gliomas. VEGF is the one angiogenic factor that has clearly been implicated in glioma angiogenesis, as it is highly overexpressed in most high-grade gliomas and especially in glioblastoma, where its receptor is overexpressed on glioma-associated endothelium (Berkman et al., 1993; Hatva et al., 1995). VEGF expression, however, has been demonstrated to be most pronounced in glioblastoma cells adjacent to areas of necrosis, consistent with its known hypoxia inducibility (Plate et al., 1992; Shweiki et al., 1992). These areas of necrosis and relative tumor hypoxia, however, are not the areas of the most pronounced angiogenesis or most rapid tumor cell proliferation within a malignant glioma. Rather, it is the invading border of the tumor that harbors the most prominent angiogenic reaction and tumor cell proliferation (VanMeter et al., 2001). This is the area that corresponds to the highest SCF expression.

Our interpretation of these data leads us to believe that SCF, along with VEGF, may have complementary roles in the robust angiogenic response seen in malignant gliomas. To date, there have been a number of clinical trials with small molecule inhibitors of FLK-1 (the VEGF receptor); however, none have yet to show definitive clinical benefit. Although the lack of clinical success may be secondary to the failings of the individual molecules tested, it is also plausible to postulate that angiogenic pathways other than or in addition to VEGF will need to be targeted for clinical success. As such, SCF may prove to be an important

