

# Semaphorin-3F Is an Inhibitor of Tumor Angiogenesis

Ofra Kessler,<sup>1</sup> Niva Shraga-Heled,<sup>1</sup> Tali Lange,<sup>1</sup> Noga Gutmann-Raviv,<sup>1</sup> Edmond Sabo,<sup>2</sup> Limor Baruch,<sup>3</sup> Marcelle Machluf,<sup>3</sup> and Gera Neufeld<sup>1</sup>

<sup>1</sup>Department of Cell Biology and Anatomy, Rappaport Family Institute for Research in the Medical Sciences, The Bruce Rappaport Faculty of Medicine, Technion, Israel Institute of Technology; <sup>2</sup>Department of Pathology, Carmel Medical Center; and <sup>3</sup>Department of Biotechnology and Food Engineering, Technion-Institute of Technology, Haifa, Israel

## ABSTRACT

The neuropilin-1 (np1) and neuropilin-2 (np2) receptors form complexes with type-A plexins. These complexes serve as signaling receptors for specific class-3 semaphorins. Np1 and np2 function in addition as receptors for heparin-binding forms of vascular endothelial growth factor (VEGF), such as VEGF<sub>165</sub>. Human umbilical vein endothelial cells (HUVEC) express tyrosine-kinase receptors for VEGF and basic fibroblast growth factor (bFGF), as well as np1, np2, and several type-A plexins. We have found that semaphorin-3F (s3f), a semaphorin which signals through the np2 receptor, was able to inhibit VEGF<sub>165</sub>, as well as bFGF-induced proliferation of HUVECs. Furthermore, s3f inhibited VEGF as well as bFGF-induced phosphorylation of extracellular signal-regulated kinase-1/2. Our experiments indicate that bFGF does not bind to neuropilins, nor does s3f inhibit the binding of bFGF to FGFRs. It is therefore possible that s3f inhibits the activity of bFGF by a mechanism that requires active s3f signal transduction rather than by inhibition of bFGF binding to FGFRs. s3f also inhibited VEGF<sub>165</sub>, as well as bFGF-induced *in vivo* angiogenesis as determined by the alginate micro-encapsulation and Matrigel plug assays. Overexpression of s3f in tumorigenic human HEK293 cells inhibited their tumor-forming ability but not their proliferation in cell culture. The tumors that did develop from s3f-expressing HEK293 cells developed at a much slower rate and had a significantly lower concentration of tumor-associated blood vessels, indicating that s3f is an inhibitor of tumor angiogenesis.

## INTRODUCTION

Neuropilin-1 (np1) is a receptor for the axonal guidance factor semaphorin-3A (s3a), a member of the class-3 semaphorin subfamily (1, 2). The related neuropilin-2 (np2) receptor is a receptor for semaphorin-3F (s3f), a semaphorin which was originally identified as a tumor suppressor whose function is lost in small cell lung carcinoma (3, 4). Expression of recombinant s3f in several types of tumorigenic cell lines inhibited their tumor-forming ability in nude mice, confirming that s3f is a tumor suppressor (5). s3f was subsequently found to function as an axon guidance factor that repulses np2-expressing growth cones during the development of the nervous system (1, 6). COS cells expressing np2 and either plexin-A1 or plexin-A2, but not np2 alone, contract in response to s3f, indicating that these plexins are essential for s3f signal transduction (7). s3f binds to np1 with an affinity that is ~10-fold lower than its affinity to np2. However, s3f is unable to repulse neuronal growth cones expressing np1, indicating that s3f cannot transduce biological signals via this receptor (8). Other class-3 semaphorins also bind to np1, np2, or both receptors, but these semaphorins have been less extensively studied (9).

The 165 amino acids form of vascular endothelial growth factor (VEGF<sub>165</sub>) also binds to both neuropilins. In contrast, VEGF<sub>145</sub> is able to bind to np2, but not np1, whereas VEGF<sub>121</sub> does not usually bind to neuropilins (10–12). VEGF<sub>165</sub> binding to np1 enhances VEGF<sub>165</sub> signaling mediated by the VEGF receptor (VEGFR)-2 (10). Additional factors belonging to the VEGF family, such as placenta growth factor, VEGF-B, and VEGF-C, are also able to bind to specific neuropilins (13–15). In early chick and mouse embryos, np1 is mainly expressed in arteries, whereas np2 is expressed in veins. At later stages of development, np2 expression appears in lymphatic vessels, and the expression in veins is down-regulated (16–18). Gene-targeted mice lacking functional np1 display severe cardiovascular and neuronal abnormalities (19). In contrast, np2 gene-targeted mice are viable. They display neuronal abnormalities, but the cardiovascular system is normal, except for some abnormalities in peripheral lymph vessels (18). However, mice in which both neuropilin genes were inactivated display cardiovascular defects that are much more acute than those generated by the inactivation of a single neuropilin, thereby confirming a role for both neuropilins in vasculogenesis and developmental angiogenesis (20).

These studies indicate that semaphorins may perhaps be able to function as modulators of vasculogenesis, angiogenesis, and possibly lymphangiogenesis. Indeed, s3a was found to inhibit capillary sprouting in an *in vitro* assay of angiogenesis and VEGF<sub>165</sub>-induced migration of endothelial cells. s3a inhibits the binding of VEGF<sub>165</sub> to np1, and it was therefore postulated that the s3a inhibits angiogenesis by inhibiting the potentiating effect that VEGF<sub>165</sub> binding to np1 confers on VEGFR-2-mediated signal transduction (10, 21). In addition, it was recently shown that s3a can inhibit the adhesion of endothelial cells to extracellular matrix constituents, such as fibronectin, and inhibit developmental angiogenesis in chick embryo (22, 23). These effects of s3a may or may not be linked to its ability to induce apoptosis (24). Surprisingly, there were no gross effects on vascular system organization in mice that were gene targeted for s3a, except for heart malformations (25).

In contrast to s3a, which competes with VEGF<sub>165</sub> for binding to np1, s3f does not compete with VEGF<sub>165</sub> for binding to np2, although both factors bind to np2 (12). The vascular systems of mice that lack functional *s3f* genes appear relatively normal, and the mice were viable (26), indicating that on its own, s3f does not fulfill a critical function during the organization of the embryonic vascular system. s3f had been originally isolated as a tumor suppressor of small cell lung carcinoma (3, 5) and had been shown to affect the proliferation and migration of tumor cells that express s3f receptors (27). In the present study, we have examined the effects of s3f on the proliferation of human umbilical vein derived endothelial cells (HUVECs). The proliferation and survival of these cells *in vitro* depend on the addition of exogenous angiogenic growth factors, such as VEGF or basic fibroblast growth factor (bFGF). s3f inhibited the mitogenic effects of VEGF<sub>165</sub> and bFGF in these cells, indicating that s3f may be able to function as an inhibitor of angiogenesis. Indeed, we show that s3f inhibits VEGF<sub>165</sub> and bFGF-induced angiogenesis *in vivo*. Furthermore, s3f also inhibited the formation of tumors derived from human embryonic kidney (HEK)293 cells, although s3f was not able to

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**Note:** O. Kessler and N. Shraga-Heled contributed equally to this work.

