Hormonal Regulation and Distinct Functions of Semaphorin-3B and Semaphorin-3F in Ovarian Cancer

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Abstract

Semaphorins comprise a family of molecules that influence neuronal growth and guidance. Class-3 semaphorins, semaphorin-3B (SEMA3B) and semaphorin-3F (SEMA3F), illustrate their effects by forming a complex with neuropilins (NP-1 or NP-2) and plexins. We examined the status and regulation of semaphorins and their receptors in human ovarian cancer cells. A significantly reduced expression of SEMA3B (83 kDa), SEMA3F (90 kDa), and plexin-A3 was observed in ovarian cancer cell lines when compared with normal human ovarian surface epithelial cells. The expression of NP-1, NP-2, and plexin-A1 was not altered in human ovarian surface epithelial and ovarian cancer cells. The decreased expression of SEMA3B, SEMA3F, and plexin-A3 was confirmed in stage 3 ovarian tumors. The treatment of ovarian cancer cells with luteinizing hormone, follicle-stimulating hormone, and estrogen induced a significant upregulation of SEMA3B, whereas SEMA3F was upregulated only by estrogen. Cotreatment of cell lines with a hormone and its specific antagonist blocked the effect of the hormone. Ectopic expression of SEMA3B or SEMA3F reduced soft-agar colony formation, adhesion, and cell invasion of ovarian cancer cell cultures. Forced expression of SEMA3B, but not SEMA3F, inhibited viability of ovarian cancer cells. Overexpression of SEMA3B and SEMA3F reduced focal adhesion kinase phosphorylation and matrix metalloproteinase-2 and matrix metalloproteinase-9 expression in ovarian cancer cells. Forced expression of SEMA3F, but not SEMA3B in ovarian cancer cells, significantly inhibited endothelial cell tube formation. Collectively, our results suggest that the loss of SEMA3 expression could be a hallmark of cancer progression. Furthermore, gonadotropin- and/or estrogen-mediated maintenance of SEMA3 expression could control ovarian cancer angiogenesis and metastasis. Mol Cancer Ther; 9(2): 499–509. ©2010 AACR.

Introduction

Semaphorins are a large class of secreted or membrane-associated proteins that act as chemotactic cues for cell migration and axon guidance (1–3). Although best characterized in the nervous system (4–6), they are widely expressed in other tissues where they have a role in angiogenesis, organogenesis, and immune cell regulation (7, 8). There is a growing body of evidence suggesting that semaphorins and their receptors may play a regulatory role in carcinogenesis (9–11).

Two receptor families have been implicated in mediating the actions of class 3 semaphorins: the neuropilins (NP) and plexins (10, 11). The nine known plexins are divided into four subfamilies (A through D) based on structure. Most semaphorins bind directly to plexins to activate plexin-mediated signal transduction, but class 3 semaphorins, however, do not appear to bind plexins directly. Rather, the functional receptors for these semaphorins are complexes of NP and A-type plexins, with the former serving as the ligand-binding moiety and the latter as the signal-transducing component (2, 12). Neuropilins are membrane receptors. There are two NPs (NP-1 and NP-2), which bind class 3 semaphorins and vascular endothelial growth factors, and regulate two diverse systems, neuronal guidance and angiogenesis (13–16).

Two related secreted semaphorins, semaphorin-3B (SEMA3B) and semaphorin-3F (SEMA3F), were isolated from a region on human chromosome 3p21.3 commonly deleted in cancer (17, 18). Loss of heterozygosity in this region is frequent in cancers of the lung, breast, ovary, and other organs (17–20). Thus, both genes are candidate tumor suppressors (21, 22). Both semaphorins (SEMA3B and SEMA3F) normally show high levels of expression in normal tissues and low-grade tumors but are downregulated in highly metastatic tumors in the lung, melanoma cells, bladder carcinoma cells, and prostate carcinoma (23, 24). Reexpression of SEMA3B and SEMA3F increases...
apoptosis and inhibits cell growth in human lung, breast, and ovarian cancer cells, and in A9 mouse fibrosarcoma cell line (22, 25, 26). These findings strongly implicate SEMA3B and SEMA3F as functional inhibitors of tumor cell growth.

Tse et al. (21) and Xiang et al. (22) showed that ovarian cancer cells expressing SEMA3B or SEMA3F exhibited a diminished tumorigenicity in nude mice. Functional redundancy between these proteins has hindered the dissection of their individual contribution in the inhibition of tumor growth. The aforementioned studies did not address the role of SEMA3B and SEMA3F in invasion, angiogenesis, and metastasis, nor did they address the mechanisms underlying these inhibitory activities. To the best of our knowledge, no information is available about whether key reproductive hormones regulate the expression of semaphorins in malignant ovarian surface epithelial cells. The aim of the present study was to examine the regulation of SEMA3B and SEMA3F by hormones and to decipher whether SEMA3B and SEMA3F play different roles in tumorigenesis. Our results indicated that both semaphorins were upregulated by estrogen and, when overexpressed, inhibited cell motility and invasive-ness through decreased focal adhesion kinase (FAK) phosphorylation and inhibition of matrix metalloprotei-nase (MMP)-2 and MMP-9 expression. However, tube for-mation and inhibition of tumor growth. The aforementioned studies did not address the role of SEMA3B and SEMA3F in invasion, angiogenesis, and metastasis.

