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Semaphorin-3A and Semaphorin-3F Work Together to Repel Endothelial Cells and to Inhibit Their Survival by Induction of Apoptosis*

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Semaphorin-3A (sema3A) is a neuropilin-1 (np1) agonist. It inhibits the binding of the 165-amino acid form of VEGF ($VEGF_{165}$) to np1 and was reported to inhibit angiogenesis as a result. However, we find that sema3A concentrations that inhibit the mitogenic effects of $VEGF_{165}$ do not inhibit $VEGF_{165}$-induced phosphorylation of VEGF receptor-2 (VEGFR-2). Furthermore, sema3A inhibits the biological effects of $VEGF_{121}$, a VEGF form that does not bind to neuropilins and basic fibroblast growth factor, a growth factor whose activity, unlike that of VEGF, is not inhibited by small interfering RNA directed against np1. Therefore, the mechanism by which sema3A inhibits $VEGF_{165}$ activity does not depend on competition with $VEGF_{165}$ for binding to np1. Sema3A induced rapid disappearance of focal contacts followed by collapse of the actin cytoskeleton in human umbilical vein-derived endothelial cells. HEK293 cells expressing sema3A repel human endothelial cells and at high concentrations induce their death by apoptosis. Furthermore, sema3A inhibited the formation of tubes from endothelial cells in an *in vitro* angiogenesis assay. Similar effects are induced by the neuropilin-2 (np2) agonist sema3F. These inhibitory effects are abrogated by small interfering RNAs directed against np1 or np2, respectively. The anti-proliferative effects of sema3A and sema3F are additive when the semaphorins are added as pure proteins. However, when sema3A and sema3F were co-expressed in HEK293 cells their pro-apoptotic and cell repellant activities appeared to be synergistic. These observations suggest that combinations of sema3A and sema3F may be able to inhibit tumor angiogenesis more effectively than single semaphorins.

Semaphorins are axon guidance factors that induce localized collapse of neuronal growth cones (1). They are characterized by the presence of a sema domain located close to their N termini. The sema domain is essential for semaphorin signaling and determines the specificity of binding (2). Class 3 semaphorins are the only secreted vertebrate semaphorins and are distinguished in addition by the presence of a basic domain at their C termini. Most of the class 3 semaphorins bind to one or to both of the receptors belonging to the neuropilin family. The class 3 semaphorin sema3A[3] binds to np1 (3, 4), whereas the related sema3F binds to np2. The receptors of the plexin family play an important role in class 3 semaphorin signaling. Because the intracellular domain of the neuropilins is too short to enable signal transduction, neuropilins associate with plexins that serve as the signal transducing elements in neuropilin/plexin holoreceptors. Four type-A plexins as well as plexin-D1 have been found to associate with neuropilins (5–7).

In addition to their indispensable role in the shaping of the nervous system the neuropilins were also found to play important roles in developmental angiogenesis as revealed by gene targeting experiments that revealed major vascular defects in mice that lack either np1 or both np1 and np2 (8, 9). It was found that the neuropilins also function as receptors for heparin binding forms of the angiogenic factor VEGF and are expressed in endothelial cells (10–12). Experiments in which the native np1 receptor of mice was replaced by a np1 variant that binds VEGF but not sema3A indicate that the cardiovascular abnormalities observed in mice lacking functional np1 receptors are probably caused by impaired VEGF signal transduction rather than impaired sema3A signaling (13, 14).

These observations do not mean that sema3A and sema3F cannot affect angiogenesis. Sema3A inhibits $VEGF_{165}$-induced endothelial cell proliferation and migration. Sema3A inhibits the binding of $VEGF_{165}$ to np1 and it is assumed that this is the cause of the inhibitory effects. Furthermore, sema3A inhibited sprouting in the rat aortic ring *in vitro* angiogenesis assay (15). Further studies revealed that $VEGF_{165}$ can in turn inhibit sema3A-induced cell contraction (16), that implantation of sema3A releasing beads can inhibit developmental angiogenesis in developing limbs of chick embryos (17), and that sema3A release from endothelial cells regulates branching of blood ves-

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[3] The abbreviations used are: sema3A, semaphorin-3A; HUVEC, human umbilical vein-derived endothelial cells; np1, neuropilin-1; np2, neuropilin-2; sema3F, semaphorin-3F; siRNA, small inhibitory RNA; VEGF, vascular endothelial growth factor; $VEGF_{165}$, 165-amino acid long form of VEGF (other VEGF forms are labeled similarly); VEGFR-2, VEGF receptor-2; ERK, extracellular signal-regulated kinase; bFGF, basic fibroblast growth factor; HEK, human embryonic kidney; FCS, fetal calf serum; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorter.

sels in the developing chick embryo brain (18). However, it is not yet known whether sema3A can affect tumor development and tumor angiogenesis. In contrast, the gene encoding the np2 agonist sema3F was initially characterized as a tumor suppressor that is lost in small cells lung carcinoma (19–21). There is evidence indicating that sema3F is an inhibitor of angiogenesis (22, 23). The anti-angiogenic effects of sema3F do not seem to depend upon competition with $VEGF_{165}$ for binding to np2 because sema3F seems to initiate np2-dependent signaling that results in inhibition of VEGF-induced ERK1/2 phosphorylation and cell proliferation (22). Additional evidence indicates that sema3F also affects the behavior of some tumor cells directly, by inhibiting their migration and attachment (23–25).

We report that sema3A inhibits the proliferation and induces apoptosis of endothelial cells. It does that using a mechanism that does not require competition with $VEGF_{165}$ for a shared receptor because it is also able to inhibit the survival promoting effects of $VEGF_{121}$, a VEGF form that does not bind to neuropilins. We show that the effects of sema3A and sema3F are synergistic at low concentrations. We also show that sema3A inhibits the spontaneous organization of endothelial cells into tube-like structures in an *in vitro* angiogenesis assay.

EXPERIMENTAL PROCEDURES

Materials—Chemicals were from Sigma unless otherwise indicated. The silver stain kit was from ICN. Mediums and sera for cell culture were from Biological Industries Inc. (Kibbutz Beth-Haemek, Israel). Lipofectamine and Oligofectamine were from Invitrogen. Antibodies directed against c-myc, VEGFR-2, phosphorylated Y-951 of VEGFR-2, phosphorylated ERK1/2, and ERK2 (total ERK) were purchased from Santa Cruz Biotechnology Inc. (San Diego, CA). Antibodies directed against phosphorylated Y-1175 of VEGFR-2 were purchased from Cell Signaling. Antibodies against β-actin, FLAG, anti-FLAG M2 affinity resin, and AP-conjugated agarose beads were purchased from Sigma. Antibodies against active caspase-3 were purchased from MBL (Woburn, MA). Antibodies directed against human placental alkaline phosphatase were purchased from DAKO (Glostrup, Denmark). Antibodies directed against human semaphorin-3A were purchased from R&D Systems (Minneapolis, MN). The AP-sema3A encoding cDNA was kindly given by Dr. Tessiere-Lavinge (Genentech, South San Francisco, CA). The fluorescent vital dye DiI was purchased from Molecular Probes (Eugene, OR). Cy2-conjugated donkey anti-mouse antibodies were from Jackson ImmunoResearch. Antibodies directed against vinculin were from Sigma. Alexa-conjugated phalloidin was from Molecular Probes. bFGF was produced as previously described (26).

Cells—HUVEC and HEK293 cells expressing sema3A, sema3F, or empty vector were cultured as previously described (22). Radial artery and saphenous vein-derived endothelial cells were a kind gift from Dr. Flugelman (Lady Davis Carmel Medical Center, Haifa). Human radial artery and sapheneous vein-derived endothelial cells were cultured as previously described for HUVEC cells (10). Endothelial cells were not used beyond passage 8. HEK293, which express sema3A, were generated by transfecting cells with the sema3A-FLAG/pcDNA3.1/Hygro plasmid. To simultaneously express sema3A and sema3F, sema3A expressing HEK293 cells were transfected with the sema3F-myc/pcDNA3.1/neo plasmid (22). Clones expressing sema3F at similar levels to those found in the parental sema3F expressing HEK293 cells were selected using G418 (0.5 mg/ml), followed by isolation of clones and Western blot screens for characterization of sema3A and sema3F expression levels.

Production and Purification of Sema3A—The sema3A cDNA was ligated in-frame to a FLAG epitope tag inserted in-frame before the stop codon. The cDNA was ligated into the pcDNA3.1/hygro plasmid to generate the sema3A-FLAG/pcDNA3.1/hygro expression plasmid. HEK293 cells were transfected using Lipofectamine and stable sema3A-FLAG expressing clones were isolated using hygromycin (0.3 mg/ml). HEK293 cells transfected with the sema3A expression vector or with empty expression vector were cultured to 90% confluence. The medium was changed to serum-free Dulbecco's modified Eagle's medium. Sodium butyrate (5 mM) was added after 24 h to the control and to the sema3A expressing cells, and the medium was collected after an additional 24 h. HEPES buffer (10 mM, pH 7.3) and protease inhibitors (phenylmethylsulfonyl fluoride, 0.2 mg/ml; leupeptin, 5 μg/ml; aprotinin, 2 μg/ml, and 1 mM EDTA) were added. The medium was incubated overnight at 4 °C with anti-FLAG M2 affinity gel (0.5 ml beads/0.5 liter of medium). The column was washed thoroughly with TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.02% Tween 20) and bound sema3A eluted using 0.1 M glycine (pH 3.0) into a neutralizing volume of 1 M Tris/HCl (pH 8.0) to obtain a final concentration of 150 mM Tris/HCl (pH 7.3). Sema3A containing fractions and the corresponding fractions from the control were frozen in liquid nitrogen.