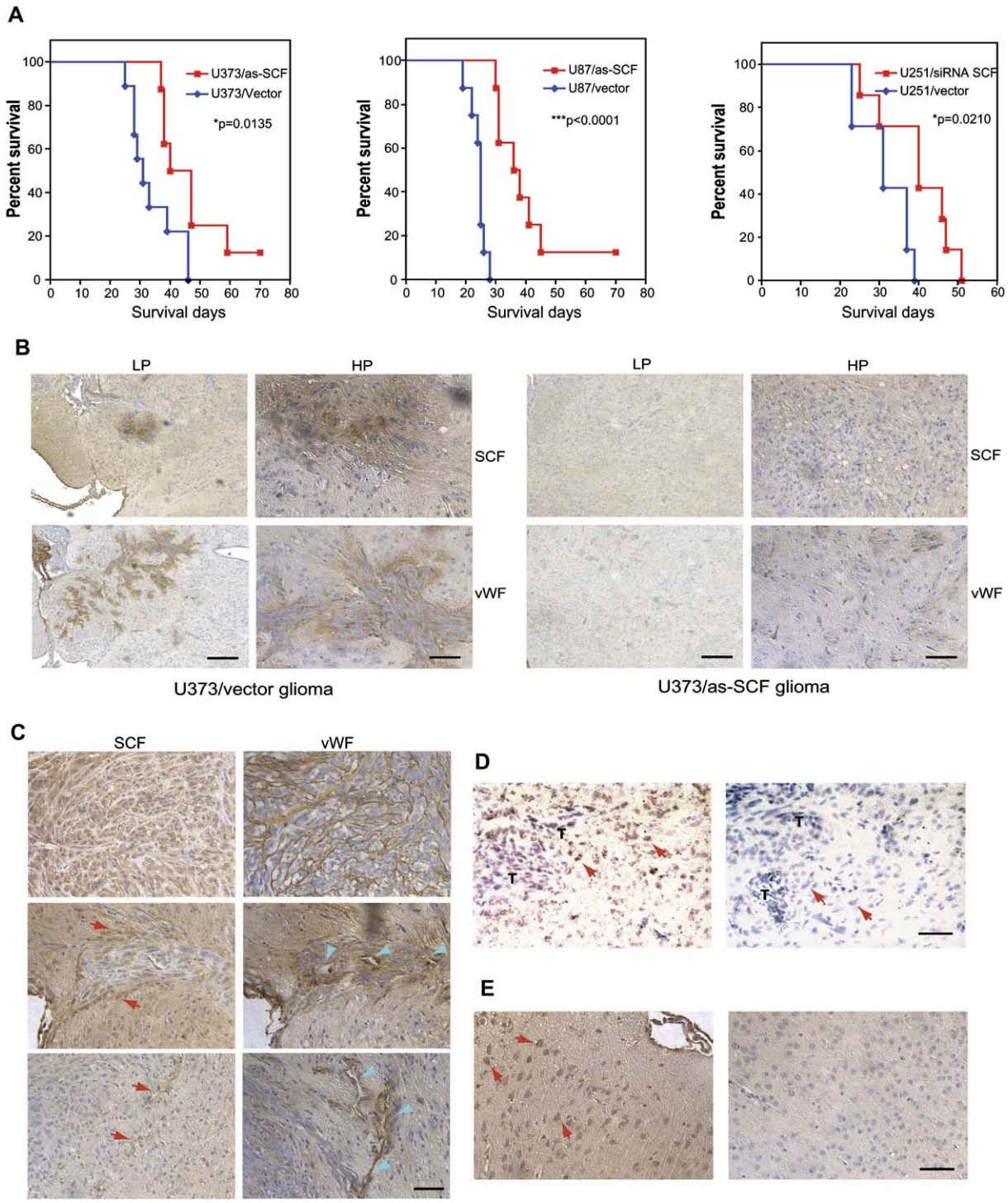

Figure 6. Downregulation of SCF expression in glioma cells impedes tumor angiogenesis and prolongs survival in an intracranial tumor model

A: Kaplan-Meier survival curve shows that downregulation of SCF expression in U373/as-SCF, U87/as-SCF, and U251/shRNA SCF tumors significantly improves survival of tumor-bearing mice compared with their vector controls, respectively (log-rank test $p < 0.05$).

B: Downregulation of SCF expression attenuates SCF-induced angiogenesis in implanted glioma tumors. In contrast to U373/vector gliomas, the U373/as-SCF gliomas demonstrate little SCF staining and a markedly lower density of blood vessels. Scale bars, 200 μM (LP); 50 μM (HP).

C: Colocalization of neovasculature with SCF in U373/vector tumors. Tumor tissue shows strong SCF expression and a high density of collapsed vessels (upper panels). Along the tumor invasion border (middle panels), abundant SCF surrounds tumor cluster with multiple small vessels in the center. In brain tissue adjacent to the tumor mass (lower panels), SCF is expressed both by tumor cells and normal cells within the brain. Scale bar, 50 μM.

D and E: Intracranial tumor induces SCF expression in affected neurons. **D:** *SCF*-positive neurons (arrows) are seen in the cerebral cortex infiltrated by tumor (T) as detected by in situ hybridization using a *SCF* antisense probe (left). The negative control using a hybridized sense probe is seen on the right. **E:** SCF-expressing cells are present in the cerebral cortex close to U373/as-SCF injection (left) as compared to the contralateral side of the brain (right) as analyzed by immunohistochemistry. Arrows: red, SCF staining; blue, vWF+ blood vessels. Scale bars, 50 μM.