RNA Isolation and Reverse Transcription-PCR Analysis. Total RNA was isolated using the TRI-reaagent (Sigma) according to the protocol provided by the manufacturer. Hot-start PCR using AmpliTaq Gold DNA polymerase (Applied Biosystems) was used in all amplification reactions. The PCR program for human SEMA3B (Forward 5′-TGCCGCCGCTTCGAGGGAAGA-3′, Reverse 5′-GGCCACCTCCTGGCCCTGGT-3′), SEMA3F (Forward 5′-TGAAGAACCCTGTCATTTACG-3′ Reverse 5′-AA-TAGTGTAGCAGCGTATTATGGA-3′) and β-actin (Forward 5′-CTAAACGGGAAGGCTGGAAGT-3′, Reverse 5′-CAACCGGGAGAAATTTCTTAA-3′ and Reverse 5′-CTAGTGATGATGCTGTTG-3′), and β-actin (Forward 5′-CAACCGGGAGAAATTTCTTAA-3′ Reverse 5′-CAACCGGGAGAAATTTCTTAA-3′) were 20 cycles for SEMA3B, 22 cycles for SEMA3F, 35 cycles for plexin-A3, and 18 cycles for β-actin at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, and final extension at 72°C for 7 min. The sizes of the PCR products were 392, 354, and 193 bp for SEMA3B, SEMA3F, and plexin A3, respectively.

Immunoblot Analysis. Cellular extracts were prepared as previously described (30). Briefly, cell lysates were prepared in radioimmunoprecipitation assay buffer, and proteins extracted (20 μg) from each cell culture were separated by electrophoresis on 7.5% or 10% SDS-polyacrylamide gels. The blots were incubated overnight at 4°C in blocking solution with either SEMA3B (Abcam, Inc.; 1:5,000), SEMA3F (Millipore; 1:5,000), NP-1 (Abcam, Inc.; 1:1,000), NP-2 (Santa Cruz Biotechnology, Inc; 1:1,000), plexin-A1 (Cell Signaling Technology; 1:1,000), plexin-A3 (Novus Biologicals; 1:250), MMP-2 (Abcam, Inc.; 1:200), MMP-9 (Cell Signaling; 1:1,000), p-FAK (Abcam, Inc.; 1:1,000), FAK (BD Transduction Laboratories; 1:2 μg/mL), or β-actin antibody (Sigma-Aldrich; 1:25,000). After washing with Phosphate Buffered Saline Tween-20 (PBST), the membranes were then incubated with the secondary antibody at a 1:3,000 dilution in 5% nonfat dry milk in Phosphate Buffered Saline Tween-20 for 2 h at room temperature. After washing, bound antibodies were detected by using an enhanced

Materials and Methods

Cell Lines and Culture Conditions. The human ovarian surface epithelium (HOSE) cell lines (HOSE 6-8 and HOSE 642) were generated by scrapping and immortalization of epithelial cells from the surface of normal human ovaries (27). The ovarian cancer cell lines (ovarian cancer 420 and ovarian cancer 429) were established from patients with late-stage serous ovarian cancer (28). We have described the culture conditions and phenotypes of these lines in our previous publications (29, 30). Briefly, all cell lines were grown in MCDB105/Medium 199 (1:1; Sigma-Aldrich) supplemented with fetal bovine serum (Invitrogen) and antibiotics (penicillin/streptomycin). All cell lines were grown in 5% CO2 in air.

Hormonal Treatment of Ovarian Cancer Cell Lines. The ovarian cancer (2 × 10^6) cells were seeded in a T-25 flask and were allowed to attach for 24 h. In experiments in which the effects of hormones were studied, the cells were cultured in a medium supplemented with 10% charcoal-stripped fetal bovine serum for 48 h before hormone treatment. To study the dose- and time-dependent effect of hormones on SEMA3B and SEMA3F expression, the cells were either exposed to various doses of hormones (0–20 nmol/L) for 24 h or to 10 nmol/L follicle-stimulating hormone (FSH; Calbiochem), 10 nmol/L luteinizing hormone (LH; Calbiochem), 10 nmol/L 17β-estradiol (E2; Sigma), 10 nmol/L testosterone (Sigma), or 10 nmol/L progesterone (P4; Sigma) for 3, 6, 24, 48, and 72 h, respectively. Steroid solutions were in absolute ethanol, and gonadotropin solutions were in aqueous saline. The final concentration of ethanol in the medium was 0.1%. Untreated control cultures were exposed to equal concentrations of ethanol or aqueous saline vehicle alone. For a set of experiments, ovarian cancer cell lines were cultured with hormones in the presence or absence of the respective receptor or signaling antagonist. One dose of receptor antagonist was used to block the action of the hormone. For FSH and LH, 0.1 nmol/L of the protein kinase A–selective inhibitor H89 (N-[2-(bromocinnamyl)amino]ethyl]-5-isooquinolinesulfonamide; 2HCL; Calibiochem) was added 30 min before treatment with 10 nmol/L of the gonadotropins. For estrogen receptor–specific antagonists ICI 182,780, 0.1 nmol/L (a generous gift from Zeneca Pharmaceuticals) was used. The cell cultures were treated daily with hormones or hormone antagonists for a period of 3 d, cells were collected, and protein was extracted.