Endothelial Proliferation and Repulsion Assays—HUVEC (passages 4–8) were seeded in gelatin-coated 48- or 24-well dishes at a concentration of 10^4 or 2×10^4 cells/well, respectively, in M199 medium supplemented with 10% FCS. Angiogenic growth factors and either sema3A (0.3 μg/ml) or a corresponding volume from a control fraction were added after the cells adhered. On day 2, the factors were re-added. Adherent cells were counted on day 4 using a Coulter counter. In proliferation or repulsion experiments using semaphorin-secreting HEK293 cells, endothelial cells were seeded in gelatinized 48- (2×10^4 cells/well) or 24- (5×10^4 cells/well) well dishes in M199 containing 20% FCS and 5 ng/ml bFGF. The following day up to 5% HEK293 cells expressing various semaphorins were seeded on top of the endothelial cells in M199 containing 10% FCS with or without 0.5 ng/ml bFGF. In some of the experiments the cells were incubated prior to seeding with 5 μg/ml of the fluorescent vital dye DiI for 30 min to enable easy detection of the cells in mixed cell cultures. Cells were photographed using an inverted phase-contrast/fluorescence microscope after 24–48 h and counted in a Coulter counter.

ERK1/2 and VEGFR-2 Phosphorylation—HUVEC cells were seeded in 6-well gelatinized dishes at a concentration of 4×10^5 cells/well in growth medium containing 10% FCS. Cells were allowed to attach and were incubated 16 h at 37 °C. The cells were transferred to room temperature and incubated 15 min with sema3A (0.5 μg/ml) or a corresponding volume of a control fraction purified similarly from cells transfected with

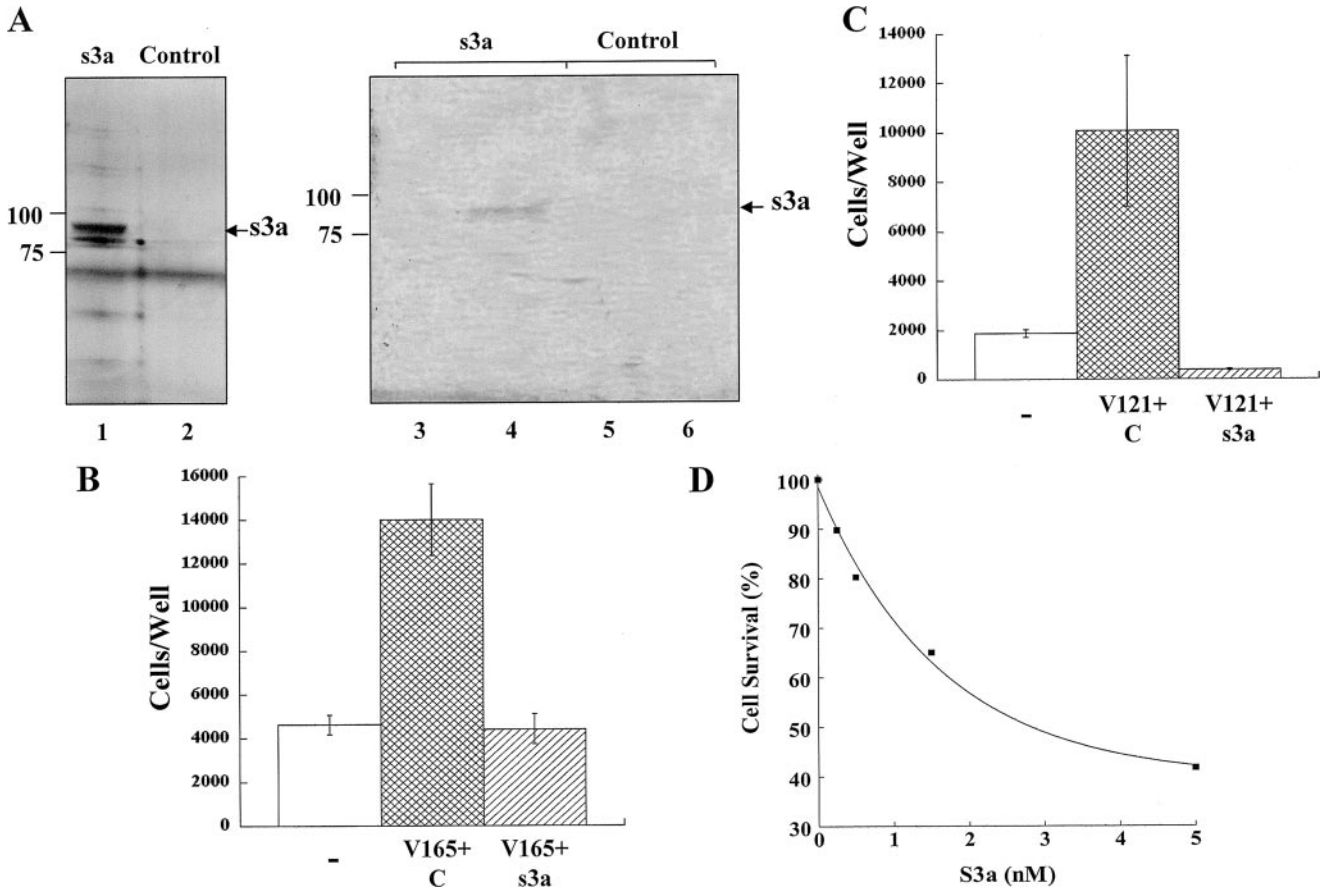

FIGURE 1. **Purification of recombinant sema3A and its effect on $VEGF_{165}$, $VEGF_{121}$, and bFGF-induced proliferation and survival of HUVEC cells.** *A*, shown on the *left* is a silver-stained gel (*lanes 1* and *2*) containing the peak fraction derived from an anti-FLAG affinity column that was loaded with serum-free conditioned medium from sema3A-expressing HEK293 cells (*s3a*) as well as a purified corresponding fraction eluted from a similar affinity column loaded with conditioned medium of empty vector-transfected HEK293 cells (*Control*). On the *right* is shown a Coomassie Blue-stained gel containing the sema3A containing fraction derived from the affinity column (*lane 4*) and the preceding fraction (*lane 3*). Also shown are the two corresponding fractions that were purified similarly from the conditioned medium of empty vector-transfected HEK293 cells (*lanes 5* and *6*). *B* and *C*, HUVEC cells were seeded in gelatinized 48-well dishes (1×10^4/well) in growth medium containing 10% FCS. $VEGF_{165}$ (3 ng/ml) (*panel B*) or $VEGF_{121}$ (10 ng/ml) (*panel C*) and either sema3A (3 nM)(*s3a*) or equal volumes of the corresponding fraction purified similarly from empty vector-transfected cells (*C*) were added to the cells on the first and third days. Cells were counted in a Coulter counter on the fourth day. *D*, HUVEC cells were seeded in gelatinized 48-well dishes (1×10^4/well) in medium containing 10% FCS. Increasing concentrations of sema3A or corresponding volumes of the control fraction were added to wells every other day. On the fourth day the cells were washed to remove dead cells, trypsinized, and counted in a Coulter counter. Each point represents the ratio of cells surviving in 3 wells treated with sema3A and cells surviving in wells that received instead the control fraction. The experiment was repeated twice with similar results.

empty expression vector. Subsequently, $VEGF_{121}$ (10 ng/ml) or $VEGF_{165}$ (3 ng/ml) were added or not and the cells were incubated for 10 more minutes. The cells were then washed with ice-cold PBS and lysed with 0.03 ml of lysis buffer containing HEPES (50 mM, pH 7.4), 4 mM EDTA, 1% Triton X-100, 0.5 mg/ml Na_3VO_4, 4.5 mg/ml $Na_4P_2O_7$, and fresh protease inhibitors (phenylmethylsulfonyl fluoride, 0.2 mg/ml; leupeptin, 5 μg/ml; and aprotinin, 2 μg/ml). The cells were scraped off, nonsoluble debris was removed by low speed centrifugation at 4 °C, and aliquots of cell lysate containing 20–60 μg of protein separated on an SDS-PAGE gel. Proteins were blotted onto a nitrocellulose filter and probed with an antibody directed against phosphorylated ERK1/2 or phosphorylated Y-951 of VEGFR-2. The blot was then stripped and re-probed with an antibody directed against ERK2 (total ERK) or VEGFR-2 (Total VEGFR-2). Quantification of band intensity was performed using a Fuji Film image reader LAS-3000 machine and the ratio between phosphorylated protein and the total amount of a target protein determined using the Multi-Gauge program.

Down-regulation of Neuropilin Expression in HUVEC Using siRNA—The np1-specific siRNA r(AAGGAAACCUUGGUGGGAU)d(TT) and the np2-specific siRNA r(CCAGAAGAUUGUCCUC AAC)d(TT) or a control siRNA r(UUCUCCGAACGUGUCACGU)dTdT were transfected into HUVEC using Oligofectamine at a final concentration of 120 nM. The cells were trypsinized 1 day following transfection, and seeded at desired concentrations. To verify down-regulation of neuropilins, cells were lysed 72 h following transfection, 40 μg of protein from the cell lysates were separated on an SDS-PAGE gel, blotted onto nitrocellulose, and probed with antibodies directed against np1 or np2. To verify that the amount of protein in each lane is similar the membrane was stripped and re-probed with antibodies directed against β-actin.

Silver Staining—Silver staining was performed according to the instructions of the vendor.

