Progesterone and 1,25-Dihydroxyvitamin D₃ Inhibit Endometrial Cancer Cell Growth by Upregulating Semaphorin 3B and Semaphorin 3F

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Class 3 semaphorins (SEMAS), semaphorin 3B (SEMA3B) and semaphorin 3F (SEMA3F), are secreted proteins that regulate angiogenesis, tumor growth and metastasis by binding to their transmembrane receptor complex consisting of plexins and neuropilins. Expression of SEMAs and their receptors was assessed in tissue microarrays by immunohistochemistry. SEMA3B, SEMA3F and plexin A3 were expressed strongly in normal endometrial tissues whereas grade-dependent decreases were found in endometrial carcinomas. No change was observed in the expression of plexin A1, neuropilin 1 (NP1) and neuropilin 2 (NP2) in normal versus endometrial cancer tissues. Endometrial cancer cells showed decreased expression of SEMA3B, SEMA3F and plexin A3 compared to their normal counterparts. Treatment of cancer cells with progesterone (P4) and 1,25Dihydroxyvitamin D$_3$ (1,25(OH)$_2$D$_3$) for a period of 72 hours induced a significant upregulation of SEMA3B and SEMA3F and inhibited growth of cancer cells by increasing caspase-3 activity. Co-treatment of cell lines with P4 or 1,25(OH)$_2$D$_3$ and their respective antagonists confirmed the specificity of their actions. Transfection of siRNA-targeting SEMA3B and SEMA3F in endometrial cancer cells attenuated P4 or 1,25(OH)$_2$D$_3$-induced growth inhibition. Restoration of SEMA3B or SEMA3F expression in cancer cells caused growth inhibition, reduced soft agar colony formation and cell invasiveness by inhibiting expression of matrix metalloproteinase-2, matrix metalloproteinase-9, integrin αvβ3 and pro-angiogenic genes and by upregulating anti-angiogenic genes. Thus, we have identified two new P4 and 1,25(OH)$_2$D$_3$-regulated antitumor genes for endometrial cancer. These results suggest that the loss of SEMAs contribute to the malignant phenotype of endometrial cancer cells, and that re-expression of SEMAs by ectopic expression or with anticancer agents P4 or 1,25(OH)$_2$D$_3$ can be a promising therapeutic treatment against endometrial cancer.
INTRODUCTION

Endometrial cancer is the fourth most common gynecological cancer among women in the Western world (1, 2). Although advances have been made in the field, the American Cancer Society (2) estimated that 43,406 new cases of endometrial cancer will be diagnosed in 2010 and approximately 7,950 women will die from this disease.

The pathogenesis of endometrial cancer involves a multistep process of genetic and molecular changes (3, 4). Studies have shown that a genomic aberration in human chromosome 3p is the most frequent and earliest genetic event in endometrial tumorigenesis (5-8). Genetic changes result in the clonal expansion of cells with selective growth advantages, including the activation of proto-oncogenes and the inactivation of tumor suppressor genes (9).

Semaphorins are glycoproteins that were originally described as mediators of neuronal guidance (10, 11). Class 3 semaphorins (SEMA3) serve as key regulators of cellular processes, such as cell survival, proliferation, apoptosis and migration (11, 12). Both SEMA3F and SEMA3B are mapped to chromosome 3p21.3, a region of frequent loss in lung, breast and endometrial cancers (13-15). Most semaphorins directly bind to plexins which elicit intracellular signals (16). However, secreted SEMA3s are recruited to plexins by neuropilin (NP) receptors (17, 18). NP1 preferentially binds to SEMA3B, whereas NP2 has higher affinity for SEMA3F (19). We (20) and others (21-23) have shown that SEMA3B and SEMA3F are either absent or are expressed at very low levels in ovarian, lung and breast cancer cells. When expression is restored, they function as potent inhibitors of tumor growth and angiogenesis. There is no
published information on the status and regulation of SEMA3B and SEMA3F in endometrial cancer.