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chemiluminescence detection system (Pierce). The SEMA3B antibody recognizes both the long and short form of SEMA3B. SEMA3F is alternatively spliced and expressed as a short form and long form. However, the Millipore anti-SEMA3F antibody only recognizes the long form of SEMA3F. Thus, the whole amount of SEMA3F made by the cells is not shown by this antibody.

Transfection of SEMA3B and SEMA3F in Ovarian Cancer Cells. The vectors containing SEMA3B and SEMA3F (short form, 755 amino acid) were kindly provided by Dr. John D Minna (Hamon Center for Therapeutic Oncology Research, University of Texas Southwestern Medical Center, Dallas, TX) and Dr. Gera Neufeld (Cancer Research and Vascular Biology Center, The Bruce Rappaport Faculty of Medicine, Technion, Israel Institute of Technology, Haifa, Israel). Ovarian cancer (ovarian cancer 420 and ovarian cancer 429) cells were transfected with SEMA3B or SEMA3F vectors using the Lipofectamine 2000 reagent (Invitrogen). The overall transfection efficiency for ovarian cancer cells assessed by the 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside staining assay against p5Vg-galactosidase vector-transfected cells was 67% to 71%. Cellular extracts were prepared for Western blotting and caspase-3 analyses.

Cell Viability Assay. Cell viability was determined using the CellTiter96 AQueous One Solution cell viability assay (Promega) according to the instructions of the manufacturer. Cell viability was evaluated after transfection of ovarian cancer cells with SEMA3B or SEMA3F vectors, or empty vectors. On the fourth day, 20 μL of Cell Titer 96 Aqueous one solution reagent were added into each well of the 96-well assay plate containing the samples in 100 μL of culture medium. Absorbance was measured at 490 nm using a microtiter plate reader. Relative cell viability was expressed as percent change of transfected cells over empty vector–transfected cells.

Soft Agar Assay. The effects of ectopic expression of SEMA3B and SEMA3F on anchorage-independent growth were measured as their abilities to form colonies on soft agar. Ovarian cancer 420 and ovarian cancer 429 cells were transfected with SEMA3B or SEMA3F vectors (1 μg/mL), or the empty vectors as described above. After 18 h, transfected cells were cultured at 5,000 cells per 100-mm plate (four plates per sample) in 0.3% agar above an underlayer of 0.6% Noble agar, both containing complete medium (31). Number of colonies was counted after 14 d of culture.

Invasion Assay. The Biocoat Matrigel Invasion Chambers (BD Biosciences) were used to assess the effects of the ectopic expression of SEMA3B or SEMA3F on the invasive property of ovarian cancer cells. Matrigel chambers were rehydrated at 37°C for 2 h. Ovarian cancer cells (2.5 × 10⁴ cells; ovarian cancer 420 and ovarian cancer 429), transfected with an empty vector or a vector carrying an expression plasmid of SEMA3B or SEMA3F, were seeded in the inserts of the Matrigel Invasion Chambers. Serum added to the bottom chamber was used as the chemoattractant. At 24 h after plating, noninvading cells were removed and invading cells were counted in five fields per slide as previously described (30). All slides were coded to avoid biased counting. The assay was run in triplicate.

Adhesion Assay. Adhesion assays were done as previously described (32). Both SEMA3B- and SEMA3F-transfected, and empty vector–transfected ovarian cancer cells were collected after trypsinization in medium containing 1% serum. Briefly, 1 × 10⁴ cells were plated in triplicate on 96-well plates coated with fibronectin, collagen type-1, or laminin (10 μg/mL) at 37°C for 90 min. Cells were then washed thrice with PBS to remove nonadhering cells, and the adherent cells were fixed with 100% methanol for 5 min at room temperature. Cells were stained with 0.5% crystal violet for 15 min. Stained cells were washed with PBS and were incubated with 2% SDS at room temperature for 30 min. Cell adhesion was determined by measuring absorbance at 590 nm.

Endothelial Tube Formation Assay. The human umbilical vascular endothelial cells (HUVEC) were cultured in EBM-2 medium containing serum and endothelial cell supplements according to the instructions of the endothelial tube formation assay kit manufacturer (Chemicon International). The cells were washed twice with PBS and were trypsinized, and then 5 × 10⁴ HUVEC cells per well were cultured in 24-well plates coated with Matrigel in the presence of conditioned media (CM) from ovarian cancer cells overexpressing SEMA3B or SEMA3F. The controls of experiments were CM from cells transfected with their respective empty vectors. After 18 h, tube formation was assessed under an inverted light microscope (magnification, ×100) and cultures were photographed. The experiment was repeated thrice, with duplicate samples.

