FULL LENGTH SEMAPHORIN-3C IS AN INHIBITOR OF TUMOR LYMPHANGIOGENESIS AND METASTASIS

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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 anti-angiogenic 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 up-regulated 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 umbilical vein derived endothelial cells (HUVEC). 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 pro-tumorigenic 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 anti-tumorigenic 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 (4). 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, CXC12 (SDF-1) and CCL1 that recruit tumor cells to lymph vessels (4). VEGF-D was also found to promote the dilation of collecting lymphatics by up-regulation of prostaglandin production thereby promoting the passage of tumor cells to lymph nodes (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 if 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 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 up-regulated 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 pro-tumorigenic factor.
MATERIALS AND METHODS

Cell lines: HUVEC were isolated and cultured as previously described (26). Neonatal human lymphatic microvascular endothelial cells (LEC) were purchased from Lonza (Cat. CC-2812) and cultured in medium supplied by the vendor (Cat. CC-3162). LM2-4 cells were kindly provided by Dr. Kerbel (Toronto, Canada) (27). HEK293, U87MG and A549 cells were purchased from ATCC. HEK293-FT were purchased from Invitrogen. These cell lines were cultured as described (27-29).

Antibodies and Reagents: Di-Asp was from Molecular Probes. Heparin Sepharose CL-6B from GE Healthcare. Recombinant bFGF was produced as described (30). VEGF-C was from Peprotech. The expression plasmid encoding the extracellular domain of the sema6A cDNA was kindly provided by Dr. Behar, Hebrew University, 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 materials section.

Production of sema3C variants: The human sema3C cDNA was purchased from Dharmacon-GE Life Sciences, USA. 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 are 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 24 h in serum free medium. Conditioned medium was loaded on a protein-A column at 4°C. The beads were washed with 20mM Tris pH-8 containing 150mM NaCl and eluted with 100mM glycine pH-3 which was immediately neutralized with Tris base (1M).
**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 (28).

**Proliferation assays:** HUVEC (2x10⁴ cells/well) or LEC (4x10⁴ cells/well) were seeded in LEC medium in the presence or absence of semaphorins (1µg/ml). The number of adherent cells was determined after 3 days using either the WST-1 kit according to the vendor instructions or 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 (3x10⁴ 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 section.

**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 6-7 week old female scid/nod mice (2x10⁶ cells/mouse). After 30 days, tumors and lymph nodes were excised and weighted. 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 phaloidin tagged with fluorescent probes was done as previously described (17). More details are provided in the supplemental 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/sec/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 hematoxilin.

**Heparin-sepharose affinity chromatography:** Heparin-sepharose affinity chromatography was done as described (32).

**Statistical analysis:** One tailed unpaired student's 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 manuscript have given their informed consent to the manuscript. The animal studies were all conducted according to NIH guidelines and were approved by the institutional review board of the Technion.

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 (Fig. 1A). 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 (Fig. 2S), 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. 1A). 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 (19) (Fig. 1B). The expression patterns of the type-A plexins were similar between HUVEC (and other primary human vascular endothelial cells (Fig. 1C) 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 (Figs. 1B).

Class-3 semaphorins are susceptible to cleavage by proteases of the furin like pro-protein convertase (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 up-regulate production of FPPCs (25). Sema3C was efficiently cleaved when it was expressed in HEK293 cells or in several other types of tumor cells including LM2-4 breast cancer cells (Fig. 2B & 2C). 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 was deleted. Fc or myc epitope tags were then fused in frame upstream to a stop codon to generate FR-sema3C/Fc and FR-sema3C/myc (Fig. 2A). In contrast with sema3C, FR-sema3C/Fc remained mostly intact when expressed in HEK293 cells or in LM2-4 cells (Figs. 2B-2D & 4A). Purified FR-sema3C/Fc induced contraction of LEC (Fig. 2E) while a purified FPPC resistant point mutated form of sema3E (UNCL-sema3E/Fc) (35) failed to induce contraction of LEC (Fig. 2E). FR-sema3C/Fc also induced the contraction of HUVEC while p65-Sema3C/Fc did not (Fig. 2SD).

Sema3C binds to the extracellular matrix and can be released from it by heparin and by digestion with ADAMTS1 (Fig. 2A) (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-sepharose and is released from it with 0.5 M NaCl (Fig. 2F). Interestingly, sema3C lacking the basic domain and the ADAMTS1 site still binds to heparin and is active while p65-Sema3C looses 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 (Figs 3A & 3B, & Supp. Movies 3-4) and the incorporation of BrdU into the cells (Fig. 4S). The LEC eventually died (Supp. Movies 3-4) 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 (Figs. 3A & 3B, & Supp. Movies 1-2).