Apoptosis Assays—For fluorescence-activated cell sorter (FACS) analysis, HUVEC cells were seeded in gelatinized 6-cm dishes (6×10^5 cells/dish). The following day HEK293 cells

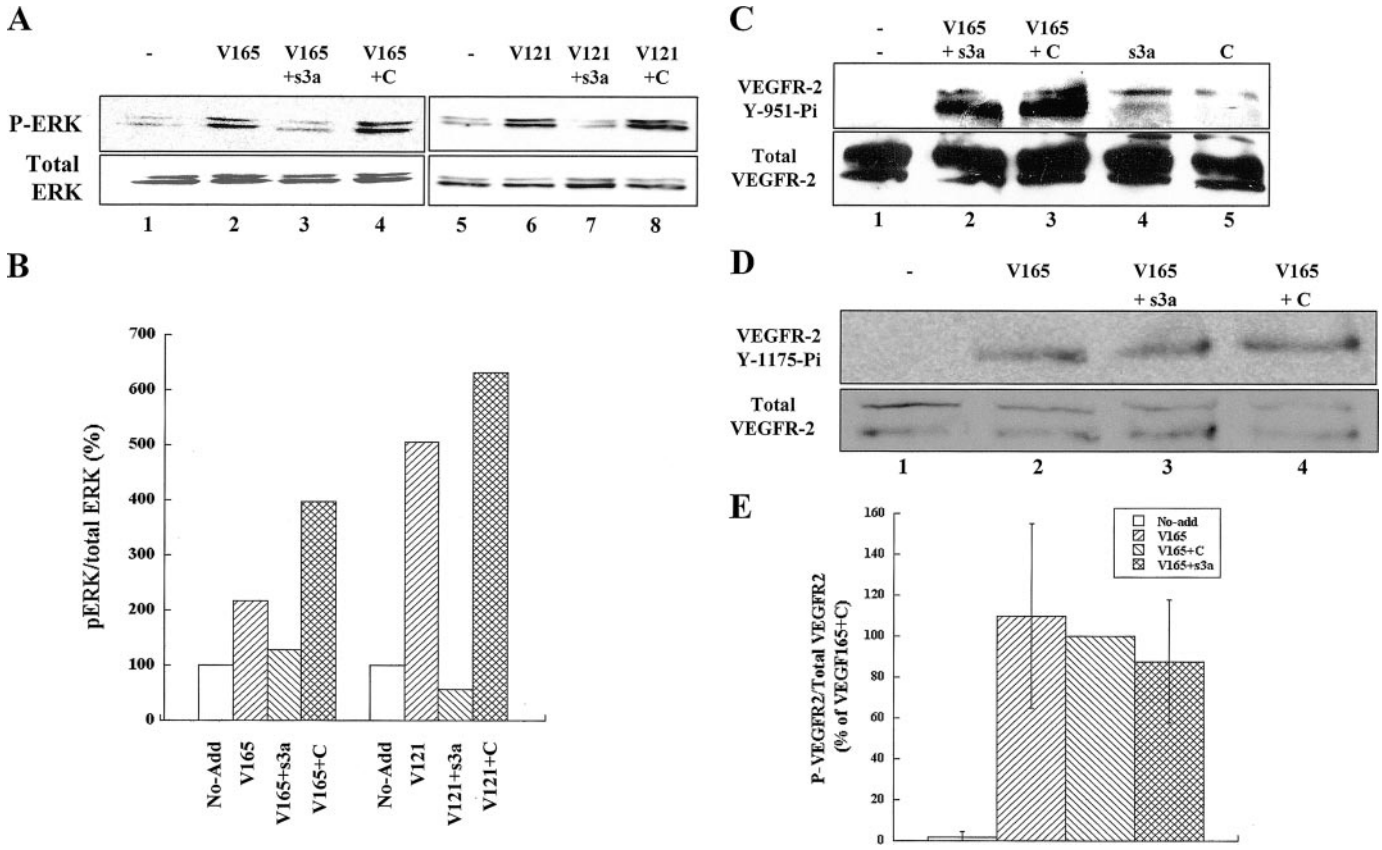

FIGURE 2. **The effect of sema3A on $VEGF_{165}$- and $VEGF_{121}$-induced phosphorylation of ERK1/2 and on $VEGF_{165}$-induced phosphorylation of VEGFR-2.** *A*, HUVEC cells were seeded in gelatinized 6-well dishes (4×10^5 cells/well) in medium containing 10% FCS. After 16 h sema3A (5 nM) (*lanes 3* and *7*) or an equal volume of a corresponding fraction purified similarly from empty vector-transfected cells (*C*) (*lanes 4* and *8*) were added. Following a 15-min preincubation at room temperature, $VEGF_{165}$ (3 ng/ml) (*lanes 2–4*) or $VEGF_{121}$ (10 ng/ml) (*lanes 6–8*) were added or not (*lanes 1* and *5*) to the cells. The experiment was terminated after 10 more minutes. $VEGF_{165}$- or $VEGF_{121}$-induced phosphorylation of ERK1/2 (*P-ERK*) was measured using anti-pERK antibodies as described (22). The blot was stripped and total ERK1/2 was detected using an antibody directed against ERK2 (*Total ERK*). *B*, the ratio between phosphorylated ERK1/2 and the total amount of ERK1/2 in *A* was quantified as described under "Experimental Procedures." *C*, HUVEC cells (4×10^5 cells/well) were not stimulated (*lanes 1*, *4*, and *5*) or stimulated with $VEGF_{165}$ (3 ng/ml) in the presence of 5 nM sema3A (*lane 2*) or in the presence of a control fraction purified identically from empty vector-transfected cells (*C*) (*lane 3*). Other cells were incubated with sema3A (*lane 4*) or with the control (*C*) (*lane 5*). VEGFR-2 phosphorylation was measured using antibodies directed against phosphorylated Y-951. Total VEGFR-2 content was assessed following stripping of the blot using an antibody directed against VEGFR-2. *D*, a similar experiment to the experiment described under *C* except that phosphorylation of Y-1175 was measured. The concentrations of $VEGF_{165}$ and sema3A used were identical as was the experimental procedure (*E*). The ratio between Y-1175-phosphorylated VEGFR-2 and the total amount of VEGFR-2 in samples was quantified as described under "Experimental Procedures." Shown is the average of four independent experiments. The average induction obtained with $VEGF_{165}$ plus the control fraction was designated as 100%. *Error bars* represent the S.D. ± mean. Sema3A did not inhibit significantly $VEGF_{165}$-induced phosphorylation of VEGFR-2 in these experiments.

(2.5×10^4 cells/dish) co-expressing sema3A and sema3F or HEK293 cells expressing sema3A, sema3F, or empty vector-transfected cells (5×10^4 cells/dish) were seeded on top of HUVEC cells in M199 medium containing 10% FCS. After 28 h the supernatant and the trypsinized cells from each plate were centrifuged, washed once in cold PBS, and cells fixed in ice-cold 70% EtOH overnight. The next day the cells were incubated with 0.2 mg/ml RNase A and 20 μg/ml propidium iodide for 30 min at 37 °C. FACS analysis was performed using a Callibur flow cytometer (BD Biosciences). The proportion of cells with hypodiploid DNA content was quantified using the CellQuest program. To quantify apoptosis by active caspase-3 content HUVEC cells were seeded in gelatinized 6-well dishes (4×10^5 cells/well) in medium containing 20% FCS and 5 ng/ml bFGF. The following day, the medium was aspirated, replaced with fresh medium lacking bFGF, and 1 μg/ml of sema3A or sema3F or corresponding volumes of control fractions were added. Serum-starved cells were used as a positive control and cells treated with 5 ng/ml bFGF were used as a negative control. The supernatant and the trypsinized cells from each well were lysed 24 h later and 100 μg of protein were loaded on a 14% SDS-PAGE gel, blotted onto nitrocellulose, and probed with an antibody directed against active caspase-3. Blots were then stripped and the total amount of protein assessed using antibodies directed against β-actin. Microscopic determination of DNA fragmentation by the TUNEL method was performed using a commercial kit according to the manufacturer's instructions (DeadEnd™ Colorimetric TUNEL System, Promega). Briefly, HUVEC were seeded in gelatinized 24-well dishes (5×10^4/well) in M199 medium containing 20% FCS and various factors were added. After 36 h the cells were fixed with 4% paraformaldehyde in PBS for 30 min and permeabilized by incubation with 0.2% Triton® X-100 in PBS. DNA strand breaks were labeled with biotinylated nucleotide mixture using terminal deoxynucleotidyl transferase. Horseradish peroxidase-conjugated streptavidin was bound to the biotinylated nucleotides incorporated into the 3′-OH end of damaged DNA and detected by diaminobenzidine staining. Positive cells were