Gelatin Zymography. Subconfluent monolayers of ovarian cancer cells, transfected with SEMA3B or SEMA3F, were grown in T-25 tissue culture flasks and were serum starved in serum-free media overnight. Cells were cultured for an additional 24 h. Cell culture CM were collected, cleared by centrifugation, and concentrated 5-fold using Centricon centrifugal filters (Millipore). CM equivalent to 200 μg of ovarian cancer cells was analyzed by gelatin zymography. CM were electrophoresed on a 10% SDS-polyacrylamide gel containing 0.1% to 0.2% gelatin. Gels were washed twice with washing buffer [50 mmol/L Tris-HCl (pH 7.5), 100 mmol/L NaCl, and 2.5% Triton X-100]. Gels were then treated with incubation buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 10 mmol/L CaCl₂, 0.02% NaN₃, and 1 μmol/L ZnCl₂] at 37°C for 18 to 36 h, were stained (0.05% Coomassie blue, 10% isopropanol, and 10% acetic acid), and were destained (10% isopropanol and 10% acetic acid). MMPs were detected as transparent bands on the blue background of Coomassie blue–stained slab gels.

Caspase-3 Activity Assay. The ovarian cancer cells transfected with SEMA3B or SEMA3F vectors were collected from each culture and suspended in 500 μL of...
ice-cold lysis buffer provided with the Caspases Assay kit (MLB International). After sonification, the cell lysates were centrifuged for 20 min at 14,000 g at 4°C. The resulting supernatants were analyzed for protein concentrations by the Bradford dye-binding assay and stored at −20°C until use. Colorimetric caspase-3 enzyme activity assay was done according to the manufacturer’s instructions.

**Statistical Analyses.** Statistical analysis was carried out using ANOVA, followed by Tukey’s *post hoc* test. Values are presented as the mean ± SEM and are considered significant at a *P* value of <0.05.

**Results**

**Expression of SEMA3B, SEMA3F, and Their Receptors in HOSE and Ovarian Cancer Cells.** The expression of SEMA3B and SEMA3F was examined in CM and cellular extracts of a panel of HOSE and ovarian cancer cell lines by Western blot analysis. Both semaphorins were strongly expressed in CM and extracts of HOSE cell lines, whereas a marked decrease in the SEMA3B and SEMA3F expression was observed in CM and extracts of ovarian cancer cells (Fig. 1A). Mouse cerebellum and rat brain lysates were used as positive controls for SEMA3B and SEMA3F, respectively (Fig. 1B and C). Lysates probed with only secondary antibody served as negative controls (Fig. 1B and C). SEMA3 family members act through a receptor complex composed of NP-1, NP-2, plexin A-1, and plexin A-3 receptor (2, 33, 34). In both human U87MG glioma cells and human umbilical vein cells, plexin-A1 forms a complex with NP-2 (34). SEMA3 binds to NPs (NP-1 or NP-2), then the complex of SEMA3 and NP binds to plexin (A1 or A3). Therefore, we also analyzed the expression of NP-1, NP-2, plexin-A1, and plexin-A3 in HOSE and ovarian cancer cell lines. NP-1, NP-2, and plexin-A1 receptors were uniformly expressed in all normal and cancer cell lines, whereas the expression of plexin A-3 was significantly lower in ovarian cancer cell lines than HOSE cells (Fig. 1A). We next analyzed the expression levels of SEMA3B, SEMA3F, and plexin-A-1 in different grades (G0, G1, and G3) of ovarian tumors by semiquantitative reverse transcription-PCR to compare

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**Figure 1.** Downregulation of SEMA3B, SEMA3F, and plexin A-3, but not NP-1, NP-2, and plexinA1 in ovarian cancer cells. **A,** normal immortalized HOSE cells (HOSE 6-8, and HOSE 642), and ovarian cancer cells (ovarian cancer 420 and ovarian cancer 429) were evaluated by Western blot for the expression of SEMA3B, SEMA3F, plexin-A1, plexin A-3, NP-1, and NP-2. Cells and CM were harvested, and 20 μg of protein from the whole-cell extract or CM were loaded in each lane. The blot was probed with the indicated antibody. β-Actin was used as a loading control. **B,** mouse E16 cerebellum and (C) rat brain lysates were used as positive controls for SEMA3B and SEMA3F, respectively. Lysates probed with only secondary antibody (rabbit IgG) were used as negative controls. **D,** suppression of SEMA3B, SEMA3F, and plexin A-3 expression in G3 ovarian tumors. Total cellular RNA (1 μg) was isolated from epithelial ovarian tumor tissues of different grades varying from G0 to G3. Semiquantitative reverse transcription-PCR was used to compare SEMA3B, SEMA3F, and plexin A-3 mRNA expression in control normal ovarian tissue (normal, *n* = 3), benign tumor (G0, *n* = 3), borderline tumor (G1, *n* = 3), and high-grade tumor (G3, *n* = 3). SEMA3B, SEMA3F, and plexin A-3 expression levels were significantly lower in G3 ovarian tissues.
and establish the expression levels in silico. SEMA3B expression was suppressed in G1 and G3 tumors, whereas SEMA3F and plexin A-3 was downregulated in G3 ovarian tumors (Fig. 1D).

