Full-Length Semaphorin-3C Is an Inhibitor of Tumor Lymphangiogenesis and Metastasis

Yelena Mumblat, Ofra Kessler, Neta Ilan, and Gera Neufeld

Abstract

Semaphorins play important regulatory roles in diverse processes such as axon guidance, angiogenesis, and immune responses. We find that semaphorin-3C (sema3C) induces the collapse of the cytoskeleton of lymphatic endothelial cells (LEC) in a neuropilin-2-, plexin-D1-, and plexin-A1-dependent manner, while most other semaphorins, including antiangiogenic semaphorins such as sema3A do not. Sema3C is cleaved, like other class-3 semaphorins, by furin-like pro-protein convertases (FPPC). Cleaved sema3C (p65-Sema3C) was unable to induce the collapse of the cytoskeleton of LEC. FPPC are strongly upregulated in tumor cells. In order to examine the effects of full-length sema3C on tumor progression, we therefore generated an active point mutated furin cleavage-resistant sema3C (FR-sema3C). FR-sema3C inhibited potently proliferation of LEC and to a lesser extent proliferation of human umbilical vein–derived endothelial cells. FR-sema3C also inhibited VEGF-C–induced phosphorylation of VEGFR-3, ERK1/2, and AKT. Expression of recombinant FR-sema3C in metastatic, triple-negative LM2-4 breast cancer cells did not affect their migration or proliferation in vitro. However, tumors derived from FR-sema3C–expressing LM2-4 cells implanted in mammary fat pads developed at a slower rate, contained a lower concentration of blood vessels and lymph vessels, and metastasized much less effectively to lymph nodes. Interestingly, p65-Sema3C, but not FR-sema3C, rendered A549 lung cancer cells resistant to serum deprivation, suggesting that previously reported protumorigenic activities of sema3C may be due to p65-Sema3C produced by tumor cells. Our observations suggest that FR-sema3C may be further developed into a novel antitumorigenic drug.

Introduction

Tumor metastasis to lymph nodes represents the first step of dissemination in head and neck tumors and in breast cancer tumors, and is a major prognostic indicator for disease progression (1–4). From there, tumor cells can then enter the vascular circulation via the thoracic lymphatic duct (5). The metastatic spread of tumor cells to lymph nodes is enhanced following the induction of tumor lymphangiogenesis, which is driven by lymphangiogenic factors such as VEGF-C and VEGF-D (5–7). The metastatic spread of tumor cells to lymph nodes can also be enhanced by cytokines such as CCL21, CXCL12 (SDF-1), and CCL1 that recruit tumor cells to lymph vessels (8). The class-3 semaphorins were initially characterized as axon guidance factors (9). Class-3 semaphorins, with the exception of sema3E, which binds directly to the plexin-D1 receptor, bind to one of the two receptors of the neuropilin family, which subsequently associates with type-A plexin receptors or with plexin-D1 to transduce signals (10, 11). The identification of the neuropilins as receptors for the angiogenic factor VEGF (12–14) suggested that class-3 semaphorins also regulate angiogenesis. Indeed, sema3A, sema3B, sema3E, and sema3F have been identified as inhibitors of angiogenesis, and consequently as inhibitors of tumor development (15). Class-3 semaphorins can inhibit angiogenesis by competition with angiogenic factors for binding to shared receptors, such as neuropilins, as well as by the activation of inhibitory signaling cascades that inhibit signal transduction induced by angiogenic factors (16–18).

Lymphatic endothelial cells (LEC) express the neuropilin-2 receptor (19) and proliferate in response to VEGF-C and VEGF-D that signal using the VEGFR-3 receptor (20). VEGFR-3 forms complexes with neuropilin-2, which is required for the transduction of VEGF-C signals (20, 21). These observations suggest that class-3 semaphorins that use the neuropilin-2 receptor, such as sema3F, or sema3G (22, 23) may inhibit lymphangiogenesis. Indeed, sema3F was reported to repel LEC and to inhibit metastasis of melanoma cells to lymph nodes, although inhibition of tumor lymphangiogenesis was not demonstrated and it is thus uncertain whether the inhibition of lymph node metastasis observed was indeed due to inhibition of lymphangiogenesis (24).