Clinical, epidemiologic and basic science evidence suggest that progestins and vitamin D may be highly effective endometrial cancer preventive agents. Routine use of progestin lowers endometrial cancer risk and the protective effect increases with increasing progestin potency. In premenopausal women, use of estrogen-progestin combination oral contraceptives (OCs) for a period of at least 12 months confers a 30-50% reduction in the risk of endometrial cancer, a protective effect which lasts for 10-20 years after discontinuation of use (24, 25). In addition, progestin-potent OCs have enhanced endometrial cancer protective effects compared to OCs containing weak progestins (26, 27). Finally, high-dose progestin therapy has been shown to reverse preexisting PTEN-inactivated endometrial latent precursors, as well as endometrial hyperplasia and even low grade endometrial cancer in some women (28, 29). With regard to vitamin D, several lines of evidence suggest a chemopreventive role in the endometrium: 1) there is a significant inverse correlation between UVB exposure and endometrial cancer risk (30); 2) the endometrium has been shown to express the 1-alpha hydroxylase enzyme (CYP27B1), and is thereby capable of synthesizing 1,25 (OH) 2D 3 from circulating 25 (OH) 2 D 3; 3) vitamin D has been shown to modulate the PTEN pathway and inhibit carcinogenesis in PTEN-related neoplasms (31); 4) it has been shown that high dietary intake of vitamin D is associated with a decrease in endometrial cancer risk (OR 0.43; 95% CI 0.23-0.80; p= 0.01) (32), and 5) vitamin D has been shown to inhibit carcinogenesis in a mouse model of endometrial cancer (33).

Although the biological mechanism(s) that underlies the protective effect of progestins and 1,25(OH)2D3 have not been well characterized, a growing body of evidence has implicated
apoptotic signaling events as important mediators of the chemopreventive effects in the endometrium (29, 34).

The purpose of the study was to characterize the status of tumor suppressors SEMA3B and SEMA3F in endometrial cancer and to determine whether the chemopreventive effects of progesterone and 1,25(OH)₂D₃ were mediated by modulation of SEMA3B and SEMA3F levels in endometrial cancer cells. Understanding how SEMAs regulate cell invasion and metastasis could lead to the development of new anti-cancer therapies for endometrial cancer patients.

Our results demonstrated a decrease in the expression of SEMA3B and SEMA3F in endometrial tumors and in cancer cell lines as compared to the normal endometrium. Furthermore, restoration of SEMA3 expression by ectopic expression or by P4 or 1,25(OH)₂D₃ treatment resulted in the reduction of cell proliferation, colony formation and invasion that was associated with decreased expression of matrix metalloproteinases (MMPs), integrins, αvβ3 and pro-angiogenic genes and increased expression of anti-angiogenic genes, implicating a role of SEMAs in the inhibition of tumorigenesis.

MATERIALS AND METHODS

Immunohistochemistry. Endometrial tissue arrays, EMC962 consisting of normal (12 cases/24 cores) and cancerous (36 cases/72 cores) tissues of the endometrium in duplicates were obtained from US Biomax (Rockville, MD). Immunohistochemistry was done on formalin-fixed, paraffin-embedded endometrial cancer tissue arrays. The array slides were deparaffinized and antigen retrieval was carried out by using a Retriever 2100 (Electron Microscopy Sciences,
Fort Washington, PA) in 10 mmol/L of sodium citrate buffer (pH-6). The staining kits used were Vectastain Elite ABC kit and DAB (Vector Laboratories Inc., Burlingame, CA). The primary antibodies were SEMA3B (Abcam, Inc, 1:100), SEMA3F (Millipore, 1:50), plexin-A1 (Cell Signaling Technology, 1:50), plexin-A3 (Santa Cruz, 1:50), NP1 (Abcam, Inc, 1:100) and NP2 (Abcam, Inc, 1:50). Images were captured using a Nikon Eclipse E-800 microscope. Expression levels within tumor tissue was quantified across cores as a product of the staining intensity (0 = negative, 1 = weak, 2 = moderate, 3 = strong) x percentage cells stained. Staining intensity was quantified under high magnification (x20). Expression patterns were correlated with a range of clinical parameters such as tumor classifications (tumor stage and grade).

**Cell lines and culture conditions.** Normal epithelial endometrial (EC-E6/E7/HERT) cells established and characterized by Kyo et al. (35) and endometrial cancer cell lines (Ishikawa, HEC-1B and RL-95) from ATCC (Manassas, VA) were maintained and propagated in DMEM (EC-E6/E7/HERT), DMEM:F12, Eagle MEM (HEC-1B) and DMEM:F12 (RL-95) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin (Life Technologies) and 10 μg/ml insulin (Sigma, Chemical Co, St. Louis, MO) at 37°C in a humidified atmosphere of 5% CO₂, 95% air.