VEGF-C promotes the proliferation of LEC as well as lymphangiogenesis (36, 37). We therefore determined if 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 co-receptor 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 (Fig. 3SA) determined how the silencing affects FR-sema3C/Fc induced cell contraction using the xCELLigence machine as shown (Fig. 3SB). The 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 (Figs. 4C & 4D). LM2-4 cells express very little neuropilin-2 which may explain why they do not respond to FR-sema3C/Fc (Fig. 7SB).

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). While the expression of FR-sema3C/myc in the LM2-4 cells did not change their behaviour 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 (P=0.05) by about 50% when measured in whole tumor sections as compared to the concentration of lymph...
vessels in tumors that developed from control cells (Figs. 5C & 5D), indicating that FR-sema3C/myc inhibits tumor lymphangiogenesis. It was reported that intra-tumoral lymph vessels do not contribute to tumor metastasis (38). We therefore determined if FR-sema3C reduces preferentially the concentration of peripheral lymph vessels in a peripheral band with a thickness of about 10% of the average diameter of the tumor (Fig. 5SA). 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 expressing cells (Fig. 5SB). 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 (Fig. 5SB). These changes did not reach statistical significance, possibly because we analysed 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 (Figs. 5E & 5F) and by determination of the concentration of CD31 positive cells in the tumors by FACS analysis (P=0.0018) (Fig. 3SC). 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+ positive 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 (Fig. 3SD).

We have excised the proper axillary, lumbar aortic, and subiliac lymph nodes from mice harbouring 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 (Fig. 6S C&D). The presence of metastases in lymph nodes was also verified by staining lymph node sections for human class-1 HLA (Fig. 6B). While 95% of the mice harbouring control tumors developed at least one metastase in lymph nodes, only 39% of the mice harbouring FR-sema3C/myc tumors had at least one lymph node metastase (Fig. 6C). While metastases were
detected in 70% of the lymph nodes excised from mice harbouring control tumors, only 19% of the lymph nodes excised from mice harbouring FR-sema3C/myc tumors had metastases. Furthermore, the average size of metastases found in lymph nodes of mice harbouring FR-sema3C/myc expressing tumors was only 13% of the average size of metastases found in lymph nodes derived from mice harbouring control tumors (P<0.001) (Fig. 6D). Tomato red RFP labelled tumor cells could easily be seen in lymph vessels draining into lymph nodes in mice harbouring control tumors (Fig. 6SA). Notably, at 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 harboured size matched control or FR-sema3C expressing tumors (Fig. 6SB). 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 (Figs. 6S C&D). 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 pro-tumorigenic 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 since 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 pro-tumorigenic properties. Indeed, recombinant p65-Sema3C promoted significantly the survival of A549 lung cancer cells while FR-sema3C was completely devoid of such activity (Fig. 7SA). This preliminary experiment suggests that the reported pro-tumorigenic 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 cancer. 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 since 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 which 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 (17), FR-sema3C promoted the death of the LEC by a caspase-free 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 harbouring tumors derived from FR-sema3C expressing LM2-4 cells. Similar results were obtained even when the incidence of metastasis was compared between mice harbouring 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 which suggest that is an inhibitor of tumor progression, sema3C expressed in tumors was reported to function as a pro-tumorigenic factor (39-41). These observations are reminiscent of the similar pro-tumorigenic activity displayed by p61-sema3E which is the FPPC cleavage product of sema3E which 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 pro-tumorigenic 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.
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FIGURE LEGENDS

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 minutes incubation at 37°C they were fixed, stained with Cy3 conjugated anti-vinculin antibodies (red) and Cy2 conjugated Phalloidin (green) as described (mag. x40). Arrows designate 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 designate vinculin containing focal contacts. C. HUVEC and LEC were seeded on gelatin (HUVEC) or fibronectin (LEC) coated plates. HEK293 cells stained with Di-Asp (Arrows) and infected with empty vector (control) or expressing sema3C were seeded on endothelial cells. Phase contrast photographs were taken after 48h (mag. x10). Cell free "holes" produced as a result of repulsion are marked by a yellow border.