We find that of the class-3 semaphorins only sema3F and semaphorin-3C (sema3C) induce the collapse of the cytoskeleton of LEC. We show that sema3C inhibits VEGF-C–induced signal transduction, and strongly inhibits the proliferation of LEC. Furin-like pro-protein convertases (FPPC) cleave sema3C and are strongly upregulated in tumor cells (25). We show that ectopic expression of a furin cleavage-resistant point mutated...
sema3C (FR-sema3C) in tumors derived from LM2-4 breast cancer cells implanted in mammary fat pads inhibits tumor lymphangiogenesis and the metastatic spread of tumor cells to lymph nodes. Finally, we show that the major FPPC cleaved form of sema3C, p65-Sema3C, but not FR-sema3C, promotes the survival of neuropilin-2–expressing tumor cells. This observation may explain why sema3C was previously described as a protumorigenic factor.

Materials and Methods

Cell lines
Human umbilical vein–derived endothelial cells (HIVEC) were isolated and cultured as previously described (26). Neonatal human lymphatic microvascular endothelial cells (LEC) were purchased from Lonza (cat. no. CC-2812) and cultured in medium supplied by the vendor (cat. no. CC-3162). LM2-4 cells were kindly provided by Dr. Robert Kerbel (Biological Sciences Platform, Sunnybrook Research Institute, Toronto, ON, Canada; ref. 27). HEK293, U87MG, and A549 cells were purchased from the ATCC. HEK293-FT were purchased from Invitrogen. These cell lines were kindly provided by Dr. Robert Kerbel (Biological Sciences Platform, Sunnybrook Research Institute, Toronto, ON, Canada; ref. 27). HEK293, U87MG, and A549 cells were purchased from the ATCC. HEK293-FT were purchased from Invitrogen. These cell lines were cultured as described previously (27–29).

Antibodies and reagents
Di-Asp was from Molecular Probes. Heparin Sepharose CL-6B from GE Healthcare. Recombinant fibroblast growth factor 2 was produced as described previously (30). VEG-F-C was from PeproTech. The expression plasmid encoding the extracellular domain of the sema6A cDNA was kindly provided by Dr. Behar (Hebrew University of Jerusalem, Jerusalem, Israel). The various lentiviral shRNA expression vectors were purchased from Sigma. A detailed list of the antibodies that were used can be found in the Supplementary Data.

Production of sema3C variants
The human sema3C cDNA was purchased from Dharmaco-GE Life Sciences. The construction of the cDNAs encoding FR-sema3C and the other sema3C-derived variants and of the lentiviral expression vectors, as well as the plasmids used, is described in detail in the Supplementary Methods. Conditioned media containing the various sema3C variants and the additional semaphorins were collected from HEK293 cells expressing the recombinant semaphorins as previously described (28, 29, 31).

Purification of FR-sema3C/Fc and p65-Sema3C/Fc
HEK293 cells expressing FR-sema3C/Fc or p65-Sema3C/Fc were incubated for 24 hours in serum-free medium. Conditioned medium was loaded on a protein-A column at 4°C. The beads were washed with 20 mmol/L Tris pH-8 containing 150 mmol/L NaCl and eluted with 100 mmol/L glycine pH-3, which was immediately neutralized with Tris base (1 mol/L).

Cytoskeletal contraction assay
Cytoskeletal contraction of cells in response to semaphorins was performed as previously described (28). Quantitative cell contraction was performed as described in the Supplementary Methods.

Repulsion assay
Cell repulsion assays were performed as described previously (28).

Proliferation assays
HUVEC (2 × 10^4 cells/well) or LEC (4 × 10^4 cells/well) were seeded in LEC medium in the presence or absence of semaphorins (1 μg/mL). The number of adherent cells was determined after 3 days using either the WST-1 kit according to the vendor instructions or using a coulter-counter as previously described (29).

Migration assay
Migration assays were performed using the xCELLigence machine. Cells were seeded in fibronectin-coated wells of CIM-plate 16 dishes (3 × 10^4 cells/well). Cells that passed through the membrane were then quantified according to the instructions of the vendor.

Phosphorylation assays
The phosphorylation assays were performed as described in the Supplementary Methods.

Tumor formation assays
Tomato-red RFP-expressing LM2-4 cells infected with empty lentiviral vector (control) or with lentiviruses directing expression of FR-sema3C/myc were washed, suspended in 50 μL of PBS, and injected into the mammary fat pads of 6- to 7-week-old female scid/nod mice (2 × 10^6 cells/mouse). After 30 days, tumors and lymph nodes were excised and weighed. All the animal experiments were approved by the Technion ethics committee.