**Treatment of endometrial cell lines.** The antiproliferative effects of progesterone (P4, 99.9% pure, Sigma) and 1,25-(OH)₂D₃ (Sigma) on HEC-1B, Ishikawa and RL-95 cells were determined using CellTiter96 AQueous One Solution cell viability assay. Cells (2 × 10³/well) were plated into 96-well plates and incubated overnight to allow cell adherence. The media were removed, and progesterone (12.5-200 μmol/L) or 1,25(OH)₂D₃ (50-400 nmol/L) was added in a total
volume of 200 μL. Cells were treated for 72h. In order to avoid the toxicity associated with higher doses of progesterone in most experiments we used the lowest dose of progesterone that has a potent effect on the growth of endometrial cancer cells. Since high doses of 1,25(OH)2D3 are associated with hypercalcemia in vivo, we therefore used a concentration of 1,25-(OH)2D3 that would not be thought to induce hypercalcemia in vivo (36).

For a set of experiments, HEC-1B, Ishikawa and RL-95 cells were harvested when 80% confluent and washed with phosphate-buffered saline (PBS). Then 2 x 10^5 cells were seeded per T-25 flask and allowed to attach for 24 h. The cells were exposed to either progesterone receptor antagonist mifepristone at 100 nmol/L or the vitamin D receptor (VDR) antagonist telmisartan (Sigma) at 10 μmol/L 1 h prior to treatment with P4 (25 μmol/L) or 1,25(OH)2D3 (100 nmol/L) respectively for 72 h. Cells were collected and protein was extracted.

**Transfection of SEMA3B and SEMA3F in endometrial cancer cells.** The vectors containing SEMA3B and SEMA3F 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). Endometrial cancer (HEC-1B, and Ishikawa) cells (8x10^4) were seeded in six well plates and transfected the following day with the pcDNA3 vector alone or with pcDNA3 vector containing SEMA3F or SEMA3B (0.25-1.0 μg) using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). To study if SEMA3B and SEMA3F have additive effect on proliferation cells were cotransfected with SEMA3B (0.5 μg/ml) and SEMA3F (0.5 μg/ml). The effect of SEMA overexpression was
assessed on cell proliferation, soft agar colony formation and invasion. To establish that SEMAs are mediators of P4 and 1,25(OH)2D3-induced growth inhibition, cells were transfected with siRNAs targeting SEMA3B, SEMA3F (5nM; Santa Cruz Biotechnology, Inc) or nontargeting siRNA and treated 24 h later with P4 or 1,25-(OH)2D3 for 72 h. To better understand the role of SEMA3B and SEMA3F in the inhibition of proliferation, cells were transfected with siRNAs directed against NP-1 and NP-2 (5nM; Santa Cruz Biotechnology, Inc) or scrambled siRNA and treated 24 h later with SEMA3F-CM or SEMA3B-CM for 72 h. The overall transfection efficiency for endometrial cells assessed by X-Gal staining assay against pSV-ßgalactosidase vector-transfected cells was 70-74%. Cellular extracts were prepared to analyze the expression of SEMAs and some cells were used for cell viability assay.

Cell viability assay. Viability of P4 or 1,25(OH)2D3-treated or SEMA-transfected cells was evaluated using the CellTiter96 AQueous One Solution cell viability assay (Promega) according to the instructions of the manufacturer. 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 cells 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 treated/transfected cells over control/empty vector-transfected cells.

Invasion assay. The Biocoat Matrigel Invasion Chambers (BD Biosciences) were used to assess the effects of the ectopic expression of SEMA3B or SEMA3F on endometrial cancer cell invasiveness. Matrigel chambers were rehydrated at 37°C for 2 h. Endometrial cancer cells (2.5 × 10⁴ cells), 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 (37). The assay was run in triplicate.

**Soft agar assay.** The effects of ectopic expression of SEMA3B and SEMA3F on anchorage-independent growth were measured by the ability of transfected cells to form colonies on soft agar. HEC-1B and Ishikawa 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 5000 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 (38). Number of colonies was counted after four weeks of culture.

**Western blotting.** Cellular extracts were prepared as previously described (37). 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; 1:5000), SEMA3F (Millipore; 1:5000), NP-1 (Abcam Inc; 1:1000), NP-2 (Abcam Inc; 1:1000), plexin-A1 (Cell Signaling Technology; 1:1000), plexin-A3 (Novus Biologicals; 1:250), MMP-2 (Abcam Inc; 1:200), MMP-9 (Cell Signaling; 1:1000), αvβ3 (Cell Signaling; 1:1000) or β-actin antibody (Sigma-Aldrich; 1:25000). After washing with Phosphate Buffered Saline Tween-20 (PBST), the membranes were then incubated with the respective secondary antibodies at a 1:3000 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 chemiluminescence detection system (Pierce).