Dose- and Time-Dependent Effect of Gonadotropins and Steroid Hormones on the Expression of SEMA3B and SEMA3F in Ovarian Cancer Cells. Next, we determined the dose-dependent effects of hormones on the activation of SEMA3B and SEMA3F in ovarian cancer cells. Cells were treated with indicated concentrations of hormones. Total cellular lysates were subjected to Western blot analysis. A representative Western blot of ovarian cancer 420 and ovarian cancer 429 cells treated with hormones is shown in Fig. 2A. Treatment with FSH, LH, and estradiol (5–20 nmol/L) caused a dose-dependent increase in SEMA3B (active form 83-kDa and 50-kDa cleaved form) expression in ovarian cancer cells compared with control cells (Fig. 2A). Levels of SEMA3B, however, remain unchanged following P4 and testosterone treatment (Fig. 2A). A dose-dependent increase in SEMA3F (90 kDa) expression was observed in ovarian cancer cells only with estradiol treatment. Treatment of cells with LH, FSH, P4, or testosterone did not cause any changes in the expression of SEMA3F (Fig. 2A).

To determine the time course of SEMA3B and SEMA3F expression, ovarian cancer cells were stimulated with various hormones and were harvested at various time points. The ovarian cancer (ovarian cancer 420 and ovarian cancer 429) cell lines were treated with medium alone or with FSH (10 nmol/L), LH (10 nmol/L), estradiol (10 nmol/L), testosterone (10 nmol/L), or P4 (10 nmol/L) for 3, 6, 24, 48, and 72 hours. SEMA3B and SEMA3F protein were then measured by Western blot.
Figure 3. The effect of SEMA3B and SEMA3F overexpression on ovarian cancer cell malignant phenotypes. The effect of SEMA3B and SEMA3F overexpression on the viability, colony formation, and migration was investigated in two ovarian cancer cell lines. The cell lines (ovarian cancer 420 and ovarian cancer 429) were transfected with a vector containing SEMA3B or SEMA3F. Control cells were transfected with respective empty vectors. Second control was cells without transfection. 

A, protein from transfected ovarian cancer cell lines was extracted and analyzed for the expression of SEMA3B and SEMA3F by Western blot. A significant difference in the protein levels in transfected cell lines and those observed in control cell cultures is shown in the figure. 

Ba, after 3 d of transfection, cell viability was measured by MTS assay and the viability of transfected cells was expressed as percentage growth compared with that of control cells (100%). Columns, mean of three independent experiments; bars, SEM. Statistically significant decreases in cell growth compared with those seen in control cells are indicated by *, P < 0.05.

Bb, SEMA3B and SEMA3F-transfected and control cells were cultured on soft agar, and 2 wk later, colonies were counted. The value shown is the percentage compared with that of empty vector–transfected control cells (100%). Columns, mean of three independent experiments; bars, SEM. *, statistically significant changes in colony formation, compared with those seen in control cells (P < 0.05).

Bc, ovarian cancer cells transfected with SEMA3B and SEMA3F were plated on Matrigel. After 22 h, cells that migrated through the Matrigel were counted. Columns, mean of three independent experiments; bars, SEM. *, statistically significant changes in cell invasion, compared with those seen in control cells (P < 0.05).

C, activation of caspase-3 by overexpression of SEMA3B and SEMA3F in ovarian cancer cell lines. Three days following transfection, ovarian cancer (ovarian cancer 420 and ovarian cancer 429) cells were harvested and analyzed for caspase-3 activity by measuring the cleavage of pNA-labeled caspase-3–specific substrate, DEVD/pNA. Columns, mean of three experiments; bars, SEM. *, statistical significance of difference between empty vector–transfected cells and SEMA3B– or SEMA3F–transfected group.
analysis. Levels of SEMA3B in ovarian cancer cell lines exposed to FSH, LH, or E2 for 6 hours were significantly higher than untreated control cultures (Fig. 2B). The expression of SEMA3F in ovarian cancer cells was markedly enhanced only when cells were exposed to estrogen for 24 hours (Fig. 2B). Testosterone and P4 treatment had no time-dependent effect on the expression of SEMA3B (Fig. 2B). FSH, LH, testosterone, and P4 failed to exhibit time-dependent effects on SEMA3F expression (Fig. 2B).