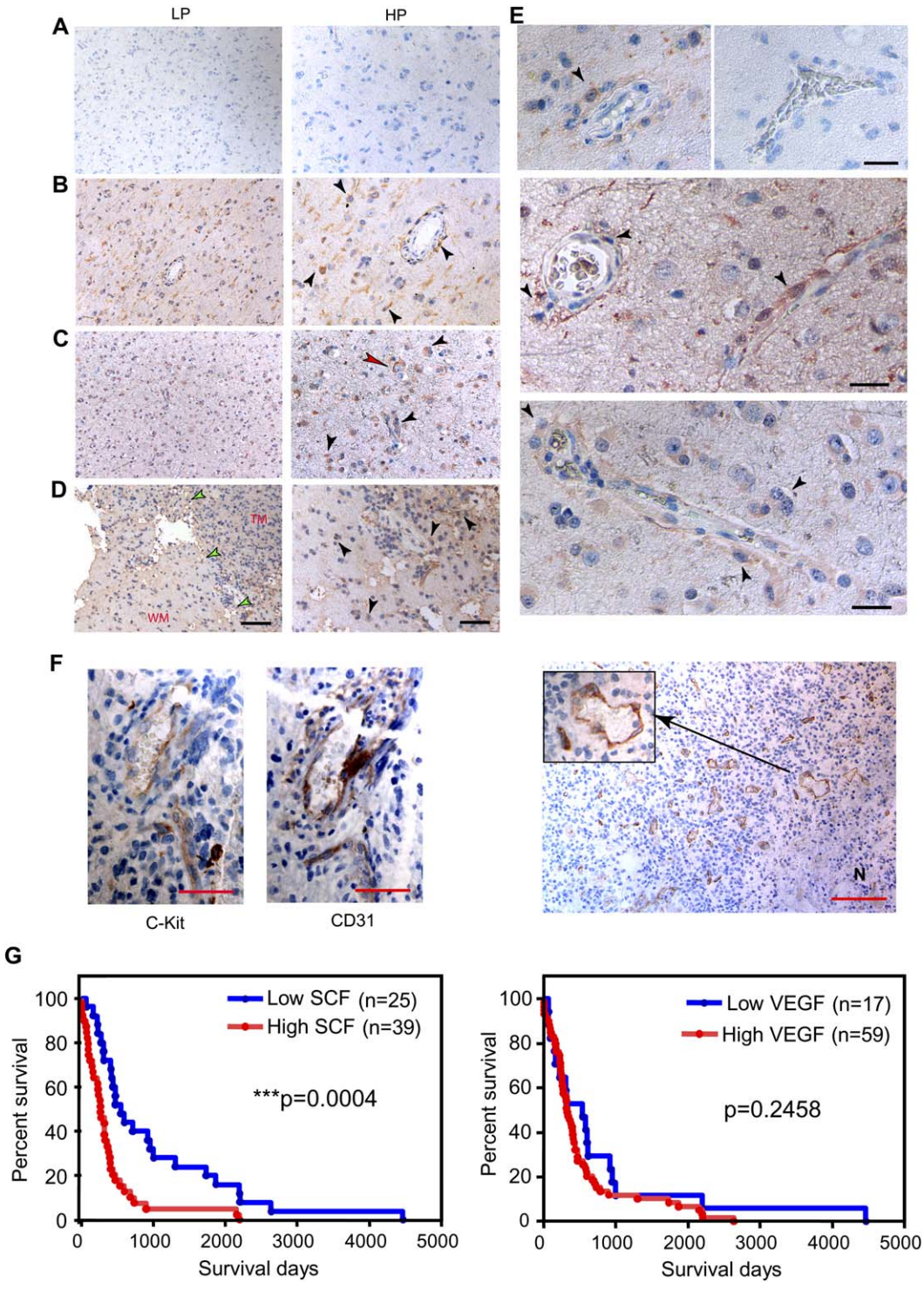

Figure 7. Distribution of SCF expression in human brain harboring a glioblastoma

A–D: SCF-expressing cells by immunohistochemistry in representative regions of brain and tumor. **A:** Center of tumor mass. **B:** Cerebral white matter infiltrated by tumor cells. **C:** Cerebral cortex with infiltration of tumor cells. **D:** Cellular tumor adjacent to white matter. Scale bars (shown in **D**), 100 μM (LP), 50 μM (HP).

E: SCF-expressing tumor cells surrounding new blood vessels are shown at higher magnification (800×). The top panels show the SCF-expressing tumor cells (left) and SCF-negative cells (right) lining blood vessels, respectively. The middle and bottom panels show SCF-expressing cells lining the full-length of the blood vessels. Arrowheads indicate cytoplasmic staining of SCF in tumor cells (black), neuron (red), and the boundary between tumor mass (TM) and tumor-infiltrating white matter (WM) (green). Note that SCF-expressing tumor cells are more pronounced in the tumor regions adjacent to normal brain and along the new blood vessels. Scale bars, 25 μM.

F: c-Kit-positive endothelial cells on the glioblastoma-associated vasculature colocalized with CD31-positive endothelial cells (left panels) throughout the tumor, including in areas adjacent to necrosis (N, right panel). Scale bars, 50 μM (left panels), 100 μM (right panel).

G: Kaplan-Meier survival curve demonstrating survival of patients with glioblastoma (grade IV gliomas) stratified by tumors expressing either low or high levels of *SCF* (log-rank analyses p = 0.0004) and expressing low or high levels of *VEGF* (log-rank analyses p = 0.2458).

therapeutic target in high-grade gliomas. There has already been one clinical trial of Imatinib (Gleevec), a small molecular inhibitor of ABL, PDGF, and c-Kit, in patients with malignant gliomas primarily based on the premise of targeting PDGF as an autocrine growth factor (Kilic et al., 2000; P.Y. Wen et al., 2002, Proc. Am. Soc. Clin. Oncol., abstract). The investigators in this trial did not observe any clinical activity from Imatinib; however, the lack of activity may have been the result of the inability of Imatinib to cross the BBB. Although it has been argued that one of the theoretical appeals of antivascular and antiangiogenic therapy for brain tumors is the nonnecessity for molecules to cross the BBB, the reality is that many of the endothelial targets may not be accessible to BBB-impermeable molecules within the bloodstream if such targets are present on the adluminal side of the EC. Indeed, given that the BBB works in both directions, a large protein such as SCF would not easily cross out of the brain back into the bloodstream. Thus, it is possible that c-Kit expression may not be expressed on the side of the endothelial cell facing the blood vessel lumen, thereby making the receptor inaccessible to BBB-impermeable drugs such as Imatinib. It remains to be seen whether some of antitumor effects of Imatinib activity against certain systemic solid tumors, such as gastrointestinal stromal tumors (GIST), and chronic myelogenous leukemia (CML) may be in part a result of its antiangiogenic as well as its direct antitumor effects (Demetri et al., 2002; Kantarjian et al., 2002; Sawyers et al., 2002).