**Requests for reprints:** Gera Neufeld, Department of Cell Biology and Anatomy, Rappaport Family Institute for Research in the Medical Sciences, The Bruce Rappaport Faculty of Medicine, Technion, Israel Institute of Technology, Haifa, 31096, Israel. Phone: 972-8523672; Fax: 972-8523947; E-mail: gera@tx.technion.ac.il.

inhibit the proliferation of these cells in cell culture. The development of the few tumors that did develop from s3f-expressing HEK293 cells was retarded, and the tumors contained significantly lower densities of blood vessels, indicating that s3f functions as an inhibitor of tumor angiogenesis.

## MATERIALS AND METHODS

**Materials.** All of the chemicals, including ultra-pure polylysine ( $M_r$  27,000), were from Sigma (St. Louis, MO), unless otherwise indicated. Ultra-pure sodium alginate (MVG) was from Pronova (Pronova Biopolymer, Drammen, Norway). Mediums and sera for cell culture and Lipofectamine were from Invitrogen-Life Technologies, Inc. (Carlsbad, CA), except for the M199 medium that was from Biological-Industries Inc. (Kibbutz Beth-Haemrk, Israel). Wheat-germ agglutinin-coupled agarose was from Vector (Burlingame, CA). BALB/c *nu/nu* female mice were obtained from Harlan, Inc. (Indianapolis, IN). DiAsp was purchased from Molecular Probes (Eugene, OR). Reverse PCR was performed using the Reverse-it kit (Abgene, London, United Kingdom). Antibody directed against phosphorylated extracellular signal-regulated kinase (ERK)-1/2 was from CST, Inc. (Beverly, MA), and the antibody directed against ERK-2 (total ERK) was from Santa-Cruz, Inc. (San Diego, CA). Antibodies directed against CD-31 were kindly donated by Dr. Neta Ilan (28). Soluble VEGFR-1 (sflt) was purchased from Sigma.

**Production and Purification of s3f.** The cDNA encoding the longer 786 amino acids splice form of s3f (s3f-1) or the shorter 755 amino acids splice form of s3f (4) was ligated in-frame into the pcDNA3.1/myc-His expression plasmid to generate expression vectors encoding myc and 6xHis epitope-tagged s3f splice forms. HEK293 and BHK-21 cells were transfected with these expression vectors using Lipofectamine and stable s3f- and s3f-1-expressing clones isolated using G418 (0.5 mg/ml) selection. To purify s3f, HEK293 cells transfected with the s3f expression vector or empty expression vector were cultured to 90% confluence. The medium was exchanged to serum-free DMEM. Sodium butyrate (5 mM) was added after 24 h to the control and s3f-expressing cells, and the medium was collected after an additional 24 h. HEPES buffer (10 mM, pH 7.3) and protease inhibitors (0.2 mg/ml phenylmethylsulfonyl fluoride, 2  $\mu$ g/ml leupeptin and aprotinin, and 1 mM EDTA) were then added. The medium was incubated overnight at 4°C with wheat-germ agglutinin agarose (1 ml of beads/0.5 liter of medium). The column was washed twice with PBS, and bound s3f was eluted using 0.5 M *N*-acetylglu-

cosamine in PBS. The s3f peak in the eluant was dialyzed overnight at 4°C against PBS in bags with a cutoff of  $M_r$  50,000. The dialyzed s3f was loaded onto an S-Sepharose column (0.5 ml of bed volume) pre-equilibrated with wash buffer [20 mM NaHPO<sub>4</sub> (pH 7.2) and 1 mM EDTA]. The column was washed twice with 5 ml of wash buffer, once with 2 ml of wash buffer containing 0.1 M NaCl, and eluted with wash buffer containing 0.5 M NaCl (Fig. 1B). s3f containing fractions were pooled and frozen in liquid nitrogen.

**Cell Culture.** HUVEC, BHK-21, and porcine aortic endothelial (PAE) cells were cultured as described previously (11, 29, 30). HUVECs were not used beyond passage 8. Np2-expressing PAE cells were described previously (11). To generate PAE cells coexpressing np2 and plexin-A1 or plexin-A1 alone, an expression vector (pcDNA3.1) containing a cDNA-encoding myc and 6xHis-tagged plexin-A1 (kindly provided by Dr. Strittmatter, Yale University) was transfected into PAE or PAE/np2 cells. Expressing clones were isolated using G418 selection (0.5 mg/ml). HEK293 cells were cultured in DMEM supplemented with 10% FCS, 2 mM glutamine, fungizone, and gentamicin. For cell proliferation assays, HUVECs (passages 4–8) were seeded in gelatin-coated wells of 48- or 24-well dishes at a concentration of  $10^4$  or  $3 \times 10^4$  cells/well, respectively. VEGF or bFGF and either s3f or a corresponding control fraction was added as indicated after the cells adhered. On day 2, the factors were readied, and adherent cells were counted on day 4 using a Coulter counter. The control fractions inconsistently (depending on the batch) produced some nonspecific inhibitory effects by themselves, but when observed, that inhibition was always much smaller than the effect of s3f.

**Cell Contraction.** Cells were incubated for 30 min at 37°C with 5  $\mu$ g/ml DiAsp in serum-free medium. The cells were washed with HBSS and incubated in CHO-S-SFM II medium containing s3f or s3f-1 or with a control-conditioned medium from empty vector-transfected cells for 1 h at 37°C, after which the medium was aspirated. Alternatively, the experiment was done using purified s3f and respective control fractions. The cells were fixed with 4% paraformaldehyde and photographed using an inverted fluorescent microscope. PhotoShop in conjunction with the scion image program were used to measure the circumference of 80 randomly selected cells from several representative fields from each experiment.