To ascertain whether the observed gonadotropin- or steroid-stimulated SEMA3B or SEMA3F expression was mediated through a receptor-mediated pathway, ovarian cancer cell lines were treated with FSH, LH, or E2 (each at 10 nmol/L) for 24 hours in the presence of the protein kinase A–selective inhibitor H-89 at 0.1 mmol/L (for FSH and LH), the antiestrogen ICI 182,780 (ICI) at 0.1 mmol/L (for E2), or with vehicle alone. Addition of H-89 markedly decreased the gonadotropin-stimulated expression of SEMA3B in both cell lines. However, in ovarian cancer 420, SEMA3B expression levels did not go down to control levels in the presence of H-89 (Fig. 2C). When ovarian cancer cells were exposed to a 24-hour treatment with E2 in the presence of ICI 182,780, a marked attenuation in E2-induced SEMA3B and SEMA3F was observed in cultures exposed to the antiestrogen (Fig. 2C). ICI alone had no effect on SEMA3B and SEMA3F expression (Fig. 2C). H-89 alone had no effect on SEMA3B (Fig. 2C). We analyzed and compared the expression of SEMA3B and SEMA3F in estrogen-nonresponsive (SKOV-3) cells, to that of ovarian cancer 420 and ovarian cancer 429. Both semaphorins were expressed in SKOV-3 cell line albeit at low levels, but comparable with ovarian cancer 420 and ovarian cancer 429. Estrogen treatment of SKOV-3 had no effect on SEMA3B and SEMA3F expression (Fig. 2D).

** Forced Expression of SEMA3B or SEMA3F Alters Cell Viability, Colony Formation, and Cell Invasion.** To study the biological functions, SEMA3B and SEMA3F were ectopically expressed in ovarian cancer cells. Ovarian cancer cells transfected with the expression plasmids of SEMA3B and SEMA3F showed strong expression of the respective semaphorin. As expected, ovarian cancer cells transfected with control vector showed little or no expression of SEMA3B or SEMA3F (Fig. 3A). The effect of SEMA3B or SEMA3F overexpression on cell viability was analyzed in two ovarian cancer cell lines. In three independent growth assays, ovarian cancer cell overexpressing SEMA3B showed dramatically reduced cell numbers (~70% inhibition) compared with the control empty vector–transfected cells, whereas overexpression of SEMA3F did not affect the viability of ovarian cancer cells. The inhibition of ovarian cancer cell adhesion and metastasis by the ectopic expression of SEMA3B and SEMA3F. A, the effect of SEMA3B and SEMA3F overexpression on adhesion to ECM components (fibronectin, collagen type 1, and laminin) was investigated in two ovarian cancer cell lines. SEMA and mock-transfected cells (1 × 10⁴ cells per well) were transferred in wells coated with ECM components and then incubated for 90 min. After gentle washing with warm PBS, cell adhesion was determined by measuring absorbance at 590 nm. Columns, mean of three independent experiments done in triplicate; bars, SEM. *, P < 0.05 (mock-transfected versus SEMA-3–transfected cells). B, restoration of SEMA3B and SEMA3F expression in ovarian cancer cells decreases MMP-2 and MMP-9 protein expression. Total cellular protein extracts were extracted as described in Materials and Methods. Representative picture from three independent experiments is shown. β-Actin serves as loading control. C, the gelatinolytic activity of MMP-2 and MMP-9 in CM of ovarian cancer cells. Ovarian cancer cells overexpressing SEMA3B or SEMA3F or mock empty vector–transfected cells were serum starved for 12 h, and then incubated in the serum-free culture medium for 24 h. CM were collected and then subjected to gelatin zymography.
Overexpression of SEMA3B or SEMA3F in ovarian cancer cell lines significantly reduced colony formation efficacy in soft agar (Fig. 3B, B). The effect of SEMA-3 restoration on invasive capabilities of ovarian cancer cells was investigated using invasion chambers. Ectopic expression of SEMA3B and SEMA3F reduced the invasive potential of ovarian cancer cells through Matrigel. No change in tumor cell phenotypes was observed in ovarian cancer cells transfected with empty vectors (Fig. 3B, A–C). To investigate whether semaphorin overexpression activates apoptosis, we measured the activation of caspase-3 in ovarian cancer cells by a colorimetric substrate assay. The ectopic expression of SEMA3B increased caspase-3 activity in ovarian cancer cell lines (Fig. 3C). Caspase-3 activity was not affected in ovarian cancer cell lines following ectopic expression of SEMA3F (Fig. 3C). To show specificity, the active cell lysates were preincubated with a caspase-3 inhibitor [DEVD-FMK, Z-Asp(OH3)-Glu(OH3)-Val-Asp(OH3)-CH2F] before the addition of the substrate. DEVD was able to abolish the SEMA3B-induced activation of caspase-3 activity (Fig. 3C).

**Expression of SEMA3B and SEMA3F Decreases Cell Adhesion.** Adhesion and invasion are two of the key components of the metastatic cascade. We examined the adhering ability of ovarian cancer cells that overexpress either SEMA3B or SEMA3F to extracellular matrix (ECM) components, fibronectin, collagen type 1, and laminin. Of the $1 \times 10^4$ cells plated on plates coated with different ECM components, marked reduction in adhesion was seen in cells ectopically expressing SEMA3B or SEMA3F in both ovarian cancer cell lines compared with receptive controls (Fig. 4A). Although the intensity of suppression of tumor cells to ECM component varied, nonetheless, SEMA3B and SEMA3F significantly reduced the adhesion of ovarian cancer cells to surfaces coated with different ECM components (Fig. 4A).