In conclusion, we have presented data that demonstrate SCF to be a potent angiogenic factor both in systemic tissue and within the brain. Additionally, SCF appears to be an important angiogenic factor in high-grade gliomas, both through direct tumor cell expression of SCF and through the induction of its expression by normal host neurons within areas of brain infiltrated by tumor. Normal neuronal expression of SCF in response to traumatic brain injury also raises the disturbing possibility that standard invasive procedures such as surgical biopsies or partial tumor resections may be inducing a proangiogenic response, or trigger, within the brain. As such, SCF represents a potentially important therapeutic target for malignant gliomas. Finally, the ability of normal host neurons to express SCF in response to injury, as well as the potent angiogenic properties of SCF within the brain, leads one to speculate that SCF may have important therapeutic potential as a proangiogenic factor in regenerative tissue repair strategies for various disease states such as stroke and brain and spinal cord injury as well as neurodegenerative diseases.

Experimental procedures

Cell culture

Primary ECs (Clonetics, Walkersville, MD) and human astrocytes (Sciencell Research Laboratories, San Diego, CA) were used at passage 8 or earlier. HBMEC (Sciencell Research Laboratories) was used at passage 1 or 2. All malignant glioma cells except for 1321N (Europe collection of cell cultures, Sigma-Aldrich Corp., St. Louis, MO), bone marrow neuroblastoma cell, SK-N-SH, and NIH 3T3 were purchased from American Type Culture Collection (Manassas, VA). All cells were passaged and cultured in conditions recommended by their suppliers.

Thymidine incorporation

After ECs (3×10^3/well) were adhered to 96-well gelatin-coated plates (BD Discovery Labware, Bedford, MA), the EC growth medium was replaced by SCF (1.10 ng/ml) (R&D Systems, Minneapolis, MN) suspended in treatment medium (basal medium with 0.1% BSA) for an additional 48 hr (HUVEC and HMVEC-d) or 72 hr (BMVEC-b) with renewal of medium at 24 hr interval. ECs were cultured for 4 hr in 0.25 μCi of [^{3}H]thymidine (Amersham Pharmacia Biotech, Piscataway, NJ) at the end. High molecular mass [^{3}H]-radioactivity was precipitated and counted.

EC proliferation

The HBMEC and HUVEC were seeded on 48-well and 24-well plates (poly-L-lysine-coated plates for HBMEC) at 5×10^3 or 2×10^4 per well, respectively. After cells adhered to the well (6 hr), growth medium was replaced by CM. The CM collected from U251 glioma cell cultures were pretreated with SCF or VEGF neutralized antibodies (R&D systems) at the indicated concentrations for 1 hr at 37°C before being added to the HUVEC. After 48 hr culture in the treated media, dead floating cells were washed away, and attached living cells were trypsinized and counted with hematocytometer.

EC differentiation assay

After the Matrigel (BD Biosciences) formed a solid gel in 24-well plates, serum-starved BMVEC-b cells were seeded on the gel surface at 10^5 cells per well and cultured in treatment medium in the presence or absence of SCF (20 ng/ml). Primary HBMEC were placed on the gel directly at 24×10^3 per well. Tubular formation was observed using an inverted phase contrast microscope, and images were captured at 4 and 18 hr after cell seeding. Each experiment was repeated two or three times.

Wound healing assay

The BMVEC-b (3×10^5/well) was seeded on collagen I-coated plates (24-well). After cells adhered to the plates (3 hr), growth medium was replaced by SCF and β-FGF (R&D Systems) or vehicle in treatment medium. After 6 hr of incubation, confluent monolayers of BMVEC-b were wounded with a pipette tip and incubated in the same condition. The cells were fixed with 4% paraformaldehyde 18 hr after being wounded, and images were captured. The cells that had migrated across the edge of the wound were observed microscopically (magnification, ×20) and counted in three fields for each well. All assays were repeated three times in duplicates or triplicates.