**ERK-1/2 Phosphorylation.** HUVECs were seeded at a concentration of  $5 \times 10^5$  cells in 6-well gelatinized dishes in growth medium containing 10% FCS. The cells were allowed to attach and incubated 16 h in a humidified incubator at 37°C. The experiment was initiated by the addition of increasing concentrations of s3f or corresponding volumes of a corresponding control

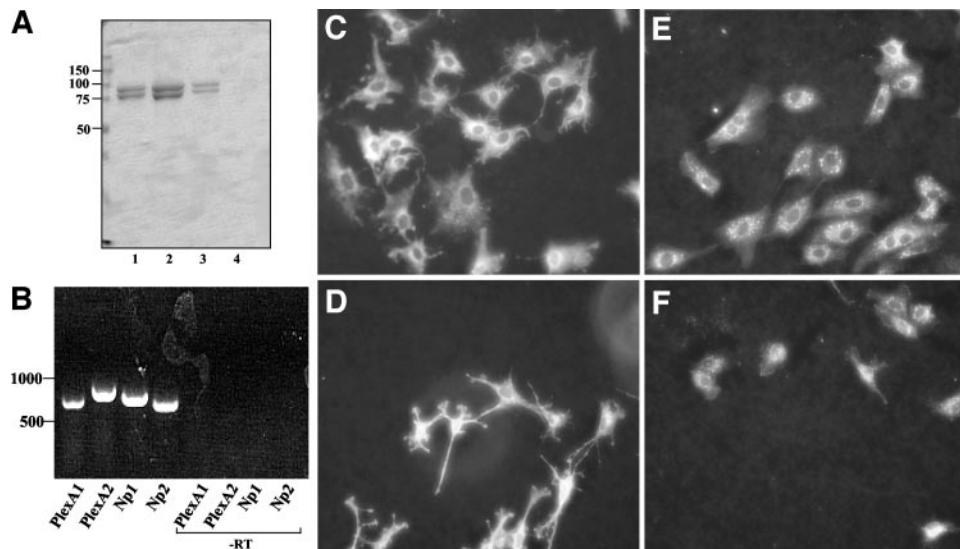


Fig. 1. Purification of recombinant semaphorin-3f (s3f) and its effect on the cell shape of endothelial cells. A, a Coomassie Blue-stained gel of three consecutive column fractions of purified S3f obtained from the last step of s3f purification (Lanes 1–3) and of a control column fraction corresponding to the s3f fraction shown in Lane 2, which was purified similarly from empty vector-transfected cells (Lane 4). B, expression of the mRNA species encoding Plexins A1 and A2, as well as np1 and np2 in HUVEC cells as determined by reverse-PCR. The primers used correspond to the following nucleotides: Np1: 5' 241–261, 3' 913–933; Np2: 5' 265–285, 3' 850–872; Plexin-A1: 5' 3699–3718, 3' 4338–4358; plexin-A2: 5' 1970–1991; 3' 2715–2735. Left four lanes, with reverse transcriptase. Right four lanes, controls without reverse transcriptase. In C and D, DiAsp-labeled PAE/np2/plexin-A1 cells were stimulated with s3f (D) or a control fraction derived from empty vector-transfected human embryonic kidney (HEK)293 cells (C). Cells were photographed after 1 h at 37°C using an inverted fluorescent microscope. E and F, DiAsp-labeled HUVECs were stimulated with s3f containing conditioned medium (F) or with control conditioned medium (E). Cells were photographed after 1 h at 37°C using an inverted fluorescent microscope.

fraction purified similarly from control cells transfected with empty expression vector. After 10 min at room temperature, bFGF (2 ng/ml) or VEGF<sub>165</sub> (10 ng/ml) was added or not added to dishes. The experiment was terminated after 10 more min by a wash with ice cold PBS. The cells were lysed with 0.03 ml of lysis buffer containing 50 mM HEPES (pH 7.4), 4 mM EDTA, 1% Triton X-100, 0.5 mg/ml Na<sub>3</sub>VO<sub>4</sub>, 4.5 mg/ml Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, and fresh protease inhibitors (0.2 mg/ml phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, and 2 μg/ml aprotinin). The cells were scraped off, and nonsoluble debris was removed by centrifugation at 4°C. Aliquots of supernatant containing 10 μg of protein were loaded on an SDS-PAGE gel, blotted onto a nitrocellulose filter, and probed with an antibody directed against phosphorylated ERK-1/2. The blot was then stripped and reprobed with an antibody directed against ERK-2 (total ERK). Quantification of band intensity was performed using a phosphorimager (Image Master, Amersham), and the ratio between phosphorylated ERK and total ERK was determined using the Tina program.

**Alginate Microencapsulation Angiogenesis Assay.** BHK-21 cells transfected with empty expression vector, BHK-21 cells expressing either s3f-l or s3f, or BHK-21 cells expressing VEGF<sub>165</sub> (30) were encapsulated in polylysine-coated microspheres composed of Ca<sup>2+</sup>-alginate as described previously (29, 31). To evaluate the concentration and viability of the encapsulated cells, capsules containing cells were incubated with 5 mg/ml 3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide for 5 h in 37°C, washed three times with 10 mM HEPES (pH 7.3), and incubated for 24 h in isopropanol containing 0.4% HCl at room temperature. Absorbance at 570 nm was then measured. After a week in culture, conditioned medium was assayed for recombinant s3f or s3f-l (using anti-myc epitope antibodies) or VEGF<sub>165</sub> (using anti-VEGF<sub>165</sub> antibodies; Ref. 32). Capsules containing VEGF<sub>165</sub>-producing cells were mixed with capsules containing s3f- or s3f-l-expressing cells or control cells and injected s.c. as described previously (29).

**Matrigel Plug Angiogenesis Assay.** Matrigel containing 80 ng/ml bFGF and either s3f (33 nM) or a corresponding volume from the corresponding control fraction was prepared and used for the Matrigel plug angiogenesis assays as described (33). Matrigel (0.6 ml/animal) was injected s.c. into female immune-deficient mice (BALB/c *nu/nu*). The animals were sacrificed, and the Matrigel plugs were removed and photographed after 7 days. Plugs were embedded in paraffin blocks and sectioned. Sections (5-μm thick) were reacted with an antibody directed against CD-31 as described (28). The sections were counterstained with hematoxylin, and CD-31-positive blood vessels that have invaded the Matrigel were visualized. The microvessel density of CD-31-positive vessels in fields of equal area was measured. Seven fields from three control and s3f-treated plugs were quantified. Microvessel density was measured using the hot spot method according to which the most vascularized fields in different samples are assessed for vascular density (34). The quantification of the blood vessel density was performed using the ImagePro Plus version 4.5 software (MediaCybernetics, Silver Spring, MD). This method was also used to determine the density of blood vessels in sections derived from tumors in a similar fashion. Vascular density was expressed as a percentage of area relative to the area of the microscopic field. All animal experiments were approved by the Technion ethics commission.

**Cross-Linking of <sup>125</sup>I-bFGF to Cells.** BHK-21 or PAE cells were grown to confluence in gelatin-coated, 5-cm dishes. The production of recombinant bFGF from bacteria-expressing recombinant bFGF, iodination of the bFGF, and the binding and cross-linking of 3 ng/ml <sup>125</sup>I-bFGF to cells was done as described previously (35, 36) in the presence or absence of 15 nM s3f.