Immunocytochemistry and immunofluorescence
Detection of vinculin, actin, CD-31, or podoplanin using appropriate antibodies or of phallolidin tagged with fluorescent probes was done as previously described (17). More details are provided in the Supplementary Methods.

Quantification of metastatic load in lymph nodes
Lymph nodes were excised and imaged ex vivo using the IVIS-200 imaging system or the Maestro in vivo imaging system. The normalized photon density (photon/s/cm²/sr) emitted from tomato-red RFP-labeled LM2-4 cells was then quantified. Sections from lymph nodes were stained with an anti-HLA antibody to detect metastases and counterstained with hematoxylin.

Heparin–sepharose affinity chromatography
Heparin–sepharose affinity chromatography was done as described previously (32).

Statistical analysis
A one-tailed unpaired Student t test was used in all the experiments unless otherwise indicated. Cell proliferation and cell survival experiments were performed in triplicates and the variation between triplicates did not exceed 10%. Error bars represent the standard error of the mean. Statistical P values are indicated. In cases in which the P value was smaller than 0.001 three asterisks are depicted. All experiments were repeated independently at least three times unless otherwise stated.

Ethics statement
All the authors of this article have given their informed consent to the article. The animal studies were all conducted according to the NIH guidelines and were approved by the Institutional Review Board of the Technion.

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More details and additional materials and methods used in supplementary experiments are described in the Supplementary Materials and Methods.

Results

Sema3F and sema3C induce the collapse of the cytoskeleton of LEC

We screened the class-3 semaphorins for their ability to induce the collapse of the cytoskeleton of cultured LEC characterized by the expression of podoplanin (Supplementary Fig. S1A). The only semaphorins that induced the collapse of the LEC cytoskeleton were sema3C and sema3F (Fig. 1A). All the other semaphorins we tested were active and induced the cytoskeletal collapse of other cell types (Supplementary Fig. S2) but, nevertheless, did not induce cytoskeletal collapse in LEC. Contrary to our expectations, sema3G, a class-3 semaphorin that signals using the neuropilin-2 receptor (23), failed to induce cytoskeletal collapse of LEC (Fig. 1B). Sema3C also induced the collapse of the cytoskeleton of HUVEC (Fig. 1B) and in repulsion assays cells expressing recombinant sema3C repulsed both HUVEC and LEC (Fig. 1C). Unlike HUVEC, which express both neuropilins, LEC, expressed, as expected, much more neuropilin-2 (Supplementary Fig. S1B; ref. 19). The expression patterns of the type-A plexins were similar between HUVEC [and other primary human endothelial cell types (Supplementary Fig. S1C)] and LEC except that plexin-A4 was not expressed at all in LEC, and plexins A1 and D1 seemed to be more abundant in LEC (Supplementary Fig. S1B).

Class-3 semaphorins are susceptible to cleavage by proteases of the FPPC family (28, 33). In the case of sema3C, cleavage by FPPC results in the generation of a 65-kDa peptide (p65-Sema3C) containing the N-terminal of sema3C up to the first FPPC cleavage site (Fig. 2A, site 1). Malignant cells upregulate production of FPPCs (25). Sema3C is efficiently cleaved when it was expressed in HEK293 cells or in several other types of tumor cells, including L2-4 breast cancer cells (Fig. 2B and C). We produced and purified a fusion protein in which p65-Sema3C is fused in frame to Fc or myc epitope tags at its C-terminal (Fig. 2A). However, p65-Sema3C/Fc failed to induce contraction of LEC, and we therefore assumed that p65-Sema3C is inactive (Fig. 2E).

Generation of an active point mutated sema3C (FR-sema3C) that resists cleavage by FPPC

To study the effects of full-length sema3C on tumor progression, we generated a point mutated sema3C that resists cleavage by FPPC. The arginines of FPPC cleavage site-1 were replaced with lysine residues and the part of the basic domain that contains the ADAMTS1 cleavage site (34) and the second FPPC cleavage site (Fig. 2A). Introduction of these mutations into sema3C reduced its susceptibility to cleavage by FPPC (Fig. 2F). A purified recombinant form of FR-sema3C/Fc induced contraction of LEC (Fig. 2E) while a purified FPPC-resistant point mutated form of sema3E (UNCL-sema3E/Fc; ref. 35) failed to induce contraction of LEC (Fig. 2E). FR-sema3C/Fc also induced the contraction of HUVEC while p65-Sema3C/Fc did not (Supplementary Fig. S2D).