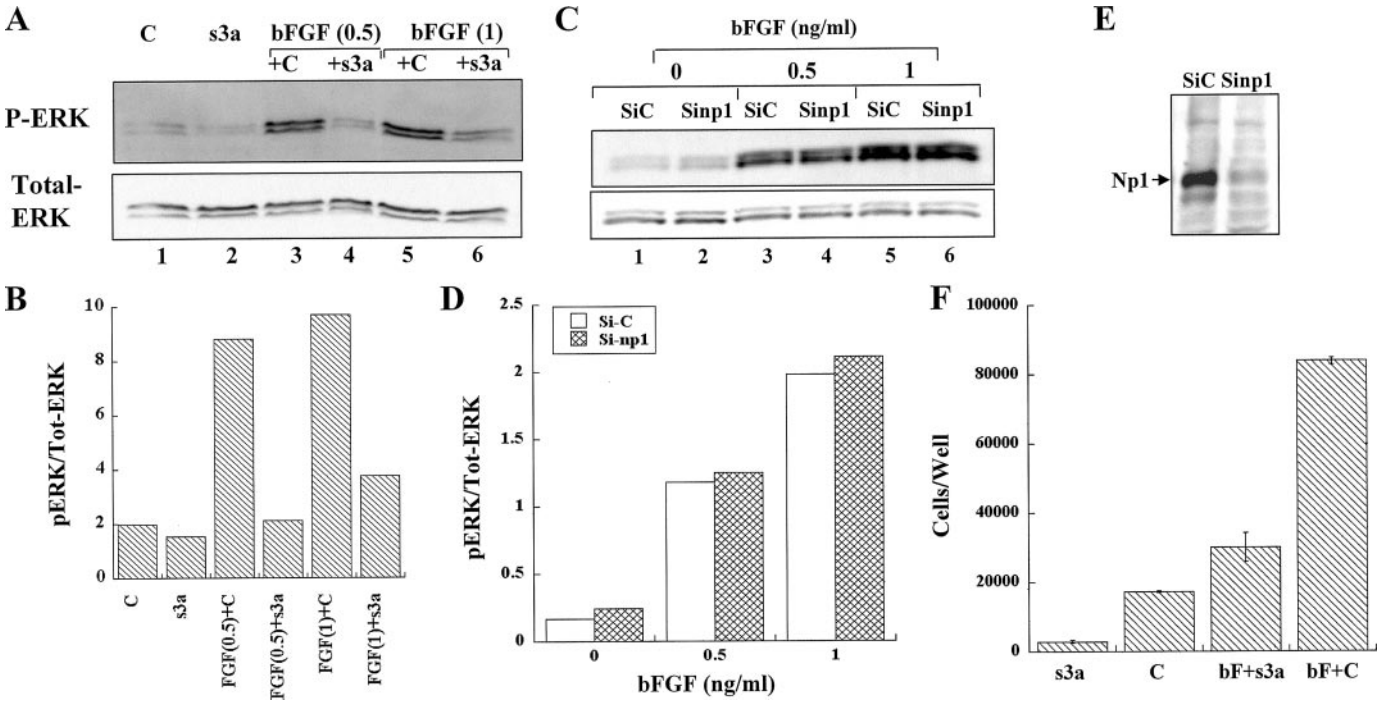

FIGURE 3. **Sema3A inhibits the activity of bFGF even though neuropilin-1 is not required for bFGF-induced signaling.** *A*, HUVEC cells were seeded in gelatinized 6-well dishes (4.5×10^5 cells/well) in growth medium containing 10% FCS. After 16 h the medium was aspirated and replaced with conditioned medium of empty vector-transfected HEK293 cells (*pcDNA*) (*lanes 1*, *3*, and *5*) or HEK293 cells expressing sema3A (*s3a*) (*lanes 2*, *4*, and *6*). For conditioning, HEK293 cells expressing or not expressing sema3A (80% confluent) were incubated 24 h in M199 containing 10% FCS. Following a 15-min preincubation at room temperature, 0.5 ng/ml bFGF (*lanes 3* and *4*) or 1 ng/ml (*lanes 5* and *6*) were added or not added (*lanes 1* and *2*) to the cells. The experiment was terminated after 10 more minutes. Phospho-ERK1/2 and total ERK1/2 were visualized as described. *B*, the ratio between phosphorylated ERK1/2 and the total amount of ERK1/2 shown in *A* was quantified as described under "Experimental Procedures." *C*, HUVEC were transfected with nonspecific siRNA (*SiC*) or with siRNA directed against np1 (*Sinp1*). ERK1/2 activation in response to the indicated bFGF concentrations was then assayed as described under *A*. *D*, the ratio between phosphorylated ERK1/2 and the total amount of ERK1/2 shown in *C* was quantified as described under "Experimental Procedures." *E*, HUVEC were transfected with nonspecific siRNA or siRNA directed against np1 and the expression of the neuropilins determined by Western blot analysis as described. *F*, HUVEC were seeded in gelatinized 24-well dished (2×10^4 cells/well) in conditioned medium from HEK293 cells expressing Sema3A (*s3a*) or in conditioned medium from empty vector-transfected HEK293 cells (*C*) in the presence or absence of bFGF (0.5 ng/ml) (*bF*). Adherent cells were counted after 3 days using a Coulter counter.

counted blindly and the results expressed as the percentage of TUNEL positive cells. The total number of cells counted in each group was at least 100.

Tube Formation Assay—Fibrin gels were prepared by dissolving bovine fibrinogen immediately before use in M199 medium to a final protein concentration of 2.5 mg/ml and filtered through a 0.22-μm filter. Fibrinogen was added to 24-well dishes (0.3 ml/well). Bovine thrombin was added to a final concentration of 0.2 units/ml and the dishes were then incubated at 37 °C for 30 min. HUVEC cells (2×10^5 cells/well) were then seeded on top of the fibrin gel. After spreading, the cells were covered with a similar coat of fibrin gel. M199 medium supplemented with 20% FCS and 1 μg/ml sema3A or a corresponding control fraction was added after polymerization of the covering gel. Developing capillaries were photographed using a phase-contrast microscope.

HEK293 Proliferation Experiments—HEK293 cells transfected with empty expression vector or HEK293 cells expressing recombinant sema3A or sema3F were seeded in 48-well gelatin-coated dishes (5×10^3 cells/well). Cells were trypsinized and counted in a Coulter counter every day.

Immunofluoresence Experiments—HUVEC were seeded in 8-well gelatin-coated chamber slides (4×10^4 cells/chamber). Following a 7-min incubation with sema3A at 37 °C, cells were washed with PBS and fixed in 4% paraformaldehyde 15 min at room temperature. The cells were washed with PBS and permeabilized using 0.5% Triton for 1 min. Following 3 more washes in PBS and blocking with 10% goat serum in PBS (1 h at room temperature), the cells were then incubated with anti-vinculin antibody followed by washes with PBS. Bound antibody was visualized using a Cy2-conjugated anti-mouse antibody and photographed using a fluorescent microscope. A similar procedure was used to stain actin fibers with Alexa-conjugated phalloidin except that the cells were stimulated with sema3A for 30 min prior to fixation.

RESULTS

Sema3A Inhibits $VEGF_{121}$ as Well as $VEGF_{165}$ and bFGF-induced Proliferation of Endothelial Cells—Sema3A was previously found to inhibit $VEGF_{165}$-induced proliferation, to compete with $VEGF_{165}$ for binding to np1, and to inhibit VEGF-induced angiogenesis in *in vitro* angiogenesis assays (15). We have previously observed that sema3F inhibits proliferation of endothelial cells and angiogenesis by a mechanism that does not require competition with VEGF for binding to shared receptors (22). Here we have used sema3A tagged at the C-terminal with a FLAG epitope tag, which we have purified on an anti-FLAG affinity resin (Fig. 1*A*, *lanes 1* and *4*). A fraction purified similarly from the conditioned medium of cells trans-