## RESULTS

**S3F Induces a Weak Contraction of HUVEC.** Class-3 semaphorins repulse axonal growth cones by inducing a localized collapse of the actin cytoskeleton (37). This effect depends on the simultaneous presence of neuropilins and plexins on the cell surface. We have produced recombinant s3f-l and s3f (4) in HEK293 cells and added to both s3f forms myc and 6xHis epitope tags at their COOH terminals. s3f was purified from serum-free conditioned medium by wheat-germ agglutinin affinity chromatography, followed by dialysis and ion exchange chromatography (Fig. 1A). PAE cells expressing recombinant plexin-A1 and np2 contract strongly and rapidly in response to s3f (compare Fig. 1, C with D). In contrast, PAE cells expressing only

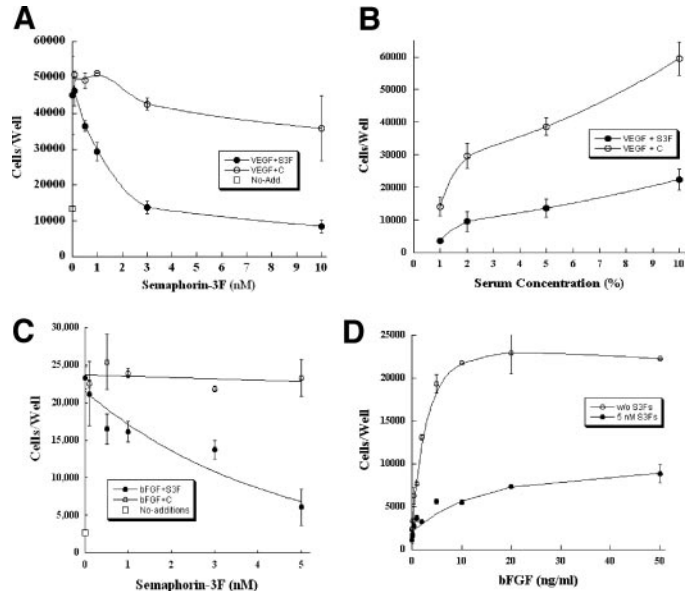


Fig. 2. The effect of s3f on vascular endothelial growth factor (VEGF)<sub>165</sub> and basic fibroblast growth factor (bFGF)-induced survival and proliferation of HUVECs. In A, HUVECs were seeded in gelatinized 24-well dishes ( $4 \times 10^4$ /well) in medium containing 1% FCS. VEGF<sub>165</sub> (10 ng/ml) and increasing concentrations of either s3f (s3f) or equal volumes of a control fraction purified similarly from empty vector-transfected cells (c) were added. These factors were added again after 2 days. After 4 days, the cells were washed to remove dead cells, trypsinized, and counted in a Coulter cell counter. Each point represents the average of three wells. Error bars represent the SD from the mean. In B, HUVECs were seeded in triplicates in gelatinized 24-well dishes ( $3 \times 10^4$ /well) in medium containing increasing concentrations of FCS. VEGF<sub>165</sub> (10 ng/ml) and either s3f (5 nM; s3f) or equal volumes of a corresponding control fraction (c) were added as described in A. Cells were counted after 4 days as described in A. Each point represents the average of three wells. Error bars represent the SD from the mean. In C, HUVECs were seeded in gelatinized 48-well dishes ( $1 \times 10^4$ /well) in medium containing 10% FCS, bFGF (5 ng/ml), and increasing concentrations of s3f (s3f) or of a corresponding control fraction (c). These factors were added twice as described in A. After 4 days, adherent cells were counted as described in A. Each point represents the average of triplicate wells, and error bars represent the SD from the mean. In D, HUVECs were seeded in gelatinized 48-well dishes ( $1 \times 10^4$ /well) in medium containing 10% FCS in the presence or absence of 5 nM s3f. Increasing concentrations of bFGF were added to wells as indicated. s3f and bFGF were added twice as described in A and counted after 4 days. Each point represents the average of three wells. Error bars represent the SD from the mean.

np2 or parental PAE cells did not contract in response to s3f (data not shown). HUVECs express np1, np2, plexin-A1, and plexin-A2 as shown by reverse PCR (Fig. 1B). However, the contraction of HUVEC in response to s3f is relatively weak (compare Fig. 1, E with F), although measurements of cell circumference using the scion image program indicate that this small contraction is statistically significant as determined using two-tailed unequal Student's *t* test ( $P < 0.01$ ; data not shown).

**S3f Inhibits VEGF<sub>165</sub> and bFGF-Induced Survival and Proliferation of HUVECs.** To find out if s3f affects the survival of HUVECs, we cultured the cells in medium containing 1% serum. Under these conditions, VEGF<sub>165</sub> acts as a survival factor. The addition of  $\leq 10$  nM s3f (4) to the medium inhibited significantly the survival-inducing effect of VEGF<sub>165</sub> (Fig. 2A). Similar results were obtained using human saphenous vein-derived endothelial cells (data not shown). Inactivation of s3f at 80°C for 5 min abolished the inhibitory effect of s3f (data not shown). The inhibitory effect of s3f was also evident in the presence of serum because s3f also inhibited strongly VEGF<sub>165</sub>-induced proliferation in  $\leq 10\%$  serum (Fig. 2B). The inhibition was specific to s3f because corresponding column fractions purified similarly from conditioned medium of HEK-293 cells transfected with empty expression vector (control fractions) failed to inhibit the effects of VEGF<sub>165</sub> (Fig. 2, A and B, controls).

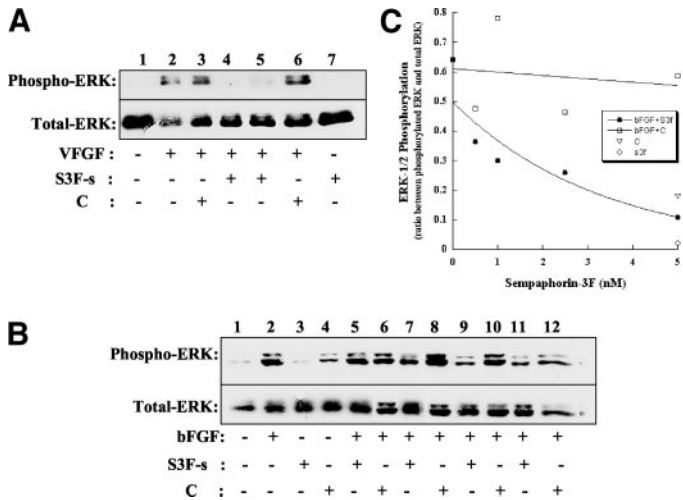


Fig. 3. The effect of s3f on VEGF and bFGF-induced phosphorylation of extracellular signal-regulated kinase (ERK)-1/2. In A, VEGF<sub>165</sub> (10 ng/ml)-induced phosphorylation of ERK-1/2 (phospho-ERK) in HUVEC cells was measured in the absence or presence of 5 nM s3f (S3F) or corresponding volumes of control fractions purified similarly from control cells (c) using antibodies directed against phosphorylated ERK-1/2 (phospho-ERK) as described in "Materials and Methods." The amounts of total ERK-1/2 were assessed using an antibody directed against ERK-2 (Total-ERK) after blot stripping as described in "Materials and Methods." In B, bFGF (2 ng/ml)-induced phosphorylation of ERK-1/2 (Phospho-ERK) in HUVECs was measured in the absence (Lane 2) or presence of 0, 0.5, 1, 2.5, and 5 nM s3f (Lanes 3, 4, 5, 7, 9, and 11, respectively) or in the presence of increasing volumes of a control fraction (c) (Lanes 6, 8, 10, and 12, respectively) as described in A. No stimulation was observed with 5 nM s3f alone (Lane 3), but some stimulation was seen with the corresponding volume of the control (Lane 4). The blot was subsequently stripped, and the content of total ERK-1/2 (Total-ERK) in each of the lanes was determined as described in "Materials and Methods." C, quantification of the phosphorylation experiment shown in B. The ratio between phosphorylated ERK-1/2 and the total amount of ERK-1/2 in samples was quantified as described in "Materials and Methods."