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

**Caspase-3 activity assay.** Endometrial cells were treated with P4 or 1,25(OH)₂D₃ or transfected with siRNA of SEMAs. After 72 h of incubation, the detached cells and the adherent cells were collected from each culture and suspended in ice-cold lysis buffer provided with the Caspases Assay kit (MLB International, Watertown, MA). Following sonification, the cell lysates were centrifuged for 20 min at 14,000g at 4°C. The resulting supernatants were analyzed for protein concentrations using a protein determination kit (Pierce) and stored at −20°C until use.
Colorimetric enzymatic activity assays for caspase-3 were performed according to the manufacturer’s instructions.

**Angiogenesis RT² profiler PCR array.** Total RNA was isolated from control, SEMA3B, or SEMA3F-overexpressing cells using TRI-reagent (Sigma). After DNase treatment, RNA was further cleaned using the Qiagen RNeasy Mini kit (Qiagen). cDNA was synthesized by RT² First Strand kit (SA Biosciences) per the company's instructions. Gene expression profiling using the Angiogenesis RT² Profiler PCR Array (SA Biosciences, Frederick, MD) was conducted. This platform is designed to profile the expression of 84 key genes in angiogenesis (for a comprehensive list of genes included in this array see [http://www.sabiosciences.com](http://www.sabiosciences.com)). Quantitative RT-PCR was conducted using the 7500 RT-PCR System (AB Applied Biosciences) based on the array manufacturer’s instructions. Relative gene expression was determined using the ΔΔCT method. Data was further analyzed with the PCR Array Data Analysis Web Portal ([http://www.SABiosciences.com/pcrarraydataanalysis.php](http://www.SABiosciences.com/pcrarraydataanalysis.php)).

**Data normalization and analysis.** Five endogenous control genes: beta-2-microglobulin (B2M), hypoxanthine phosphoribosyltransferase (HPRT1), ribosomal protein L13a (RPL13A), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin (ACTB) present on the PCR array were used for normalization. Each replicate cycle threshold (CT) was normalized to the average CT of five endogenous controls on a per plate basis. The comparative CT method was used to calculate the relative quantification of gene expression. The following formula was used to calculate the relative amount of the transcripts in the treated samples to the control group, both of which were normalized to the endogenous controls: ΔΔCT = ΔCT (treated) – ΔCT (control)
for RNA samples. ΔCT is the log$_2$ difference in CT between the target gene and the endogenous controls by subtracting the average CT of controls from each replicate. The fold-change for each treated sample relative to the control sample = 2$^{-ΔΔCT}$. Sensitivity detection and identification of expressed genes PCR array quantification was based on the CT number. CT was defined as 35 for the ΔCT calculation when the signal was below detectable limits. A list of differentially expressed genes was identified using a two-tailed student t-test. Changes in gene expression between SEMA-tranfected cells and vector controls were illustrated as a fold increase/decrease. The criteria were: a $p$ value < 0.05 and a mean difference equal to or greater than a two-fold change in expression levels after treatment. The statistical calculation was based on the web-based program for Profiler TM PCR Array Data Analysis. Alterations in mRNA levels that fitted the criteria above were considered to be up- or down-regulated. The experiments were repeated three times.

**Statistical analyses.** Statistical analysis was carried out using ANOVA, followed by Tukey's post hoc test. Values are presented as the mean ± SD and are considered significant at a $p$ value of <0.05.

**RESULTS**

**Attenuation of SEMA3B, SEMA3F and plexin A3 expression in stage III endometrial cancer tissues.** *In vivo* expression of SEMA3B, SEMA3F, plexin A1, plexin A3, NP1 and NP2 was analyzed by immunohistochemistry in TMA (US Biomax Inc, Rockville, MD). The TMA consisted of 24 normal and 72 malignant tissues. There were 22 tissues from grade I, 26 from grade II and 16 from grade III cancer. The staining of TMAs was correlated with
clinicopathological parameters of patients (Table 1). Stained TMAs were examined by three individuals in a blinded manner. The results indicated that in normal endometrial tissues, SEMA3F and SEMA3B were detected in the epithelial cells in the glands as well as in the stromal cells (Figure 1, panels 2 and 7). Expression of SEMA3F and SEMA3B was markedly decreased ($p<0.05$, Table 1) with progression of tumors to advanced grades (Figure 1, panels 5 and 10). Semaphorin receptors, plexin A1 and plexin A3, were expressed in the gland and stromal cells of normal endometrial tissues (Figure 1, panels 12 and 17). Gradual decrease in plexin A3 expression was seen in cancer specimens (Figure 1, panels 18-20). Complete loss of plexin A3 was evident in grade III endometrial cancer (Figure 1, panel 20). No changes in expression of NP1 and NP2 was noticed between normal and different grades of endometrial cancer tissues. Taken together, this data suggested that loss of SEMA3B, SEMA3F and plexin A3 expression is associated with endometrial carcinogenesis.