Flow cytometry analysis

Cells were incubated with Cy-Chrome conjugated anti-human c-Kit (CD117) antibody or PE-Cy5 mouse IgG1 as a negative control (BD Pharmingen, San Diego, CA), respectively. Dead cells were excluded by propidium iodide staining (Sigma Chemie), and expression of c-Kit on the cell surface was analyzed by FACS (Becton Dickinson, San Jose, CA). For the detection of total c-Kit including that in the cytosol, permeabilization was performed with BD cytofix/cytoperm solution (Kit from BD Biosciences) for 15 min prior to the incubation with c-Kit antibody.

Immunoprecipitation and immunoblotting

Confluent-grown ECs were treated with 50 ng/ml of SCF for 5, 10, 15, and 30 min after 24 hr of serum starvation. The ECs were collected with RIPA lysis buffer (Upstate, Charlottesville, VA) containing 50 mM NaF, 1 mM Na_3VO_4, 50 mM PMSF, and Protease Inhibitor Cocktail (Roche). The antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA) or as mentioned otherwise. All the primary antibodies were used in a 1:1000 dilution except for the anti-SCF antibody, which was used in a 1:2000 dilution. The primary antibodies were anti-phospho-c-Kit, anti-phospho-MEK1/2 and anti-MEK1/2, anti-phospho-p44/42 MAPK and anti-p44/42 MAPK, anti-phospho-SAPK/JNK and anti-SAPK/JNK, anti-phospho-p38 MAPK and anti-p38 MAPK, anti-phospho-Akt and anti-Akt, anti-phospho-mTOR and anti-mTOR, anti-phospho-4E-BP1 and anti-4E-BP1, anti-SCF and anti-VEGF (Chemicon, Temecula, CA), and anti-β-tubulin (Sigma). Reblotting was done after membranes were stripped by reblot kit (Chemicon). Tie2 detection in Matrigel plugs from the in vivo Matrigel assay was carried out by immunoprecipitation and immunoblotting. The gel plugs were carefully separated from muscle and skin and homogenized with RIPA lysis buffer. The resulting total tissue lysates (40 mg of protein) were immunoprecipitated with Tie2 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and then analyzed by immunoblot with Tie2 antibody (Zadeh et al., 2004).

ELISA

Cells were seeded at 80% of confluence, and the growth medium was changed 24 hr later to basal medium containing 0.1% BSA after washing

with PBS. Forty-eight hours later, the CM were collected, and supernates were obtained by centrifuge at 2K RPM for 5 min. SCF in the CM was measured using the human SCF ELISA kit (R&D System) in triplicate following the protocol provided by the manufactory and normalized by the total protein levels in the media of each individual sample.

Microarray analysis

All gliomas (grade II, III, and IV) were pathologically diagnosed according to WHO standard. All tumors and nontumor brain tissues of epilepsy were obtained from surgery patients and frozen immediately after operation (clinical protocol approved by the NCI IRB committee with informed consent obtained from all subjects). The frozen samples were disrupted and homogenized with TRIZOL (Invitrogen Corp., Carlsbad, CA), and the total RNA isolated from each sample was further purified using RNeasy Mini Kit (Qiagen, Valencia, CA). The integrity and quality of the RNA met the quality requirements for Human Genome U133 Plus 2.0 arrays (Affymetrix, Inc., Santa Clara, CA) recommended by the company. All experimental procedures for labeling, array hybridization, and scanning were performed using protocols recommended by Affymetrix. CEL files were generated by Affymetrix GCOS 1.2 software, and the present/absent calls were defined with global scaling to target value of 500. By dChip software (Li and Wong, 2001), the CEL files were normalized to a median-intensity array, and model-based expression values were calculated using PM/MM difference model. Based on the latest annotation from Affymetrix NETAFFX service, four probe sets for *SCF* (*KITLG*) gene were present in each chip. The signal intensities of each probe set were used for analyzing *SCF* (*KITLG*) expressions. The significance in differential *SCF* (*KITLG*) expressions in different grades of gliomas versus human nontumor brains was determined using log2-transformed expression values by standard unpaired two-tailed Student's t test of two groups without assuming equal variance between groups. The microarray data have been submitted to the Gene Expression Omnibus (GEO) public database at NCBI, and the accession number is GSE4290.