It was suggested that s3a inhibits angiogenesis in *in vitro* models by interfering with the binding of VEGF<sub>165</sub> to np1 (21). We have shown previously that VEGF<sub>165</sub> does not compete with s3f for binding to np2 receptors (12), suggesting that s3f inhibits the effects of VEGF<sub>165</sub> by a mechanism that requires active s3f-mediated signaling. However, s3f also binds to np1, although s3f does not induce the collapse of growth cones in np1-expressing axons (8). Nevertheless, s3f may perhaps inhibit, at least in part, the effect of VEGF<sub>165</sub> by inhibiting the binding of VEGF<sub>165</sub> to np1. To exclude this possibility, we examined the effects of s3f on bFGF-induced proliferation of endothelial cells. bFGF is a potent angiogenic factor (38). We have found, using <sup>125</sup>I-bFGF binding and cross-linking experiments, that it does not bind to np1 or np2 receptors overexpressed in PAE cells (data not shown). Furthermore, s3f was unable to compete with <sup>125</sup>I-bFGF for binding to FGF receptors of PAE or BHK-21 cells as determined by binding/cross-linking experiments (data not shown). Despite these observations, s3f inhibited the bFGF-induced proliferation of HUVECs (Fig. 2C), indicating that at least part of the inhibitory effect of s3f is generated by a mechanism that does not rely on the inhibition of the interaction of proangiogenic factors such as bFGF with bFGF receptors. Half maximal inhibition of VEGF<sub>165</sub> activity was observed at ~1.5 nM s3f (Fig. 2A), whereas half maximal inhibition of bFGF activity was observed at ~2 nM s3f (Fig. 2C). These measurements were obtained in the presence of saturating concentrations of VEGF<sub>165</sub> and bFGF and thus do not represent accurate IC<sub>50</sub> measurements. However, they do show that the range of s3f concentrations required for the inhibition of VEGF and bFGF-induced cell proliferation is similar. Increasing the concentration of bFGF ≤50 ng/ml did not reverse significantly the inhibitory effect produced by 5 nM s3f, although a minor reversal of ~10% could be seen (Fig. 2D).

**S3f Inhibits VEGF and bFGF-Induced Phosphorylation of ERK-1/2.** The ERK-1 and ERK-2 mitogen-activated protein kinases are positioned near the end of the ras signaling cascade and phosphorylated in response to VEGF and bFGF in HUVECs (39, 40). Purified s3f, but not control fractions, inhibited the VEGF<sub>165</sub>-induced phosphorylation of ERK-1/2 (compare Fig. 3A, Lanes 3 with 4). Similar inhibition was seen when the effects of increasing concentrations of s3f on bFGF-induced activation of ERK-1/2 were compared with the effects of correspondingly increasing volumes of a control fraction, although the inhibition was not as complete as inhibition of VEGF activity (Fig. 3B). The ratio between phosphorylated ERK-1/2 and the amount of total ERK was quantified for each of the points in this experiment, and the results are shown in Fig. 3C. The inhibition experiments were repeated six times with similar results, and when the inhibition was analyzed statistically (at 5 nM s3f), it was found to be highly significant ( $P < 0.01$  using Student's *t* test). Because the induction of ERK-1/2 phosphorylation and inhibition occur within 10 min, these results indicate that inhibition of receptor up-regulation is probably not part of the mechanism by which s3f inhibits the activities of VEGF and bFGF.

**The Mitogenic Effects of bFGF Are Not Mediated by VEGF.** It was suggested that the proangiogenic effects of bFGF might be mediated in part by VEGF produced in response to bFGF (41). If that possibility is correct, then s3f could perhaps inhibit the activity of bFGF by competing with VEGF produced in response to bFGF for binding to neuropilins. To exclude this possibility, we determined if bFGF-induced proliferation of HUVECs is inhibited by soluble-flt (sflt), a natural inhibitor of VEGF (42). Our results indicate that sflt does not affect bFGF-induced proliferation of HUVECs. However, s3f inhibited the effects of bFGF even in the presence of sflt (Fig. 4), indicating that the mitogenic activity of bFGF is not mediated indirectly by VEGF and that s3f does not inhibit the mitogenic effects of bFGF by using a mechanism that involves competition for shared receptors.

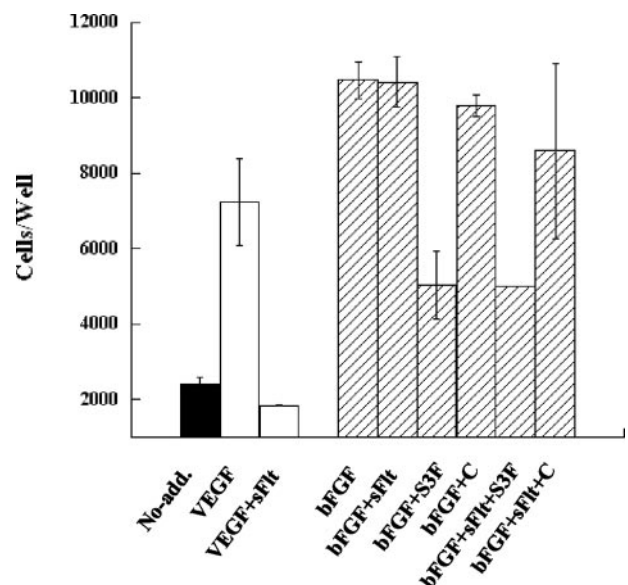


Fig. 4. The mitogenic effects of bFGF are not mediated by VEGF. HUVECs were seeded at  $10^4$  cells/well in 48-well dishes. The cells were cultured in the presence of 10% FCS and combinations of s3f (5 nM), a control fraction (c), VEGF<sub>165</sub> (10 ng/ml), bFGF (5 ng/ml), and sflt (1  $\mu$ g/ml) as indicated. All factors were added after cell adherence, and 2 days later, another addition of the factors was made. Adherent cells were counted using a Coulter counter after 4 days. The experiment was run in triplicate, and the error bars represent the SD from the mean. The experiment was performed twice with similar results.