FIGURE 2

Characterization of FR-sema3C: A. The location of the two FPPC cleavage sites, the point mutation introduced into the first FPPC site, and the structure of the various sema3C deletion mutants are depicted. Fc or myc epitope tags were fused in frame upstream of the stop codon. B. Samples of conditioned medium from HEK293 cells expressing sema3C/Fc, FR-Sema3C-Fc, or conditioned medium from control cells were analysed by western blot using an antibody directed against the N-terminal of sema3C. C. The conditioned medium samples described under B were probed with antibodies directed against the Fc tag. D. An aliquot (10 μl) of FR-Sema3C/Fc purified from conditioned medium using protein-A sepharose was separated by SDS/PAGE and stained with...
coomassie brilliant blue. E. LEC were seeded on cover slips in 12 well dishes. After 24 hours the cells were stimulated with vehicle (control) or with purified FR-Sema3C-Fc (1µg/ml), Sema3C-p65-Fc (1µg/ml) or UNCL-Sema3E/Fc (35) (1µg/ml). After 40 minutes at 37°C the cells were fixed with 4% paraformaldehyde and stained with an antibody against vinculin (red) and with phalloidin (green) (mag. x40). Arrows point at vinculin stained focal contacts. F. Medium conditioned for 48h by U87MG cells expressing sema3C/myc, sema3C-noBD/myc or sema3C-p65/myc was adsorbed to heparin-sepharose at 4°C followed by elution with increasing NaCl concentrations. The conditioned mediums (CM), flow through (FT) and eluted fractions were then subjected to Western blot analysis using anti-myc antibodies.

**FIGURE 3**

*FR-sema3C inhibits LEC proliferation and VEGF-C induced signal transduction: A:* HUVEC or LEC were seeded in 12 well dishes (2x10^4 and 5x10^4 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 h. **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 re-probed 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 non-targeting shRNA (sh-control) were seeded in wells of the xCELLigence machine (2x10^4 cells/well) and cultured for 24h as described. They were subsequently stimulated with FR-sema3C/Fc (1µg/ml). Cell contraction over time was then measured.
Maximal contraction was determined as described in Fig. 3SB. Statistical analysis was performed using one way anova. *** indicate P<0.001

**Figure 4**

*FR-sema3C/myc does not affect cultured LM2-4 cells:* A. Conditioned medium from LM2-4 cells expressing tomato red RFP and either empty vector (control) or a FR-Sema3C/myc encoding lentiviral vector was subjected to western blot analysis using anti-myc antibodies. B. HUVEC and LM2-4 were seeded in gelatin coated 12 well plates (10^4 cells/well). After 24 hours the medium was exchanged with conditioned media from LM2-4 cells infected with empty lentiviruses (control) or with conditioned medium from FR-sema3C/myc expressing LM2-4 cells and cell contraction assayed as described (mag. x10). C. LM2-4 cells infected with FR-Sema3C/myc or with empty (Control) lentiviral vectors were seeded in 24 well dishes (1x10^5 cells/well). The number of adherent cells after seeding (Day 0) or after 3 days was determined using a coulter-counter. Error bars represent the standard error of the mean derived from three independent experiments. D. The migration of LM2-4 cells infected with empty lentiviral vector (control) or with lentiviruses directing expression of FR-sema3C/myc was measured using the xCELLigence machine.

**Figure 5**

*FR-sema3C inhibits tumor development, tumor angiogenesis and tumor lymphangiogenesis:* A. Tissue samples from control and FR-sema3C/myc expressing tumors were minced and lysed using RIPA lysis buffer. Shown is a western blot probed with an anti-myc epitope antibody. B. LM2-4 cells expressing tomato red RFP and either empty vector (control) or a FR-Sema3C/myc encoding lentiviral vector (2x10^6 cells in 50 µl PBS) were implanted in mammary fat pads of in-house bred scid/nod mice. Tumors were excised after 30 days. Shown are the average tumor weights from N tumors obtained from three independent experiments. C. Representative paraffin section stained with hematoxilin and an antibody directed against LYVE-1. Arrows indicate intra-tumoral lymph vessels.
D. The entire cross-section derived from each tumor was scanned and photographed. The ratio between the average area of LYVE-1 staining and the average area of the tumor sections (derived from N tumors) was determined using ImagePro. E. Representative paraffin section from a control tumor stained with hematoxilin and an antibody directed against CD31. Intra-tumoral blood vessels are indicated (arrows). F. The ratio between the average area of CD31 staining per tumor section from control and FR-sema3C/myc expressing tumors was determined as described under C.