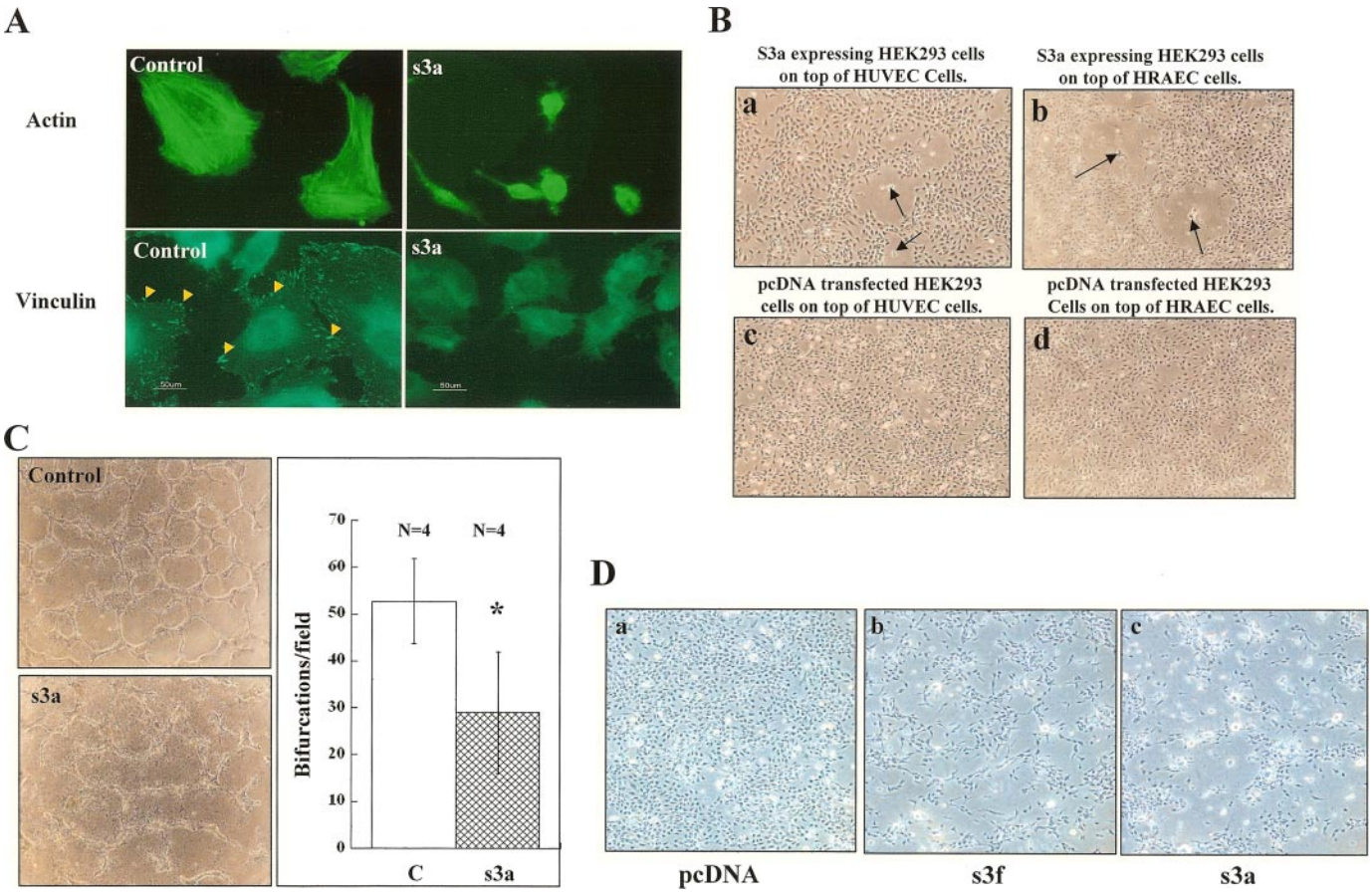

FIGURE 4. **Cells expressing sema3A repel endothelial cells at low concentrations and at high concentrations compromise endothelial cell survival.** *A*, HUVEC cells were seeded in 8-chamber gelatin-coated dishes. Conditioned medium containing sema3A (*s3a*) or conditioned medium derived similarly from empty vector-transfected HEK293 cells (*Control*) were added to the cells. The cells were fixed, permeabilized, and bound anti-vinculin antibody (*lower panels*) or bound FITC-conjugated phalloidin (*upper panels*) visualized and photographed as described under "Experimental Procedures." Focal contacts are pointed out by the *yellow arrows*. *B*, HUVEC or human radial artery (*HRAEC*) cells were seeded in gelatinized 24-well dishes (5×10^4/well) in M199 containing 20% FCS. The following day 10^3 HEK293 cells expressing sema3A (*s3a*) or empty vector-transfected HEK293 cells (*pcDNA*) were seeded on top of the endothelial cells in M199 medium containing 10% FCS. Cells were photographed 24 h later. *Arrows* point to sema3A expressing HEK293 cells. *C*, HUVEC were seeded between two layers of fibrin gel as described. Sema3A (10 nM) (*panel b*) or a corresponding volume of a control fraction purified identically from conditioned medium of empty vector-transfected cells were added (*panel a*). Spontaneously formed capillary-like tubes were photographed after 72 h. The experiment was repeated four times with similar results. The effect of sema3A on tube formation was quantified by counting bifurcation points in the tubular network. In the *right panel* are shown the average numbers of bifurcation points as counted in photographic fields derived from four independent experiments in the presence of sema3A (10 nM) or a corresponding volume of control fraction (control). *Error bars* represent the S.D. ± mean. Student's *t* test was used to determine that sema3A inhibits significantly ($p < 0.05$) the formation of tubes in these experiments. *D*, HUVEC cells were seeded in 24-well dishes (6×10^4 cells/well) in M199 containing 20% FCS and 5 ng/ml bFGF. The following day the cells were washed, and HEK293 cells expressing sema3A (*s3a*), sema3F (*s3f*), or empty vector-transfected cells (Control) were seeded on top of the HUVEC at a concentration of 5×10^3 cells/well in M199 medium containing 10% FCS. The cultures were washed after 24 h to remove non-adherent cells and photographed.

fected with empty expression vector was used as a control in all the experiments in which purified sema3A was used (Fig. 1*A*, *lanes 2*, *5*, and *6*). $VEGF_{165}$ promotes the proliferation and survival of endothelial cells. These activities were inhibited by Sema3A (Fig. 1*B*). It was reported previously that sema3A and $VEGF_{165}$ compete for a common binding site on np1 (15). However, we observed that sema3A was also able to inhibit the activity of $VEGF_{121}$, a VEGF form that does not bind to neuropilins (Fig. 1*C*) (12). Sema3A was also able to inhibit bFGF-induced proliferation of HUVEC (Fig. 3*F*) even though the mitogenic response of HUVEC to bFGF is not affected when the expression of np1 is inhibited (data not shown). Furthermore, sema3A compromised the survival of endothelial cells even in the absence of VEGF, indicating that sema3A transduces signals that compromise endothelial cell survival regardless of the presence or absence of VEGF (Fig. 1*D*). To make sure that the inhibition was due to sema3A and not due to nonspecific factors such as endotoxin we have heated the sema3A. Endotoxin is heat resistant (27) but the inhibitory activity was completely abolished by heating (data not shown). We have also tested the concentration of endotoxin in our assays and it was less than 10 pg/ml, which is a non-toxic concentration (28).

To make sure that the inhibition takes place in the absence of VEGF secreted by endothelial cells or by the sema3A producing HEK293 cells we have also repeated the experiment shown in Fig. 1*D* in the presence or absence of 20 μM of the VEGFR-2 inhibitor su1498 (29). The inhibitor itself had a marginal effect on the survival of the cells. However, sema3A inhibited efficiently the survival of the cells even in the presence of the inhibitor (data not shown).

Likewise, Sema3A inhibits $VEGF_{165}$- or $VEGF_{121}$-induced ERK1/2 activation in HUVEC cells (Fig. 2, *A* and *B*). To find out

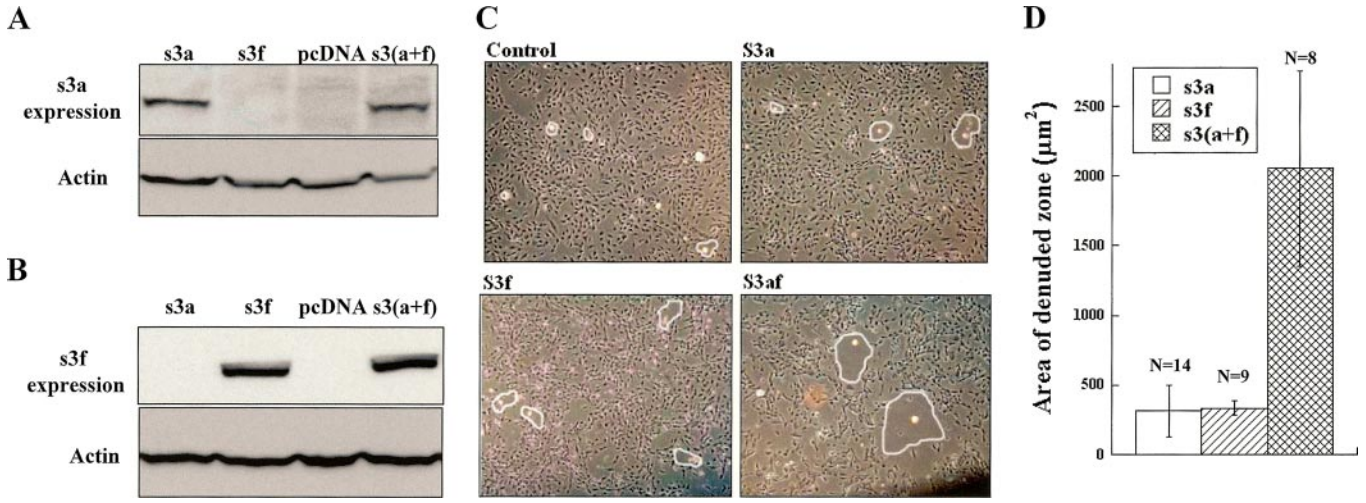

FIGURE 5. **Cells co-expressing sema3A and sema3F repel endothelial cells more potently than cells expressing corresponding concentrations of sema3A or sema3F alone.** *A* and *B*, HEK293 cells expressing sema3A (*s3a*), sema3F (*s3f*), both sema3A and sema3F (*s3*($a + f$)), or empty vector-transfected cells (*pcDNA*) were lysed, equal amounts of protein from each cell lysate were loaded on SDS-PAGE gels, and sema3A and/or sema3F content was measured using antibodies directed against sema3A (*panel A*) or antibodies directed against the Myc epitope tag contained in sema3F (*panel B*). To verify that the amount of protein in each lane is similar the membrane was stripped and re-probed with antibodies directed against β-actin. *C*, HUVEC cells were seeded in gelatinized 24-well dishes (5×10^4/well) in M199 containing 20% FCS. The following day 10^3 HEK293 cells that were pre-labeled with the fluorescent vital dye DiI and transfected with either empty vector (*control*) or expression vectors directing expression of sema3A (*s3a*), sema3F (*s3f*), or both sema3A and sema3F (*s3*($a + f$) as described above were seeded on *top* of the endothelial cells in M199 medium containing 10% FCS. Cells were photographed after 18 h. *Arrows* point to HEK293 cells in the different cultures. Denuded areas surrounding single HEK293 cells are marked by a *white line*. *D*, the area of the denuded zones surrounding the HEK293 cells in the different mixed cell cultures were computed using the Image-Pro morphometric software. Shown is the average area of the denuded zones. *N* represents the number of denuded zones quantified. *Error bars* represent the S.D. ± mean. This experiment was repeated twice with two different clones of HEK293 cells co-expressing sema3A and sema3F with similar results.

whether competition between sema3A and $VEGF_{165}$ for binding to np1 plays a role in the sema3A-induced inhibition of $VEGF_{165}$ activity, we determined if a sema3A concentration that inhibits $VEGF_{165}$-induced activation of ERK1/2 and inhibits its $VEGF_{165}$-induced proliferation/survival of HUVEC also inhibits $VEGF_{165}$-induced phosphorylation of VEGFR-2. Surprisingly, we found that at this sema3A concentration the $VEGF_{165}$-induced phosphorylation of VEGFR-2 was not inhibited significantly (Fig. 2, *C* and *D*). This result was repeated in four independent experiments (Fig. 2*E*). Similar results were obtained when VEGFR-2 phosphorylation was examined at Y-951 (Fig. 2*C*) or Y-1175 (Fig. 2*D*) phosphorylation sites. Taken together, these experiments suggest that competition with $VEGF_{165}$ for binding to np1 is not the main mechanism by which sema3A inhibits VEGF activity.