Sema3C binds to the extracellular matrix and can be released from it by heparin and by digestion with ADAMTS1 (Fig. 2A; ref. 34). These observations suggest that sema3C is a heparin-binding protein and that binding to heparin or heparan sulfates may be important to its activity. Indeed, sema3C binds to heparin-epitope tags and is released from it with 0.5 mol/L NaCl (Fig. 2F).

Figure 1. Sema3C and sema3F induce contraction of LEC. A. LEC were seeded on cover slips and incubated with conditioned media collected from HEK293 cells infected with empty vector (control) or HEK293 cells expressing designated semaphorins. After a 30-minute incubation at 37°C, they were fixed, stained with Cy3-conjugated anti-vinculin antibodies (red), and Cy2-conjugated phalloidin (green) as described (magnification, ×40). Arrows, vinculin containing focal contacts. B, HUVEC were seeded on cover slips. The cells were stimulated with control or sema3C containing conditioned medium and the effect on their actin cytoskeleton and focal adhesions determined as above. Arrows, vinculin containing focal contacts. C, HUVEC and LEC were seeded on gelatin (HUVEC) or fibronectin (LEC)-coated plates. HEK293 cells stained with Di-Asp and infected with empty vector (control) or expressing sema3C were seeded on endothelial cells. Phase contrast photographs were taken after 48 hours (magnification, ×10). Cell-free "holes" produced as a result of repulsion are marked by a yellow border.
Interestingly, sema3C lacking the basic domain and the ADAMTS1 site still binds to heparin and is active while p65-Sema3C loses the heparin-binding ability (Fig. 2F).

**FR-sema3C/Fc inhibits the proliferation of LEC and VEGF-C–induced signaling.**

FR-sema3C/Fc inhibited strongly the proliferation of LEC (Fig. 3A and B; Supplementary Movies S3 and S4) and the incorporation of bromodeoxyuridine (BrdUrd) into the cells (Supplementary Fig. S4). The LEC eventually died (Supplementary Movies S3 and S4) but we could not detect in these cells enhanced expression of activated caspase-3 (data not shown). In contrast, the proliferation of HUVEC was inhibited very moderately (by 25%), and was not accompanied by cell death (Fig. 3A and B; Supplementary Movies S1 and S2).

VEGF-C promotes the proliferation of LEC as well as lymphangiogenesis (36, 37). We therefore determined whether FR-sema3C/Fc affects VEGF-C–induced signaling in LEC. Stimulation by FR-sema3C/Fc inhibited VEGF-C–induced phosphorylation of the VEGFR-3 receptor (Tyr-1230/1231), and also VEGF-C–induced phosphorylation of ERK1/2 and AKT (Fig. 3C). These results suggest that FR-sema3C may inhibit the proliferation of LEC by inhibition of VEGF-C signaling and that FR-sema3C may compete with VEGF-C for binding to the VEGF-C coreceptor neuropilin-2 (21). To determine the relative contribution of the different neuropilins and plexins to FR-sema3C signal transduction in LEC, we silenced the expression of these receptors (Supplementary Fig. S3A) and determined how the silencing affects FR-sema3C/Fc–induced cell contraction using the xCELLigence machine as shown (Supplementary Fig. S3B).
FR-sema3C/Fc–induced collapse of the actin cytoskeleton of the LEC was inhibited in LEC silenced for neuropilin-2 (P < 0.001), plexin-D1 (P < 0.001), or plexin-A1 (P < 0.001) expression although silencing plexin-A1 inhibited cell contraction somewhat less potently (Fig. 3D).