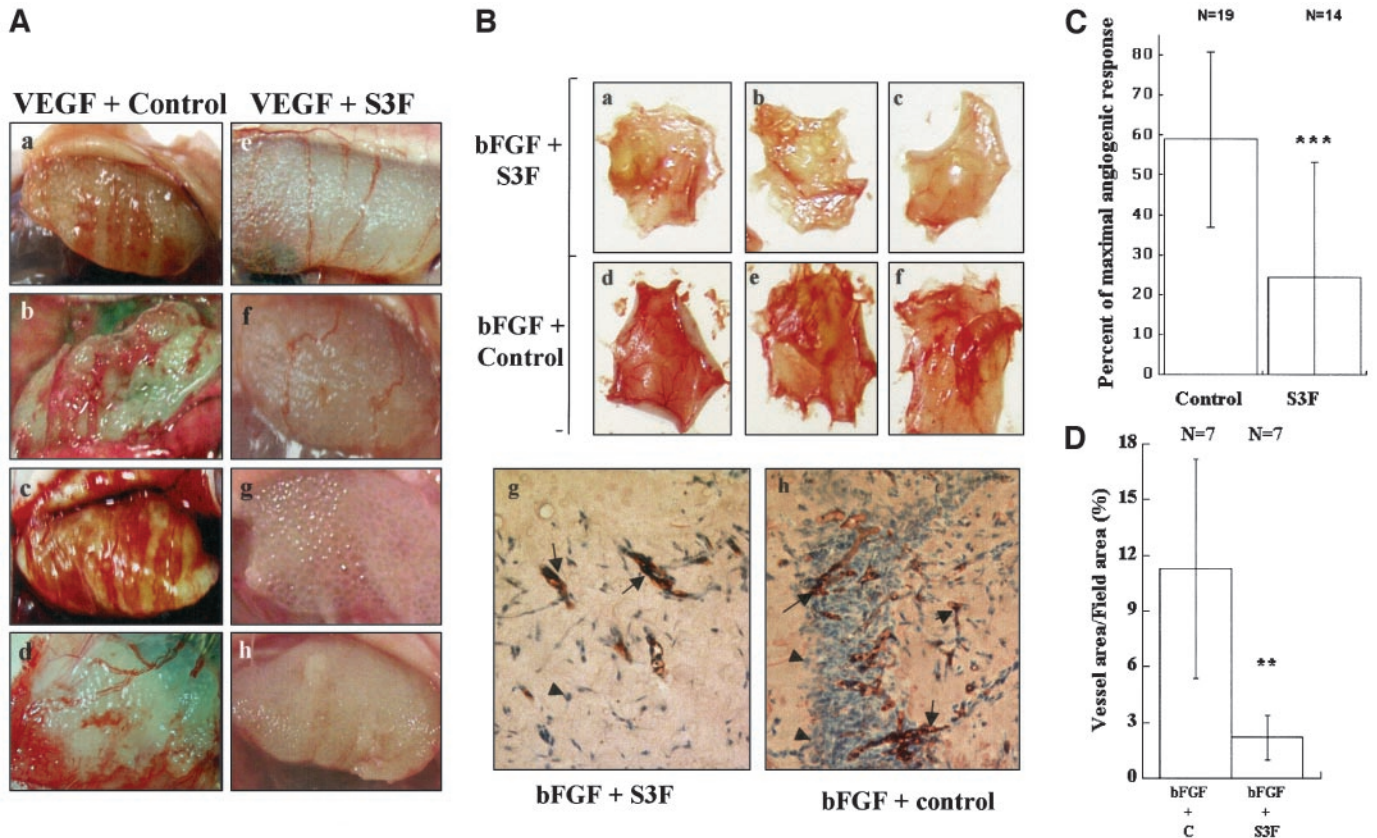


Fig. 5. Inhibition of angiogenesis by s3f. In A, athymic nude mice were injected with mixtures of alginate capsules containing BHK-21 cells expressing VEGF<sub>165</sub> (10<sup>5</sup> cells/mouse) and either BHK-21 cells transfected with empty expression vector (10<sup>6</sup> cells/mouse; a–d) or BHK-21 cells expressing the s3f-1 (10<sup>6</sup> cells/mouse; e–h). After a week, the animals were anesthetized, and the clusters of alginate capsules were photographed. In B, Matrigel plugs (0.6 ml) containing bFGF (80 ng/ml) and s3f (33 nM; a–c) or bFGF and a corresponding volume of a control fraction (c; d–f) were implanted s.c. in mice. Shown are photographs of representative Matrigel plugs excised after 7 days. Histological sections prepared from Matrigel plugs were stained with an antibody directed against CD-31. Representative sections prepared from plugs containing bFGF and s3f (g) or bFGF plus a corresponding volume of a control fraction (h) are shown (magnification: ×100). In C, pictures of clusters of the alginate capsules were given to five independent observers to evaluate blindly. The strongest response was arbitrarily given a 100% score, and the others were given a score in comparison to it. The score for each cluster of capsules was averaged. The means of these averages within each group are shown in the histogram. Error bars represent the SD from the mean. The two groups were compared using two-tailed unequal Student's *t* test (\*\*\**P* < 0.001). In D, blood vessels labeled with an antibody directed against the endothelial cell marker CD-31 were counted in fields of equal area in sections of Matrigel plugs containing either bFGF and s3f or bFGF and a corresponding volume of a control fraction (see description in B). The number of counted fields corresponds to *N*. The error bars represent the SD from the mean. The two groups were compared using two-tailed unequal Student's *t* test (\*\**P* < 0.01).

### S3f Inhibits VEGF<sub>165</sub> and bFGF-Induced Angiogenesis *in Vivo*.

Inhibitors of endothelial cell proliferation can usually function as inhibitors of angiogenesis. To find out if s3f inhibits angiogenesis, we used the alginate cell encapsulation system (31, 43, 44). BHK-21 cells expressing recombinant VEGF<sub>165</sub> (30) were encapsulated in polylysine-coated alginate capsules (29, 31). We chose to use BHK-21 cells because these cells produce a particularly quiet background (44). The encapsulated cells were mixed with similar capsules containing either BHK-21 cells transfected with an empty expression vector (control) or with capsules containing s3f- or s3f-1-producing BHK-21 cells. Mixtures of capsules were injected s.c. into nude mice. The concentrations of s3f required to inhibit completely the effects of VEGF<sub>165</sub> *in vitro* are higher than the VEGF concentrations required to induce a maximal mitogenic response. In addition, the secretion of s3f-1 from capsules containing s3f-1-expressing cells was less efficient than the secretion of VEGF<sub>165</sub>. We have therefore used a 10-fold higher number of s3f-1-expressing cells as compared with the number of VEGF<sub>165</sub>-producing cells. When capsules containing VEGF<sub>165</sub>-expressing cells were mixed with capsules containing control cells, there was no inhibition of VEGF<sub>165</sub>-induced angiogenesis (Fig. 5A, a–d). However, inclusion of capsules containing cells producing s3f-1 inhibited VEGF<sub>165</sub>-induced angiogenesis (Fig. 5A, e–h). Similar results were obtained in an experiment in which BHK-21 cells expressing s3f were used (data not shown). Implantation of capsules contain-

ing s3f-expressing cells or parental BHK-21 cells on their own did not induce angiogenesis (data not shown).