Sema3A also inhibited bFGF-induced activation of ERK1/2 (Fig. 3, *A* and *B*) even though bFGF-induced activation of ERK1/2 was not affected by siRNA-mediated inhibition of np1 expression (Fig. 3, *C*, *D*, and *E*). Because we used in these experiments submaximal concentrations of bFGF even a small effect of np1 on bFGF signaling should have been translated into a change in ERK1/2 activity. These results suggest that sema3A transduces an inhibitory signal that inhibits ERK1/2 activation downstream to the bFGF and VEGF receptor levels (Fig. 9).

Sema3A Repels Endothelial Cells—If the main mechanism by which sema3A affects endothelial cells involves the activation of a sema3A-induced signaling pathway, than sema3A should be able to induce changes in the behavior of endothelial cells even in the absence of VEGF or bFGF. When HUVEC were stimulated with sema3A they lost their focal contacts after 7 min as evidenced by staining for vinculin (Fig. 4*A*). This was accompanied after 30 min by loss of actin stress fibers and strong cell contraction (Fig. 4*A*). It was shown that the np2 agonist sema3F repels endothelial cells (23). Using a similar repulsion assay we found that single HEK293 cells expressing sema3A repel endothelial cells when seeded on top of a monolayer of endothelial cells leading to the formation of denuded areas around the sema3A-expressing cells (Fig. 4*B*). This effect was specific to sema3A because there were no such denuded areas when control HEK293 cells were used (Fig. 4*B*).

When the concentration of sema3A-expressing cells was increased further, up to a maximum of 5% of the total cell population, the survival of the endothelial cells was compromised. These cultures contained many floating dead cells and the concentration of live adherent cells was greatly reduced. Similar effects were observed when sema3F-producing HEK293 cells (22) were used. In contrast, empty expression vector-transfected HEK293 cells did not affect the survival of the endothelial cells (Fig. 4*D*).

Sema3A Inhibits in Vitro Angiogenesis—The experiments described above indicate that sema3A may function as an inhibitor of angiogenesis. We therefore determined if sema3A is capable of inhibiting the spontaneous organization of endothelial cells into tube-like structures in fibrin gels (30). Indeed, 1 nM sema3A inhibited significantly the formation of tubes from endothelial cells in these assays. The tubes that formed in the presence of sema3A appeared less organized, had fewer branching points, and dark cell debris were scattered around them in comparison to the tubes formed in the presence of control fractions purified from the conditioned medium of empty vector-transfected HEK293 cells (Fig. 4*C*).

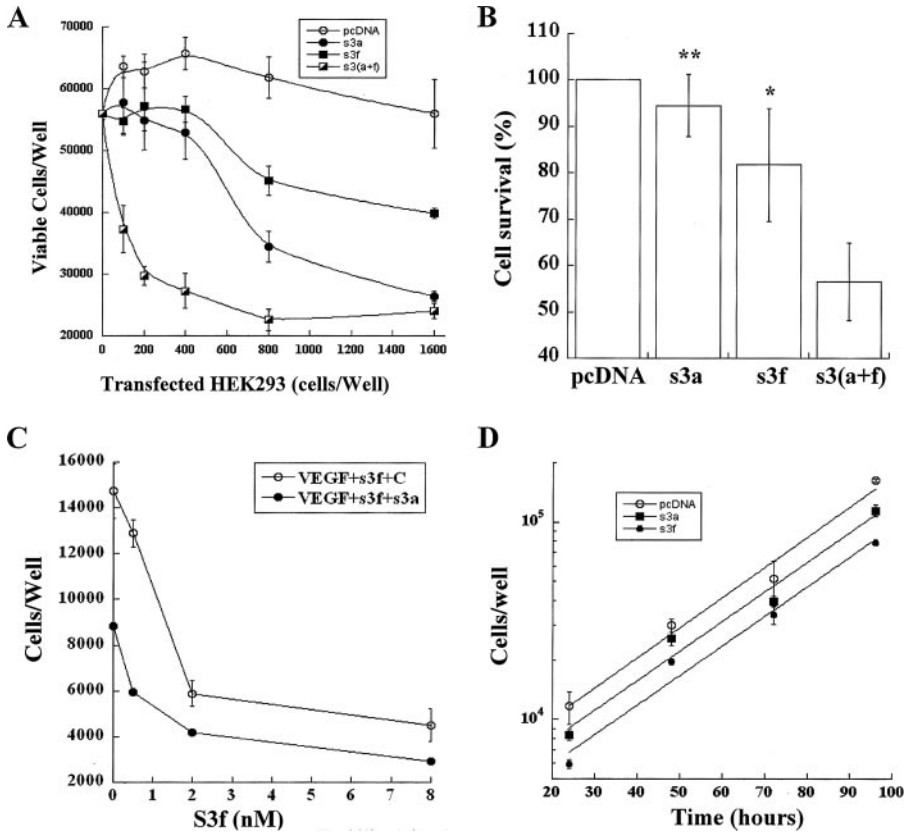

FIGURE 6. **Sema3A and sema3F work together to inhibit proliferation of endothelial cells.** *A*, HUVEC were seeded in gelatinized 48-well dishes (2×10^4 cells/well) in M199 containing 20% FCS and 5 ng/ml bFGF. The following day the medium was aspirated and increasing numbers of empty vector-transfected HEK293 cells (*pcDNA*) or HEK293 cells expressing sema3A (*s3a*), sema3F (*s3f*), or both sema3A and sema3F (*s3*(*a* + *f*)) were seeded in M199 containing 10% FCS and 0.5 ng/ml bFGF on top of the HUVEC cells. The wells were washed after 48 h to remove dead cells, trypsinized, and adherent cells counted in a Coulter counter. Each point represents the average of three wells. *Error bars* represent the S.D. ± mean. Shown is a representative experiment. The experiment was repeated four times with similar results. *B*, inhibition of HUVEC proliferation by 400 control sema3A or sema3F expressing HEK293 or 200 HEK293 cells expressing both sema3A and sema3F was measured in four independent experiments conducted as described under *panel A*. The average number of cells in cultures seeded with empty vector-transfected HEK293 cells (*pcDNA*) was taken as 100%. *Error bars* represent the S.D. ± mean. Student's *t* test was used to compare the effects of cells co-expressing sema3A and sema3F with the effects of cells expressing single semaphorins. *, $p < 0.05$; **, $p < 0.01$. *C*, HUVEC were seeded in gelatinized 24-well dishes (2×10^4 cells/well) in M199 containing 10% FCS. After the cells adhered, $VEGF_{165}$ (3 ng/ml) was added to all the wells. Sema3A was added to some of the wells to a final concentration of 1.5 nM (s3a), whereas other wells received an equal volume of a corresponding control fraction purifies similarly from empty vector-transfected cells (*C*). Increasing concentrations of sema3F (*s3f*) were then added to all the wells to the indicated concentrations immediately after seeding and again on the third day. Adherent cells were counted in a Coulter counter after 5 days. *Points* represent the average of triplicate wells and the *error bars* represent the S.D. ± mean. The experiment was repeated twice with similar results. *D*, HEK293 cells transfected with empty expression vector (*pcDNA*) or HEK293 cells expressing recombinant sema3A (*s3a*) or sema3F (*s3f*) were seeded in 48-well dishes (5×10^3 cells/well). Cells were trypsinized and counted in a Coulter counter every day. Each point represents the mean of three wells and the *error bars* represent the S.D. ± mean.

Cells Co-expressing Sema3A and Sema3F Repel Endothelial Cells More Efficiently Than Cells That Express Only One Type of Semaphorin—Sema3A is a np1 agonist and sema3F is a np2 agonist (31). Because both semaphorins repel endothelial cells and signal through different receptors, we determined if cells co-expressing sema3A and sema3F would function as better repellents of endothelial cells as compared with cells expressing only a single semaphorin. We therefore co-transfected HEK293 cells with expression vectors that direct stable expression of sema3A and sema3F. We selected two cell lines that express amounts of sema3A and sema3F that are very similar to the amounts produced by our HEK293 cells that express sema3A and sema3F alone (Fig. 5, *A* and *B*). We then seeded these cells on top of monolayers of endothelial cells and determined the areas of denuded zones that formed around single HEK293 cells after a relatively short time of 18 h at which time the denuded areas are not fully developed (Fig. 5*C*) using the ImagePro software. The areas of the denuded zones surrounding single cells that co-expressed sema3A and sema3F were substantially larger than those surrounding single cells expressing only one type of semaphorin (Fig. 5*D*), indicating that sema3A and sema3F may work together to repel endothelial cells in synergistic fashion. Similar results were also obtained with the second clone that expressed both sema3A and sema3F, although for this clone we did not find such exact matched sema3A and sema3F expressing clones as for the clone used for the experiments shown in Figs. 4–6 (data not shown).