FR-sema3C does not affect the proliferation or migration of LM2-4 breast cancer cells but strongly inhibits metastasis to lymph nodes from tumors derived from these cells. LM2-4 breast cancer cells were derived from triple-negative MDA-MB-231 cells by repeated isolation of metastasized cells from lungs (27). We expressed in them recombinant tomato-red RFP and in addition either empty expression vector (control) or FR-sema3C/myc. Conditioned medium from these cells contained almost exclusively full-length FR-sema3C/myc (Fig. 4A). FR-sema3C/myc induced the contraction of HUVEC and LEC (Fig. 4B) but failed to induce contraction of LM2-4 cells (Fig. 4B) nor did expression of FR-sema3C/myc in these cells inhibit their proliferation or migration (Fig. 4C and D). LM2-4 cells express very little neuropilin-2, which may explain why they do not respond to FR-sema3C/Fc (Supplementary Fig. S7B).

We have implanted both control LM2-4 cells and LM2-4 expressing FR-sema3C/myc in mammary fat pads of scid/nod mice. The implanted control and FR-sema3C–expressing cells also expressed in addition tomato-red RFP. FR-sema3C/myc was expressed intact in these tumors and was not cleaved (Fig. 5A). Although the expression of FR-sema3C/myc in the LM2-4 cells did not change their behavior in vitro, it inhibited significantly (P = 0.002) by about 30% the development of tumors from these cells following their implantation in the mammary fat pads (Fig. 5B). The concentration of lymph vessels in the tumors that developed from the FR-sema3C/myc–expressing cells was significantly lower.

Figure 3. FR-sema3C inhibits LEC proliferation and VEGF-C–induced signal transduction. A, HUVEC or LEC were seeded in 12-well dishes (2 × 10⁴ and 5 × 10⁴ cells/well, respectively) in LEC growth medium in the presence of vehicle (control) or FR-sema3C/Fc (2 µg/mL). Cells were photographed after 72 hours. B, LEC and HUVEC were seeded in fibronectin-coated 96-well dishes and the effect of FR-sema3C/Fc (2 µg/mL) on their proliferation was measured using the WST-1 proliferation assay as described. Shown is the average of N independent experiments. C, confluent 6-well dishes containing LEC were incubated with vehicle or FR-sema3C/Fc and stimulated with VEGF-C as described. Cell extracts were then subjected to Western blot analysis using antibodies directed against phosphorylated VEGFR-3, phosphorylated-ERK1/2, and phosphorylated AKT as described. The blots were then stripped and reblotted with antibodies against VEGFR-3, ERK1/2, and AKT. Shown is a representative experiment out of several that were performed with similar results. D, LEC silenced for the expression of the indicated receptors or control cells expressing a nontargeting shRNA (sh-control) were seeded in wells of the xCELLigence machine (2 × 10⁴ cells/well) and cultured for 24 hours as described. They were subsequently stimulated with FR-sema3C/Fc (1 µg/mL). Cell contraction over time was then measured. Maximal contraction was determined as described in Supplementary Fig. S3B. Statistical analysis was performed using one-way ANOVA. ***P < 0.001.
concentration of tumor-associated M1 and M2 macrophages.

In one experiment, we determined the positive cells in the tumors by FACS analysis (Fig. 5E and F) and by determination of the concentration of CD31-positive lymph vessels in the tumors was about half their concentration in this peripheral band in the control tumors as well as in tumors derived from FR-sema3C/myc-expressing cells (Supplementary Fig. S5A). The concentration of the lymph vessels in the internal parts of the tumors was about half their concentration in this peripheral band in the control tumors as well as in tumors derived from FR-sema3C/myc-expressing cells (Supplementary Fig. S5B). Expression of FR-sema3C reduced the concentration of the lymph vessels by 30% in the periphery and by 45% in the internal parts of the tumors (Supplementary Fig. S5B). These changes did not reach statistical significance, possibly because we analyzed only a limited subset of the tumors, but the trend was clear. In addition, tumors derived from FR-sema3C/myc-expressing LM2-4 cells contained about half the concentration of blood vessels \( (P = 0.05) \) as revealed by staining tumor sections for CD31 (Fig. SE and F) and by determination of the concentration of CD31-positive cells in the tumors by FACS analysis \( (P = 0.018) \) (Supplementary Fig. S3C). Thus, FR-sema3C also functions as an inhibitor of tumor angiogenesis, which may explain why these tumors were smaller. We also determined in one experiment the concentration of tumor-associated M1 and M2 macrophages using FACS analysis of single-cell suspensions prepared from excised tumors. These experiments indicate that the concentration of the F4-80+/CD206+ M2 macrophage subpopulation is significantly reduced by about 50% in the FR-sema3C/myc-expressing tumors \( (P = 0.048) \) while the concentration of F4-80+/CD11b+ M1 subpopulation was not altered (Supplementary Fig. S3D).