To control for variation, and because the angiogenic effects induced by VEGF<sub>165</sub> in these experiments were submaximal, we evaluated the results of three independent experiments visually using a double blind evaluation procedure. Pictures of clusters of capsules were taken before their removal from animals (to clearly visualize blood vessels). The pictures were ordered randomly and evaluated blindly by five independent uninvolved observers. The results were analyzed using two-tailed unequal Student's *t* test. The inhibitory effect of s3f was found to be highly significant by these criteria (*P* < 0.001; Fig. 5C). The s3f-induced inhibition of angiogenesis was sensitive to the ratio of the VEGF<sub>165</sub> and s3f-producing cells in the alginate bead mixtures. When the number of capsules containing VEGF<sub>165</sub>-producing cells was increased 5-fold without a corresponding change in the number of the s3f-expressing cells, we could no longer see such efficient inhibition of angiogenesis, although even under these conditions, a small effect was evident (data not shown).

To determine whether s3f is also able to inhibit the angiogenic activity of bFGF, we used the Matrigel plug angiogenesis assay (33). bFGF is not secreted efficiently from producing cells because it lacks a signal sequence for secretion, rendering the alginate cell encapsulation system unsuitable for the determination of bFGF-induced angiogenesis. It can be seen that s3f inhibited efficiently bFGF-induced

growth of blood vessels into the Matrigel plugs, indicating that s3f can inhibit bFGF-induced angiogenesis, as well as VEGF<sub>165</sub>-induced angiogenesis (Fig. 5B, a–c). In contrast, control fractions purified by an identical procedure from conditioned medium of empty vector-transfected HEK-293 cells did not inhibit bFGF-induced angiogenesis (Fig. 5B, a–c). This conclusion was verified by determining blood vessel densities in CD-31-labeled histological sections derived from control and s3f-containing Matrigel plugs, such as those shown (Fig. 5B, g and h, black arrows). Using the hot spot method (34), we have found that bFGF and control-containing plugs had a 6-fold higher blood vessel density as compared with plugs containing bFGF and s3f (Fig. 5D). It should be noted that bFGF stimulated the migration of additional cell types into the Matrigel plugs (note the density of cells that do not express CD-31 in Fig. 5h) and that s3f also inhibited partially the migration of nonendothelial cell types. However, we have not determined which other cell types were inhibited by s3f.

**Expression of s3f in HEK-293 Cells Inhibits Tumor Formation but not Cell Proliferation.** s3f was identified as a suppressor of small cell lung carcinoma (3, 5). HEK293 cells transformed by adenovirus 5 (45) form slowly developing s.c. tumors in nude mice with an incidence of ~50% (46). We have expressed in HEK293 cells the two s3f splice forms (4). The proliferation rate of cells expressing s3f-1 (data not shown) or s3f was compared with the rate of proliferation of empty vector-transfected cells. The rate of proliferation of the s3f (Fig. 6A) or s3f-1-expressing cells (data not shown) did not differ significantly from the proliferation rate of control cells, indicating that s3f does not inhibit the proliferation of these cells. It is possible that the concentrations of s3f do not build up quickly in cell culture because the initial number of the cells in such experiments is low. We have also conducted similar experiments in which we have added to the control and s3f-producing cells exogenous s3f. However, exogenous s3f did not significantly affect the proliferation rate of the control or s3f-expressing cells (Fig. 6B).

If s3f is able to inhibit angiogenesis, then s3f should be able to inhibit the development of tumors, even in cases in which s3f fails to affect the proliferation of the tumor cells. Cells expressing s3f or s3f-1 or control cells transfected with empty expression vector were therefore injected s.c. into nude mice. About 50% of the mice injected with empty expression vector-transfected cells developed tumors after 3–4 weeks. However, not even 1 of the 17 mice injected with cells expressing s3f, and only 3 of 16 mice that received cells expressing s3f-1, developed tumors (Fig. 6C). Furthermore, the three tumors that developed from cells expressing s3f-1 developed at a much slower rate as compared with tumors derived from empty vector-transfected cells (the average weight of tumors after 7 weeks was 0.3 gram, whereas the average weight of control tumors was 2 grams). These observations suggest that s3f inhibits the development of HEK293-derived tumors by inhibiting tumor angiogenesis.

These impressions were confirmed when the density of blood vessels was compared between the control tumors and the three tumors that did develop from the s3f-1-expressing HEK293 cells. The density of the blood vessels in the tumors that developed from the s3f-expressing cells was indeed significantly lower than the density of blood vessels in control tumors (Fig. 6D), indicating that s3f can function as an inhibitor of tumor angiogenesis.

## DISCUSSION

We have shown here that the two s3f splice forms inhibit the mitogenic and survival-promoting activities of VEGF<sub>165</sub> using two types of human endothelial cells. Interestingly, s3f inhibited in addition bFGF-induced proliferation of endothelial cells. Because in our hands bFGF is not able to bind to neuropilins, and because s3f does