Sema3A and Sema3F Cooperate to Inhibit the Proliferation/Survival of Endothelial Cells by a Neuropilin-dependent Mechanism—We have previously found that sema3F inhibits the proliferation of endothelial cells (22) and have now shown that sema3A can also inhibit the survival/proliferation of endothelial cells (Fig. 1). Because it seems that cells co-expressing sema3A and sema3F repel endothelial cells more efficiently than each alone, we also determined if such cells inhibit the survival/proliferation of endothelial cells more effectively than each semaphorin alone. Increasing concentrations of HEK293 cells expressing sema3A or sema3F or both semaphorins were seeded on top of a monolayer of subconfluent endothelial cells in medium containing 10% FCS in the presence of bFGF. Empty vector-transfected HEK293 cells did not inhibit cell proliferation at any of the cell concentrations used as compared with cultures in which HEK293 cells were not seeded. In contrast, the survival of the endothelial cells was compromised as a function of the relative concentration of the sema3A or sema3F expressing cells (Fig. 6*A*). However, cells co-expressing both semaphorins functioned as better inhibitors of endothelial cell proliferation/survival. At low cell concentrations the simultaneous presence of sema3A and sema3F produced a synergistic rather than an additive effect (Fig. 6*A*). Thus, 100 cells co-expressing both semaphorins produced a substantial inhibitory effect, whereas

200 cells expressing either sema3F or sema3A did not produce a substantial inhibitory effect even though the total amount of semaphorins produced by such a concentration of cells is similar to the amount produced by 100 s3(a + f) cells (Fig. 5*A*). To verify the reproducibility of these results we also show an analysis of four independent experiments in which the effects of 200 seeded HEK293 cells on the proliferation/survival of the endothelial cells were compared. In all these experiments cells expressing both semaphorins exerted a much stronger inhibitory effect (Fig. 6*B*). Similar results were obtained with a different clone of cells expressing both semaphorins (data not shown). The proliferation rate of HEK293 cells expressing semaphorins is not different from control cells, so the effects were clearly specific to endothelial cells (Fig. 6*D*). Surprisingly, when we tried to conduct similar experiments using purified sema3A and sema3F we could only see an additive effect rather than a synergistic effect (Fig. 6*C*).

These survival compromising effects of sema3A and sema3F depend upon the respective presence of np1 and np2 receptors in HUVEC. Transfection of HUVEC with siRNA species directed against np1 or np2 inhibited partially the expression of these receptors (Fig. 7*A*) and rendered the HUVEC cells less sensitive to the survival compromising effects of sema3A and sema3F, respectively (Fig. 7*B*).

Sema3A and Sema3F Promote Apoptosis of Endothelial Cells—Sema3A had previously been found to promote apoptosis of neuronal cells (32, 33). Because we have observed that both sema3A and sema3F compromise endothelial cell survival and proliferation, we determined if sema3A and sema3F can induce apoptosis of endothelial cells. HEK293 cells expressing recombinant sema3A or sema3F or control cells transfected with empty expression vector were seeded on top of subconfluent HUVEC cells at a ratio of 95:5% HUVEC:HEK293 cells in medium containing 10% FCS. The cells were stained with propidium iodide and FACS analysis was then performed to measure the content of hypodiploid cells (34). There was no significant increase in the percentage of apoptotic cells in mixed cultures containing empty expression vector-transfected HEK293 cells and HUVEC as compared with cultures that did not receive HEK293 cells. In contrast, similar concentrations of HEK293 cells expressing either sema3A or sema3F significantly induced apoptosis above baseline levels, whereas cells co-expressing sema3A and sema3F induced apoptosis almost as potently as the serum-free medium used as a positive control. This potent induction was observed even though the s3(a + f) cells were seeded at half the concentration of the other HEK293 variants to keep the total concentration of semaphorins more or less equal (Fig. 8*A*). This increase in apoptosis was most likely due to changes in the apoptotic state of the HUVEC cells because HEK293 cells are an immortal cell line whose rate of proliferation is not affected by sema3A or sema3F (Fig. 6*D*), and because they comprised only a minor percentage of the cell population in these experiments.

To verify these results, and to find out if purified sema3A and purified sema3F are also able to induce apoptosis, we conducted two more apoptosis assays. Both sema3A and sema3F induced DNA fragmentation in the TUNEL assay (Fig. 8*B*), and induced expression of activated caspase-3, a known apoptosis marker (Fig. 8*C*) (35). These experiments strongly suggest that sema3A and sema3F induce apoptosis of endothelial cells.

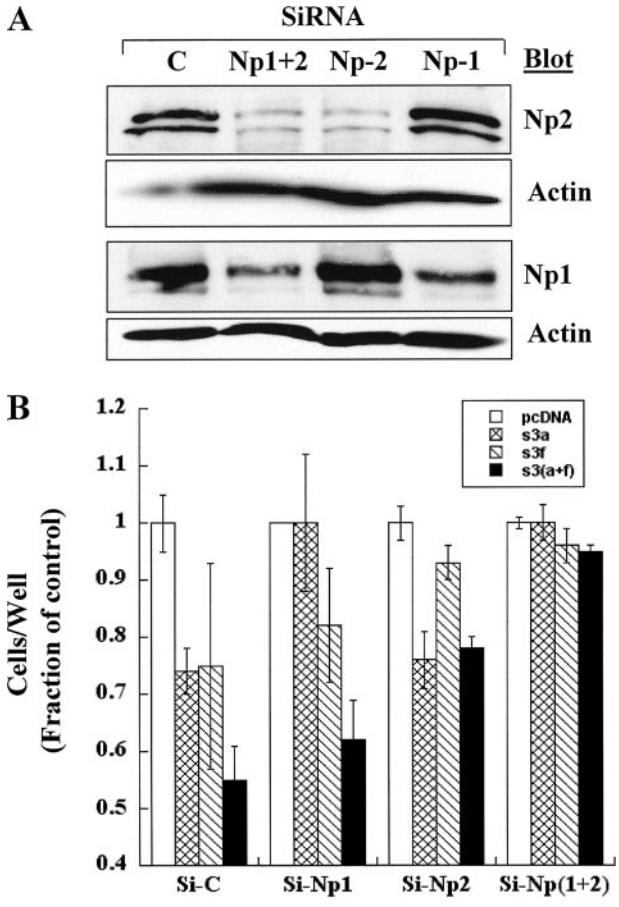

FIGURE 7. **The inhibitory effects of Sema3A and Sema3F on the survival of HUVEC are mediated by Np1 and Np2, respectively.** *A*, HUVEC were transfected with a control non-silencing siRNA (*C*), with a np1-specific siRNA (*Np1*), with a siRNA directed against np2 (*Np2*) or co-transfected with both siRNAs (*Np1 + 2*) as described. The cells were lysed 72 h following transfection and 40 μg of protein from the cell lysates were separated on an SDS-PAGE gel, blotted, and probed with antibodies directed against np1 or np2. To verify that the amount of protein in each lane is similar the membrane was subsequently stripped and re-probed with antibodies directed against β-actin. *B*, a day after transfection with siRNAs, HUVEC were seeded in 24-well dishes (6×10^4 cells/well) in M199 containing 20% FCS and 5 ng/ml bFGF. The following day HEK293 cells expressing sema3A (*s3a*), sema3F (*s3f*), both sema3A and sema3F (*s3(a + f)* cells), or empty vector-transfected cells (*pcDNA*) were seeded on top of the HUVEC. The HEK293 concentration was 3×10^3 or 1.5×10^3 cells/well in the case of cells expressing both sema3A and sema3F. The cells were seeded in M199 medium containing 10% FCS. After 24 h the cells were washed to remove non-adherent cells, trypsinized, and adherent cells were counted in a Coulter counter. Each point represents the average of three wells. *Error bars* represent the S.D. ± mean. The experiment was repeated three times with similar results.

DISCUSSION

Np1 is the major neuropilin expressed in arterial endothelial cells and potentiates VEGF signal transduction mediated by the VEGFR-2 receptor (8, 14, 36, 37). Sema3A is an alternative ligand for np1, competes with $VEGF_{165}$ for binding to np1, and activates signal transduction mediated by type-A plexins that associate with np1 (5, 6, 15). Several studies indicated that sema3A inhibits developmental angiogenesis and because sema3A inhibits $VEGF_{165}$ binding to np1 it was assumed that the inhibition is the result of competition between sema3A and