We have excised the proper axillary, lumbar aortic, and subiliac lymph nodes from mice harboring tumors derived from control and FR-sema3C/myc-expressing LM2-4 cells. Metastases in excised lymph nodes were identified and their mass measured using the IVIS-200 imaging system (Fig. 6A) or the Maestro system (Supplementary Fig. S6C and S6D). The presence of metastases in lymph nodes was also verified by staining lymph node sections for human class-1 HLA (Fig. 6B). Although 95% of the mice harboring control tumors developed at least one metastase in lymph nodes, only 39% of the mice harboring FR-sema3C/myc tumors had at least one lymph node metastase (Fig. 6C). Although metastases were detected in 70% of the lymph nodes excised from mice harboring control tumors, only 13% of the lymph nodes excised from mice harboring FR-sema3C/myc tumors had metastases. Furthermore, the average size of metastases found in lymph nodes of mice harboring FR-sema3C/myc–expressing tumors was only 13% of the average size of metastases found in lymph nodes derived from mice harboring control tumors \( (P < 0.001) \) (Fig. 6D). Tomato-red RFP-labeled tumor cells could easily be seen in lymph vessels draining into lymph nodes in mice harboring control tumors (Supplementary Fig. S6A). Notably, at
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The time the mice were sacrificed, we could not yet detect metastases in the livers or lungs of any of the mice. Taken together, our results suggest that FR-sema3C inhibits metastasis to lymph nodes.

To find out if metastasis to lymph nodes is influenced by the size of the primary tumors, we compared the incidence of lymph node metastasis in mice that harbored size-matched control or FR-sema3C–expressing tumors (Supplementary Fig. S6B). However, despite the similar size of the primary tumors in these groups, expression of FR-sema3C/myc still inhibited potently and significantly the development of metastases in lymph nodes (Supplementary Fig. S6C and S6D). Thus, although we cannot rule out completely an effect of tumor size on lymph node metastasis, it seems that most of the effect of FR-sema3C on the metastasis of tumor cells to lymph nodes is not due to differences in the size of the primary tumors.

p65-Sema3C functions as a survival-promoting factor

Several reports have characterized sema3C as a protumorigenic factor (39–41). These studies were conducted using wild-type, FPPC cleavable sema3C. It is likely that a significant portion of the sema3C found in the tumor microenvironment is present in the form of p65-Sema3C because malignant cells produce as a rule highly elevated levels of FPPC (25). We hypothesized that even though p65-Sema3C was not able to affect the cytoskeletal organization of LEC, it may nevertheless display protumorigenic properties. Indeed, recombinant p65-Sema3C promoted significantly the survival of A549 lung cancer cells while FR-sema3C was completely devoid of such activity (Supplementary Fig. S7A). This preliminary experiment suggests that the reported protumorigenic effects of sema3C may be due to p65-sema3C–specific effects. The mechanism by which p65-Sema3C exerts its survival-enhancing effect and the characterization of the effects of p65-Sema3C on tumor progression will need to be examined further in the future.

Discussion

Metastasis to lymph nodes via the lymphatic system represents the first step of metastatic dissemination in malignant melanomas, squamous carcinomas of the head and neck (HNSCC), and breast cancer. In these tumors, the identification of lymph node metastases represents a major prognostic indicator for disease progression (1–4, 42). Heightened expression of VEGF-C in breast carcinoma cells was found to induce tumor lymphangiogenesis and to enhance metastasis of tumor cells to lymph nodes (5, 43), suggesting that inhibitors of lymphangiogenesis may potentially have a beneficial effect on the progression of breast
Indeed, several inhibitors of lymphangiogenesis targeting VEGF-C and VEGF-D signaling have been recently examined (3, 44–46).