**Restoration of SEMA3B and SEMA3F Expression Attenuates MMP-2 and MMP-9 Expression in Ovarian Cancer Cells.** Given an important role of MMP-2 and MMP-9 in cancer progression and their involvement in cell-matrix interaction and tumor invasion, we examined the protein expression of MMP-9 and MMP-2 and their proteolytic activity in ovarian cancer cells overexpressing either SEMA3B or SEMA3F. The ectopic expression of SEMA3B and SEMA3F resulted in a decreased protein expression of MMP-9 and MMP-2 in ovarian cancer cells compared with vector control transfectants (Fig. 4B). Zymography done with CM from ovarian cancer 420 and ovarian cancer 429 cells overexpressing SEMA3B or SEMA3F also indicates reduced MMP activity in ovarian cancer 420 and ovarian cancer 429 cell lines (Fig. 4C).

**Overexpression of SEMA3B and SEMA3F in Ovarian Cancer Cells Is Associated with Decreased FAK Phosphorylation.** FAK is an important regulator of cell adhesion and invasion (34). Having shown that SEMA3B and SEMA3F overexpression inhibited ovarian cancer cell adhesion and invasion, we sought to determine whether FAK activation was altered in these cells. The activation of FAK requires tyrosine phosphorylation at 397 (Y397; ref. 35). Therefore, we examined FAK activation in SEMA3B- and SEMA3F-overexpressing cells by Western blotting using phosphorylo-Y397-FAK–specific antibody. As shown in Fig. 5, SEMA-3-overexpressing cells exhibited a decreased level of FAK phosphorylation compared with empty vector–transfected cells.

**Ectopic Expression of SEMA3F Inhibits Capillary-Like Tube Formation of Endothelial Cells.** To determine the effect of ectopic expression of SEMA3B or SEMA3F on angiogenesis, we examined the formation of capillary-like tube structures in an in vitro model of angiogenesis using HUVEC cells. In the absence of growth factors,
HUVEC cells did not form tube-like structures and remained round and isolated on the Matrigel bed (data not shown). The addition of CM from ovarian cancer 420 and ovarian cancer 429 cells transfected with empty vectors induced capillary-like tube formation. However, the addition of media from SEMA3F-overexpressing ovarian cancer cells partially inhibited capillary-like tube formation, whereas SEMA3B did not inhibit tube formation. Similar results were obtained from both cell lines. Results of ovarian cancer 429 are shown in Fig. 6.

Discussion

Class 3 semaphorins (SEMA3B and SEMA3F) have been extensively studied for their involvement in the progression of cancer (25, 26, 36). However, limited information is available on their role in ovarian cancer. Two studies have suggested that expression of SEMA3B and SEMA3F in ovarian cancer cells diminish tumorigenicity in nude mice (21, 22). A major goal of this research was to generate data on the status of SEMA3B and SEMA3F in normal ovarian surface epithelial cells and their regulation by hormones in ovarian cancer cells. To the best of our knowledge, the present study is the first report to document the regulation of semaphorins by hormones in ovarian cancer cells. In our studies, we found high levels of SEMA3B and SEMA3F in cell lysates and CM of HOSE cells than in ovarian cancer cells. The expression pattern of SEMA3 in cell lysates is similar to the expression of SEMA3 in CM. In addition, we found that estradiol and gonadotropins upregulated the expression of SEMA3B, whereas SEMA3F expression was exclusively enhanced by estradiol in ovarian cancer cells. The effects of gonadotropins (FSH and LH) and estrogen on the expression of the two SEMA-3s were blocked by their inhibitors (H89 and ICI, respectively), providing evidence of specificity. Our findings are partially in discord with the previous report on breast cancer cells in which increased SEMA3B and decreased SEMA3F expression was observed following estrogen treatment (37). This discrepancy may be tissue specific and could be due to differential responsiveness to hormones. To explore if there are differences in the expression of SEMA3B and SEMA3F in estrogen-responsive and estrogen-nonresponsive ovarian cancer cells, we have used SKOV-3 cell line. A 32-bp deletion in exon 1 of the estrogen receptor α is implicated in the estrogen resistance of SKOV-3 cell line (38). More importantly, our results showed the suppression of SEMA3B and SEMA3F in estrogen-responsive and estrogen-nonresponsive ovarian cancer cell lines. The expression of both semaphorins can be restored by estradiol in cell lines with intact estrogen receptor. From a clinical prospective, these findings will have implications in preventing estrogen receptor positive ovarian tumor progression.

We observed a decreased expression of SEMA3B and SEMA3F in G3 ovarian tumors. These results agree with previous studies that found diminished expression of SEMA3 in lung and ovarian tumors (17–19, 39). In the present study, we found that ovarian cancer cells exhibit low levels of plexin A-3 protein compared with normal cells. We did not find any alteration in the expression of NP-1 and NP-2 in HOSE and ovarian cancer cells. Contrary to our results, the expression of NP-1 and NP-2 was significantly increased in ovarian and breast carcinomas compared with benign and borderline tumors (39, 40). This discrepancy may be related to significant differences between NP regulation in vitro and in vivo.