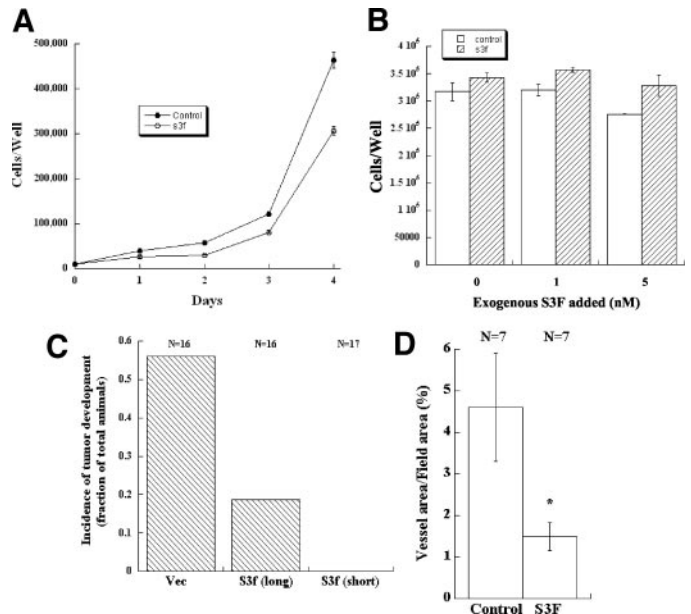


Fig. 6. The development of tumors from HEK293 cells is inhibited by s3f, although their proliferation in cell culture is not. In A, HEK293 cells transfected with empty expression vector (*Control*) or HEK293 cells expressing recombinant s3f were seeded in 24-well dishes ( $10^4$  cells/well). Cells were trypsinized and counted in a Coulter counter every day. Each point represents the mean cell number of two wells, and the error bars represent the deviation from the mean. In B, HEK293 cells transfected with empty expression vector (*Control*, empty columns) or HEK293 cells expressing recombinant s3f (*s3f*, hatched columns) were seeded in 24-well dishes ( $10^4$  cells/well). s3f was added to the final concentrations indicated, and another addition was made on day 3. Cells were counted after 4 days in a Coulter counter. Each point represents the mean cell number of three wells, and error bars represent the SD from the mean. In C, HEK293 cells transfected with empty expression vector (*Vec*) or HEK293 cells expressing recombinant s3f or s3f-1 were suspended in  $200 \mu\text{l}$  of saline and injected s.c. ( $10^7$  cells/mouse) into the lower back of 3–5-week-old BALB/c *nu/nu* female mice. Tumor growth was monitored every 3 days for 7 weeks. The number of mice in each group is indicated, and the fraction of mice that developed tumors in each of the groups after 7 weeks is shown. In D, the density of blood vessels stained with an antibody directed against CD-31 was measured in histological sections derived from tumors derived from empty vector-transfected HEK-293 cells or from s3f-1-expressing HEK293 cells. The analysis was done as described in "Materials and Methods." Error bars represent the SE. Comparison of the blood vessel density between the two groups was done using the Mann-Whitney *U* test. Two-tailed *P*s < 0.05 were taken to be statistically significant. (\**P* < 0.05).

not seem to inhibit the interaction of bFGF with FGF receptors, these results imply that the inhibitory effect of s3f is not the result of competition with bFGF for binding to FGF tyrosine-kinase receptors or neuropilins. In the case of VEGF<sub>165</sub>, s3f may also function, at least in part, by a mechanism that does not require competition of s3f and VEGF<sub>165</sub> for binding to shared receptors, such as neuropilins, although we cannot exclude that in the case of VEGF, part of the effect of s3f may be caused by competition with VEGF for binding to neuropilins.

It was reported that the angiogenic activity of bFGF is mediated in part by an up-regulation of VEGF (41) and, furthermore, that bFGF can enhance the synthesis of VEGFR-2 receptors in endothelial cells (47). It is thus possible that s3f could inhibit the activity of bFGF, at least partially, by inhibiting the interaction of VEGF produced in response to bFGF with a receptor such as np1, although the affinity of np1 toward s3f is significantly lower than the affinity of the np2 receptor (8). However, the involvement of such a VEGF/VEGFR-dependent mechanism is ruled out because sflt, a potent inhibitor of VEGF function (48), does not inhibit bFGF-induced proliferation of HUVEC and because s3f can inhibit the mitogenic effects of bFGF, even in the presence of sflt. Therefore, it is unlikely that VEGF produced in response to bFGF plays a role in the mitogenic response to bFGF *in vitro*. In addition, we have shown that s3f inhibits bFGF and VEGF-induced phosphorylation of ERK-1/2 in HUVECs. Be-

cause the phosphorylation of ERK-1/2 in response to growth factors is a very rapid response, and up-regulation of receptors, as well as synthesis of a growth factor such as VEGF, is a much slower process, it follows that the mechanism by which s3f inhibits the activities of bFGF and VEGF in HUVECs is not likely to depend on the up-regulation of VEGF expression.

The evidence gathered in this set of experiments therefore suggests that s3f inhibits bFGF-induced proliferation of HUVECs by using a mechanism that does not rely on the inhibition of the binding of bFGF to its receptors. This mechanism probably uses instead a mechanism that requires active signal transduction, most likely via the np2 and type A plexin receptors. Np1 receptors, although able to bind s3f with low affinity, do not transduce s3f signals (6, 8) and are unlikely to transduce antiproliferative s3f signals, although this will need to be verified experimentally in the future. These findings imply that s3f could function *in vivo* as a localized inhibitor of angiogenesis that may be able to inhibit the activity of a broad range of angiogenesis-inducing factors.

Proteins that inhibit the proliferation of endothelial cells *in vitro* are expected to inhibit angiogenesis *in vivo*. Indeed, by using the alginate microencapsulation and Matrigel plug angiogenesis assays, we have shown that the two splice forms of s3f inhibit significantly VEGF<sub>165</sub>, as well as bFGF-induced angiogenesis. It follows that part of the antitumorigenic properties of s3f (5) may be attributable to its anti-angiogenic effects. Most tumor cells express low levels of np1 or np2 mRNA, and it is therefore difficult to determine whether the s3f inhibition of tumor growth is the result of a direct effect on the proliferation of the tumor cells or the result of an antiangiogenic effect. Our experiments with the HEK293 cells indicate that s3f can inhibit tumor growth *in vivo*, although s3f is not able to inhibit the proliferation of the HEK293 cells in cell culture. In conjunction with the previous experiments, this experiment argues strongly that s3f can inhibit tumor development by inhibiting tumor angiogenesis. Furthermore, the few tumors that did develop from cells expressing s3f developed much slower and had a significantly lower density of blood vessels, indicating that impairment of angiogenesis contributed significantly to the retardation of their growth. However, there are tumorigenic cell types, such as the GLC45 small cell lung carcinoma cells that were reported to form tumors efficiently in nude mice, even in the presence of s3f (5). Because tumor development depends on the induction of tumor angiogenesis (49), why then is tumor development not inhibited by s3f in the case of the GLC45 cells? The answer may perhaps reside in the balance between inducers and inhibitors of angiogenesis. Our experiments indicate that the *in vivo* inhibitory effects of s3f can be partially overcome by increasing the ratio between VEGF<sub>165</sub> and s3f-producing cells in favor of VEGF<sub>165</sub>-producing cells. It is thus possible that GLC45 cells produce high concentrations of angiogenic factors, thereby overcoming the antiangiogenic effects of s3f.

It is not known whether s3f plays a role in the regulation of physiological angiogenesis. Mice lacking functional *np2* genes develop almost normally as far as the cardiovascular system is concerned, and excessive growth of blood vessels or abnormal organization of the vascular system was not noted (18, 50, 51). Likewise, mice deficient in the expression of s3f do not display gross abnormalities in their cardiovascular system (26). However, it is possible that s3f plays a role in the regulation of localized angiogenesis and especially in pathological angiogenesis. Similar observations were recently reported in relation to the angiogenic factor placenta growth factor, which has only a minimal role, if any, in developmental angiogenesis but plays a major role in pathological angiogenesis (52).

The mechanism by which s3f inhibits angiogenesis is still unclear. It is most likely that the effects are the result of an interaction with np2

receptors, which had been convincingly demonstrated to transduce s3f signals. However, it is possible that not all of the effects of s3f are mediated by np2. Although thus far signaling of s3f via np1 receptors could not be detected (6, 8), it is possible that the relatively weak interaction of s3f with np1 can nevertheless activate alternative signaling pathways. These questions will need to be addressed in the future so as to better understand the mechanisms by which s3f modulates angiogenesis and to find out whether s3f functions as a physiological modulator of angiogenesis.

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