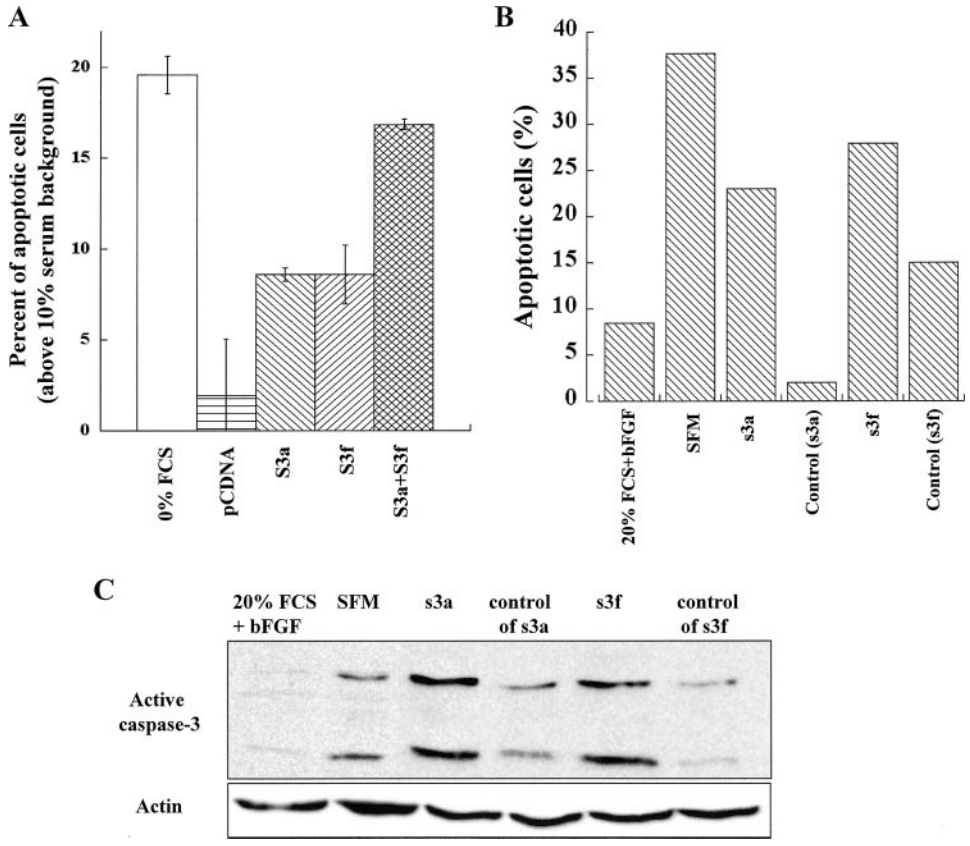

FIGURE 8. **Sema3A and sema3F promote apoptosis of endothelial cells.** *A*, HUVEC cells were seeded in gelatinized 6-cm cell culture dishes (6×10^5 cells/dish). The following day 2.5×10^4 HEK293 cells expressing both sema3A and sema3F (*s3*(*a* + *f*)) or 5×10^4 HEK293 cells expressing sema3A (*s3a*), sema3F (*s3f*), or empty vector-transfected cells (*pcDNA*) were seeded on top of HUVEC cells in M199 medium containing 10% FCS. After 28 h the cells were trypsinized, the medium and the trypsinized cells were combined and stained with propidium iodide as described. Stained cells were subjected to FACS analysis using a Callibur flow cytometer (BD Biosciences). The proportion of cells with hypodiploid DNA content was quantified using the CellQuest program. Shown is the percent of apoptotic cells above background levels determined in the presence of 10% FCS as quantified by CellQuest. *B*, HUVEC were seeded in gelatinized 24-well dishes (5×10^4/well) in M199 medium containing 20% FCS. Sema3A, sema3F (*s3f*) (5 nM), or their corresponding control fractions as well as the effect of serum-free medium (*SFM*) and 5 ng/ml bFGF were added to the wells. Following a 36-h incubation with the factors the effects on apoptosis were quantified using the TUNEL assay as described under "Experimental Procedures." The experiment was repeated twice with similar results. *C*, HUVEC cells were seeded in gelatinized 6-well dishes (4×10^5 cells/well) in M199 medium containing 20% FCS and 5 ng/ml bFGF. The next day, the medium was aspirated and either s3f or s3a were added to the cells to a final concentration of 10 nM. Cells stimulated with bFGF (5 ng/ml) were used as a negative control (*lane 1*) and serum-starved cells were used as a positive control (*lane 2*). After 24 h the cells were lysed, proteins were separated on an SDS-PAGE gel, blotted onto nitrocellulose, and probed with antibodies directed against active caspase-3. The protein concentration in each lane was assessed using antibodies directed against β-actin after blot stripping.

kinase receptors level, possibly as a result of sema3A-induced np1/plexin signaling (Fig. 9).

Sema3A repulses np1 expressing axonal growth cones (3, 4). We have shown here that sema3A induces disappearance of focal contacts followed by strong contraction of the endothelial cells. Furthermore, sema3A-expressing cells repulse several types of human endothelial cells. It is therefore possible that sema3A-expressing cells can repulse angiogenic sprouts. However, this may not be the only mechanism by which sema3A and other semaphorins such as sema3F (22, 23) inhibit angiogenesis. Sema3A induces apoptosis of neuronal cells (32, 33) and we assumed that at sufficiently high concentrations sema3A may also induce apoptosis of endothelial cells. Single cells seeded on top of endothelial cells repel the endothelial cells and do not induce apoptosis, but as the concentration of sema3A-expressing cells was increased, we observed a significant reduction in the number of surviving endothelial cells. This was accompanied by an increase in the concentration of apoptotic cells in the mixed cultures and by an increase in the activity of caspase-3, an enzyme known to be up-regulated during apoptosis. It is likely that induction of apoptosis represents a major anti-angiogenic mechanism by which sema3A and sema3F inhibit angiogenesis. Indeed, sema3A inhibited tube formation in an *in vitro* angiogenesis assay, and the inhibition was accompanied by the appearance of cell debris around the surviving tubes indicating that apoptosis may be responsible for part of the effect observed in this assay. These survival compromising effects were mediated by neuropilins as demonstrated by siRNA silencing experiments. However, it is not yet clear if the induction of apoptosis is the result of prolonged semaphorin-induced cell contraction or whether semaphorins directly activate cell death inducing pathways. This issue will need to be examined in more detail in the future.

$VEGF_{165}$ for binding to np1 (17, 18). We show here that sema3A concentrations that inhibit $VEGF_{165}$-induced cell proliferation and ERK1/2 phosphorylation do not inhibit efficiently $VEGF_{165}$-induced phosphorylation of VEGFR-2, suggesting that such competition is not the major mechanism by which sema3A inhibits VEGF activity. Furthermore, sema3A inhibits the activity of $VEGF_{121}$, a VEGF form that does not bind to neuropilins (10, 12). This last observation suggests that sema3A inhibits VEGF activity downstream to the VEGFR-2 receptor by activating np1/plexin-dependent signaling. This conclusion is also strengthened by experiments showing that sema3A inhibits bFGF-induced ERK1/2 activation and HUVEC proliferation. Because bFGF-induced ERK1/2 activation and cell proliferation are not affected by inhibition of np1 expression in HUVEC, this result also supports a model in which sema3A inhibits ERK1/2 activity downstream to the tyrosine

We expected that sema3A and sema3F will enhance each others activity because these semaphorins activate different neuropilins (3, 38). Indeed, a mixture of sema3A and sema3F displayed an additive inhibitory effect at subsaturating sema3A and sema3F concentrations. Unexpectedly, when cells co-expressing sema3A and sema3F were used instead of the purified factors, the inhibitory effects and the repulsive effects seemed

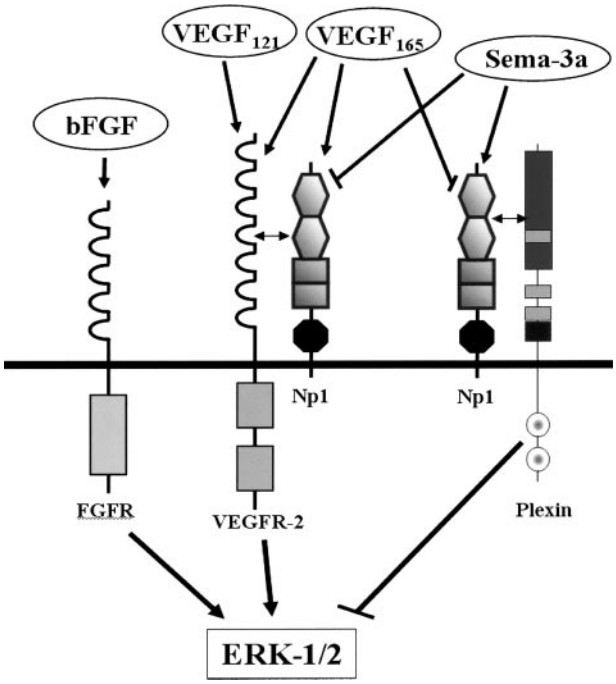

FIGURE 9. **A proposed mechanism for inhibition of bFGF and VEGF activity by sema3A.** In this scheme bFGF and VEGF induce cell proliferation by activating their respective tyrosine kinase receptors. VEGFR-2 mediated $VEGF_{121}$ and $VEGF_{165}$ signaling is strongly enhanced by np1 (39) and is not affected significantly by the binding of sema3A to np1. In contrast, bFGF activation of ERK1/2 is not affected by np1. Sema3A binding to np1 in complex with type-A plexins activates a signaling cascade that inhibits VEGF- and bFGF-induced activation of ERK1/2 downstream to the tyrosine kinase receptors. This np1/plexin-mediated inhibition can be inhibited by an excess of $VEGF_{165}$, which binds to np1 thereby competing with sema3A but not by $VEGF_{121}$, which does not bind to np1.

to be synergistically enhanced rather than being just additive. These synergistic effects were seen using two different clones of cells co-expressing sema3A and sema3F. The cell line used for most of our experiments expressed the same amounts of sema3A and sema3F per cells as the control cells that expressed only the single semaphorins. These experiments were independently repeated several times, ruling out trivial explanations such as variations in the numbers of seeded HEK293 cells. However, the reason for the different results obtained when using purified factors as opposed to cells expressing the recombinant factors are still unclear, and will need to be studied in the future. Nevertheless, these results indicate that combinations of semaphorins such as sema3A and sema3F may function as better inhibitors of angiogenesis than the single semaphorins.

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Semaphorin-3A and Semaphorin-3F Work Together to Repel Endothelial Cells and to Inhibit Their Survival by Induction of Apoptosis

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