We aimed at finding distinct functions of SEMA3B and SEMA3F in ovarian cancer. Ectopic expression of SEMA3B in ovarian cancer cell lines resulted in the significant inhibition of cell growth and invasiveness. However, no growth inhibition was seen in ovarian cancer cell lines overexpressing SEMA3F. This difference in growth inhibition was not caused by the suboptimal expression of SEMA3F in ovarian cancer cell lines, as levels of SEMA3B and SEMA3F were equally upregulated in ovarian cancer cells after transfection. This observation strongly supports our conclusion that SEMA3F has no effect on ovarian cancer cell viability, whereas SEMA3B exerts inhibitory effect on cell growth. Our results are contradictory to those reported by Xiang et al. (22) who showed that the expression of SEMA3F by the ovarian cancer cell line HEY diminished their proliferation. Our findings agree with the results of Tomizawa et al. (25) who reported that ectopic expression of SEMA3F had no effect on lung cancer cell viability. A recent study has suggested that the antitumorigenic effect of SEMA3F depends on the expression of NP receptors. SEMA3F binds to NP-1, albeit with a 10-fold lower affinity compared with its binding affinity to NP-2. SEMA3F does not inhibit the development of tumors from breast cancer MDA-MB-231 cells. This effect is attributed to the low expression of NP-2 receptors on these cells (41). Our results showed no differences in the expression levels of NP-1 and NP-2 receptors on ovarian cancer cells. Thus, the lack of effect of SEMA3F on cell viability could not be explained by the low expression of NP-2 receptors on ovarian cancer cells. It is possible that anchorage-dependent and anchorage-independent growth pathways are mediated by differential concentrations of SEMA3F. The SEMA3F ectopically expressed in ovarian cancer cells could be sufficient to mediate anchorage-independent pathway and not anchorage-dependent pathway.

It is widely known that SEMA3B and SEMA3F are modulators of metastasis (7, 10). Using in vitro cell adhesion assay, we have shown that SEMA3B and SEMA3F expression affect the adhesive ability of ovarian cancer cells. Thus, the upregulated expression of SEMA3B and SEMA3F is likely to inhibit the growth of the cancer cells in the metastatic sites due to a decrease in cell adhesive ability. FAK is found overexpressed in invadopodia of tumor cells, and FAK silencing inhibits the metastasis of cells (42–44). Phosphorylation at residue Y397 is critical for FAK activation in invadopodia. Our results showed a marked inhibition of phospho Y397 FAK in ovarian cancer cells overexpressing SEMA3B and SEMA3F, implicating...
that these two semaphorins may impair invadopodia formation, rendering them less effective for invasion.

In the present study, we have shown that the re-expression of SEMA3F, but not SEMA3B, in ovarian cancer cells inhibited tube formation of endothelial cells, implicating that SEMA3F indeed plays a role in tumor angiogenesis. It is shown that the SEMA3B produced by tumor cells is subjected to the proteolytic activity of furin-like convertases (proprotein convertases) that are upregulated in cancer cells and cleave SEMA3B into 61-kDa and 22-kDa inactive fragments that are unable to repel endothelial cells and inhibit angiogenesis (41, 45, 46). MMP, a family of zinc-dependent endopeptidases, has been associated with tumor cell invasion and metastasis due to their ability to hydrolyze a variety of ECM (47, 48). As shown in our current study, the upregulation of SEMA3B and SEMA3F significantly inhibited MMP-9 and MMP-2 expression and activity, suggesting that this could be one mechanism by which SEMAs may attenuate metastatic progression, including anchorage-independent growth, migration, and cell invasion.

Finally, our findings that the expression of SEMA3B and SEMA3F are regulated by gonadotropins and estrogens have important clinical ramifications. First, our findings may explain why ovarian cancer becomes more prevalent around the perimenopausal and postmenopausal periods of women (49). In other words, premenopausal levels of gonadotropins and estrogens may play an important role to prevent the development of an aggressive ovarian cancer phenotype. This notion, if proven, would also be in agreement with the data from the Women’s Study, which found postmenopausal hormone therapy to be promoting breast cancer but not ovarian cancer (50). Future epidemiologic or clinical studies would be worthy to validate our hypothesis.

In summary, our data show that estrogen can be used to reactivate SEMA3B and SEMA3F that are downregulated in ovarian tumors and in cell lines. Overexpression of SEMA3B and SEMA3F block ovarian cancer cell adhesion, invasion, and angiogenesis. The involvement of SEMA3B and SEMA3F in cell invasion and tube formation may be through the alteration of MMP-2, MMP-9, and FAK phosphorylation, respectively. Therefore, loss of SEMA3B and SEMA3F, possibly due to life-stage hormonal changes, could be biomarkers of ovarian cancer progression, and manipulation of SEMA3B and SEMA3F expression could control ovarian cancer angiogenesis and metastasis. Our work suggests that the restoration of SEMA3B and SEMA3F expression by gonadotropin and/or estrogen could be used in cancer therapeutics as novel strategic intervention to downregulate tumor growth.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

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