**FIGURE 6**

**FR-sema3C inhibit the spontaneous metastasis of LM2-4 cells to lymph nodes:** A. LM2-4 cells expressing tomato red RFP and either empty vector (control) or a FR-Sema3C/myc encoding lentiviral vector were implanted in mammary fat pads. The proper axillary, lumbar aortic, and subiliac lymph nodes were excised after 30 days. The size of metastases in excised lymph nodes was quantified using the IVIS-200 imaging system. Lymph nodes containing metastases (yellow arrows) or not (green arrows) are shown. B. A representative histological section derived from a metastase containing lymph node derived from a mouse harboring a control tumor is shown at two different magnifications (a: 10x and b: 40x). Also shown is a histological sections from a clean lymph node from a mouse harboring a tumor containing FR-sema3C/myc expressing LM2-4 cells (c: 10x and d: 40x). The sections were stained with Anti human-HLA-1 antibodies and counterstained with hematoxilin. C. The percentage of mice containing detectable metastases in their lymph nodes in two independent experiments is depicted. D. The relative concentration of tomato red expressing LM2-4 cells in lymph nodes containing metastases was determined using the IVIS-200 system by measurement of the normalized photon density (photon/sec/cm2/sr) emitted.
Figure 1

A

Control  Sema3C  Sema3F

Sema3A  Sema3B  Sema3D

Sema3G  Sema3E  Sema6A

B

Control  Sema3C

C

Control  Sema3C

HUVEC

LEC
Figure 2

A

<table>
<thead>
<tr>
<th>Protein</th>
<th>Fc (or myc)</th>
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<tbody>
<tr>
<td>Sema3C</td>
<td></td>
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<tr>
<td>FR-Sema3C</td>
<td></td>
</tr>
<tr>
<td>Sema3C-noBD</td>
<td></td>
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<tr>
<td>Sema3C-p65</td>
<td></td>
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</table>

B

[Image of gel electrophoresis with bands at -130 and -70 kDa]

C

[Image of gel electrophoresis with bands at -130 kDa]

D

[Image of gel electrophoresis with bands at -130 kDa]

E

[Images of fluorescent microscopy showing control and FR-Sema3C/Fc treated cells]

F

[Table showing CM FT results with NaCl concentrations (0.3, 0.5, 0.6, 0.7, 0.8, 1.0 M) and corresponding protein bands at -100 and -70 kDa for Sema3C/myc, Sema3C-noBD/myc, and Sema3C-p65/myc]
Figure 3

A

<table>
<thead>
<tr>
<th></th>
<th>LEC</th>
<th>HUVEC</th>
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<td>Control</td>
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B

![Graph showing FR-Sema3C/Fc absorbance](graph.png)

C

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<tr>
<th>LEC lysates</th>
<th>FR-s3C/Fc</th>
<th>VEGF-C</th>
<th>pVEGFR-3</th>
<th>tVEGFR-3</th>
<th>pERK1/2</th>
<th>tERK1/2</th>
<th>pAKT</th>
<th>tAKT</th>
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</tr>
</tbody>
</table>

D

![Graph showing change in cell index](graph.png)

Change in cell index after stimulation with FR-Sema3C/Fc

- Control
- Sh-Np1
- Sh-plecin-A2
- Sh-plecin-A3
- Sh-plecin-D1

**p < 0.05**
Figure 4

A

Control  FR-Sema3C/myc

-100

-35

B

Control  FR-Sema3C/myc

HUVEC

LM2-4

C

Day: 0 3 0 3

Control  FR-Sema3C/myc

Cells/Well ($\times 10^4$)

3.0 x 10^4

2.5 x 10^4

2.0 x 10^4

1.5 x 10^4

1.0 x 10^4

5.0 x 10^3

0.0 x 10^2

D

Cell index of migrated cells

0.8

0.6

0.4

0.2

0.0

Time (hours)

0 5 10 15 20

Control  FR-Sema3C/myc
Figure 5

A

Control | FR-sema3C/Fc
1 2 3 4 5 6 7 8 9 10 11 12 13

α-myc

α-vinculin

B

N=27 N=30

Tumor weight (mg)

P=0.002

Control | FR-sema3C/myc

C

N=27 N=30

Tumor weight (mg)

P=0.002

D

N=13 N=15

Concentration of Lymph vessels (LYVE1 area/tumor section area (x10^-4))

P=0.05

Control | FR-s3C/myc

E

N=13 N=15

Concentration of Blood vessels (CD31 area/Tumor section area (x10^-4))

P=0.05

Control | FR-s3C/myc
Figure 6

A

Control FR-s3C/myc

Color Scale
Min = 1.29e8
Max = 3.36e9

B

Control
FR-s3C/myc

C

N=23  N=23

Mice with lymph node metastases (% of total)

Control  FR-s3C/myc

D

Concentration of metastatic cells in positive lymph nodes (p/s/cm²/sr)

Control  FR-s3C/myc

P=0.002
FULL LENGTH SEMAPHORIN-3C IS AN INHIBITOR OF TUMOR LYMPHANGIOGENESIS AND METASTASIS

Yelena Mumblat, Ofra Kessler, Neta Ilan, et al.

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