Real-time RT-PCR

RNA isolation and cDNA synthesis were performed as described above. A human probe set covering exon 8 of *SCF* gene was purchased from Applied Biosystems (Foster City, CA). Isoform-specific probe sets were selected in the connecting region of exons 5 and 7 (for *mSCF*) and in exon 6 (for *sSCF*) (Table S1). Two-step real-time PCR was performed in duplicates or triplicates. Data were analyzed on the basis of threshold cycle values of each sample and normalized with β-actin.

Expression vector and transduction

SCF cDNA was synthesized by RT-PCR and subcloned into the pLenti6/V5-DEST vector (Invitrogen Corp.) in the antisense orientation following the supplier's protocol. The 293FT package cell was cotransfected with the expression vector and ViralPower Packaging Mixture (Invitrogen Corp.). The supernatants containing infectious virus particles from transfected 293FT were used to transduce U373 and U87 glioma cells. The stably transduced U373 and U87 cell lines were selected with 5 ug/ml of blasticidin for over 2 weeks, and the amount of SCF protein expression was assayed by immunoblot. The retroviral expression vector pSM2 with the *SCF* shRNA insert and the LinX retroviral packaging cells were purchased from Open Biosystems (Hunstsville, AL). The retrovirus was obtained from plasmid transfected LinX cells and was used to transduce U251 cells following the manufacturer's protocol. The stable shRNA expression cells were selected with puromycin at 1 ng/ml for over 2 weeks, and downregulation of SCF was verified by immunoblot.

In vivo angiogenesis assay and intracranial tumor model

All adult mice (6–8 weeks of age) were from NCI-Fredrick, and the animal experiments were conducted on an animal experimentation protocol approved by the NCI's animal protection committee (the "ACUC"). For the in vivo angiogenesis assay (Capogrossi and Passaniti, 1999), Matrigel with 150 ng of SCF and controls or 5×10^3 cells was injected into a SCID mouse, and the plugs with skin and muscle were collected 2 weeks later. For the intracranial implantation of glioma cells, stereotactic surgery was performed (1 mm anterior to the bregma and 2.5 mm lateral to the midline) using athymic *nu/nu* mice under anesthesia. U373/vector or U373/as-SCF (10^6 cell) were suspended in 5 μl of HANK and injected into the caudate nucleus. For immunohistochemical analysis, mice were sacrificed 4 weeks later, and brains were carefully recovered after perfusion.

Immunohistochemistry

The fixed brains and Matrigel plugs were prepared for paraffin sectioning and immunohistochemical analysis. The human GBM tissues were obtained from surgery patients following the clinical protocol approved by the NCI IRB committee with informed consent obtained from all subjects. After being deparaffinized, the sections were processed for immunohistochemistry with the following primary antibodies: anti-vWF (1:500 dilution), anti-c-Kit (1:200 dilution), anti-CD31 (1:20 dilution), and anti-SCF (1:100 dilution), respectively, All of the primary antibodies were purchased from Dako Corp. (Carpinteria, CA) except for the anti-SCF antibody, which was bought from Chemicon. Visualization of antibody binding was performed by a FITC-conjugated second antibody (1:500 dilution; Molecular Probes, Eugene, OR) or by the second antibody tagged with HRP (1:1000 dilution; Dako) following reaction to DAB (Molecular Probes) or AEC (Sigma).

In situ hybridization

The DIG-labeled probe hybridized to nucleotides 848–895 of the mouse *SCF* mRNA sequence (NM_013598 in GenBank) was obtained from GeneDetect company (Aucklank, New Zealand). After they were deparaffinized, mouse sections were permeabilized with target retrieval solution (Dako) and hybridized with sense or antisense DIG-labeled probe at 37°C for 18 hr. After stringent washing, the hybridized signals were detected by tyramide signal amplification system and visualized with DAB (Dako).

Statistical analysis

Student's t test was used to determine statistical significance in comparisons. Survival curves were plotted with Kaplan-Meier method and compared using log-rank test. $p < 0.05$ was considered significance. Survival trends of grade IV glioma patients ($n = 64$) were analyzed relative to *SCF* mRNA levels. The actin normalized *SCF* levels ranging from 0.64- to 10.76-fold were determined by real-time PCR; levels less than 1.8 were grouped as low SCF, and those greater than 1.8 were classified as high SCF. In the figures, columns and bars show the mean and SEM, respectively.

Supplemental data

The Supplemental Data include two supplemental figures and two supplemental tables and can be found with this article online at http://www.cancercell.org/cgi/content/full/9/4/287/DC1/.

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Accession numbers

The microarray data have been submitted to the Gene Expression Omnibus (GEO) public database at NCBI, and the accession number is GSE4290.