We find that sema3C is the only class-3 semaphorin besides sema3F that is able to induce the collapse of the cytoskeleton of LEC, and hypothesized that sema3C may function as an inhibitor of lymphangiogenesis and metastasis. To test this hypothesis, we used the highly metastatic LM2-4 breast cancer cells. These cells did not seem affected in any way directly by sema3C in cell culture, and metastasized spontaneously very efficiently from primary tumors to lymph nodes, thus enabling the discrimination between direct effects of sema3C on the tumor cells versus effects on the tumor microenvironment. However, we could not conduct this study with native sema3C because FPPC produced in abundance by malignant cells (25) cleaves sema3C, resulting in the generation of the p65-Sema3C cleavage product, which unlike full-length sema3C failed to promote the collapse of the cytoskeleton of LEC and seemed inactive. We therefore produced the point mutated FR-sema3C variant of sema3C, which is resistant to cleavage by FPPC.

FR-sema3C induced the contraction of the cytoskeleton of LEC and HUVEC. These effects were mediated in the LEC by the neuropilin-2 receptor and by the D1 and A1 plexin receptors, which associate with neuropilins to form functional class-3 semaphorin receptors (15). Surprisingly, silencing plexin-A3 had no effect on FR-sema3C/Fc–induced contraction of LEC. Thus, the mechanism by which FR-sema3C inhibits lymphangiogenesis differs from that used by sema3F, the only other class-3 semaphorin that induces in LEC cytoskeletal collapse and that transduces signals using neuropilin-2 and plexin-A3 (47). FR-sema3C also inhibited the proliferation of LEC, and inhibited in these cells VEGF-C induced signal transduction. Unlike sema3A and sema3F, which induce caspase-3–dependent apoptosis of vascular endothelial cells (47), FR-sema3C promoted the death of the LEC by a caspase-3-independent mechanism. In contrast, FR-sema3C inhibited much less effectively the proliferation of HUVEC and did not affect at all the proliferation or the cytoskeletal organization of the LM2-4 cells.

FR-sema3C–expressing LM2-4 cells formed tumors in mammary fat pads of scid/nod mice but their growth was inhibited by...
about 30% as compared with the growth rate of control tumors, possibly because FR-sema3C also functions as an inhibitor of angiogenesis. Interestingly, we have also observed a reduction in the concentration of activated M2 macrophages in the FR-sema3C-expressing tumors. M2 macrophages enhance angiogenesis and lymphangiogenesis (48–50) and it is possible that inhibition of their recruitment by FR-sema3C contributes to the FR-sema3C-induced inhibition of angiogenesis and lymphangiogenesis.

Tumors derived from FR-sema3C–expressing LM2-4 cells contained a significantly reduced concentration of lymph vessels, suggesting that FR-sema3C inhibits lymphangiogenesis. The reduction in the concentration of the lymph vessels was a bit more pronounced in the central regions of the tumors but was substantial also in the periphery. These observations suggest that the inhibition of metastasis to lymph nodes by FR-sema3C is consistent, at least in part, with inhibition of tumor lymphangiogenesis. Both the number of lymph nodes that contained metastases as well as the size of metastases that formed in positive lymph nodes were significantly reduced in mice harboring tumors derived from FR-sema3C–expressing LM2-4 cells. Similar results were obtained even when the incidence of metastasis was compared between mice harboring size-matched FR-sema3C expressing tumors and control tumors. Taken together, these results suggest that FR-sema3C could perhaps be used to inhibit the metastatic spread of breast cancer tumors to lymph nodes.

In contrast with our observations that suggest that sema3C is an inhibitor of tumor progression, sema3C expressed in tumors was reported to function as a protumorigenic factor (39–41). These observations are reminiscent of the similar protumorigenic activity displayed by p61-sema3E, which is the FPPC cleavage product of sema3E that gains the ability to activate the ErbB2 receptor and thus promotes tumor progression. However, unlike p65-sema3C, p61-sema3E still retained the cytoskeleton collapsing activity of full-length sema3E (35, 51). We have found preliminary evidence indicating that p65-Sema3C functions as a survival factor for A549 lung cancer cells, suggesting that the reported protumorigenic properties of sema3C may be due to p65-Sema3C. The properties of p65-Sema3C will therefore need to be examined in more detail in the future.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conception and design: Y. Mumblat, O. Kessler, G. Neufeld

Development of methodology: Y. Mumblat, O. Kessler, N. Ilan, G. Neufeld

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Mumblat, O. Kessler, N. Ilan

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Mumblat, O. Kessler, G. Neufeld

Writing, review, and/or revision of the manuscript: Y. Mumblat, O. Kessler, N. Ilan, G. Neufeld

Study supervision: G. Neufeld

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