Loss of SEMA3B, SEMA3F and their receptor plexin A3 expression in endometrial cancer cells. To confirm that SEMA3B, SEMA3F and their receptors are expressed in cultured endometrial carcinoma cells, expression levels of these proteins were determined by immunoblotting. Since SEMAs are secretory proteins, the expression of SEMA3B and SEMA3F was examined in conditioned media (CM) and cellular extracts of a panel of normal and malignant endometrial cell lines. Analysis of endogenous SEMA3B and SEMA3F protein expression in one normal endometrial (EC-E6/E7/HERT) and three endometrial cancer cell lines (HEC-1B, Ishikawa, and RL-95) showed decreased SEMA3B (active form 83 kDa and 50 kDa cleaved form) and SEMA3F (90 kDa) expression in CM and extract of cancer cells compared to the normal cell line (Figure 2A). SEMA3 family members act through a
membrane-associated receptor complex composed of plexin A1 or plexin A3, in addition to neuropilin 1 (NP1) and neuropilin 2 (NP2) receptors. Therefore, we also analyzed the expression of these receptors in normal and malignant endometrial cells. Normal endometrial epithelial cells (EC-E6/E7/HERT) showed higher expression of plexin A3 (220 kDa) compared to Ishikawa, HEC-1B and RL-95 cells whereas the expression of plexin A1 (211 kDa) was not altered (Figure 2A). No marked changes were observed in the expression of NP1 (130 kDa) and NP2 (116 kDa) in normal and malignant cells (Figure 2A). Mouse E16 cerebellum and rat brain lysates were used as positive controls for SEMA3B and SEMA3F respectively (Figure 2B).

**P4 and 1,25(OH)_{2}D_{3}-induced growth inhibition of endometrial cancer cells is associated with enhanced expression of SEMA3B and SEMA3F.** The effects of P4 and 1,25(OH)_{2}D_{3} on cell proliferation in two endometrial cancer lines (HEC-1B and Ishikawa) were investigated over a wide concentration range. Cells were treated with either P4 (12.5-200 µM) or 1,25(OH)_{2}D_{3} (50-400 nM) for 72 h and cell proliferation was assessed by MTS assay. Although both P4 and 1,25-(OH)_{2}D_{3} showed dose-dependent decreases in cell viability, P4 illustrated a more pronounced effect on cell growth compared to 1,25(OH)_{2}D_{3} (Figure 2C). To assess whether P4 or 1,25-(OH)_{2}D_{3}-induced growth inhibition is mediated through induction of apoptosis, we evaluated the activity of caspase-3 in cellular extracts. A concentration-dependent increase in caspase-3 activity was found in cells following P4 or 1,25(OH)_{2}D_{3} treatments (Figure 2C).

As all three endometrial cancer cell lines showed low expression of SEMA3B and SEMA3F compared to the normal cell line, we next sought to determine whether exposure of cells to endometrial cancer preventive agents P4 or 1,25(OH)_{2}D_{3} could restore the expression of
SEMA3B and SEMA3F in cells. Treatment with P4 (25 µM) or 1,25(OH)_{2}D_{3} (100 nM) for 72 h caused a marked increase in SEMA3B (active form 83 kDa and 50 kDa cleaved form) and SEMA3F expression in both normal and malignant cell lines (Figure 2D). To ascertain whether the observed P4 or 1,25(OH)_{2}D_{3}-stimulated SEMA3B or SEMA3F expression was mediated through a receptor-mediated pathway, endometrial cancer cell lines were pretreated with P4 antagonist mifepristone (100 nmol/L) or the VDR antagonist telmisartan (10 µmol/L) 1 h prior to addition of P4 or 1,25(OH)_{2}D_{3}. Addition of mifepristone or telmisartan blocked P4 and 1,25(OH)_{2}D_{3} stimulated expression of SEMA3B and SEMA3F down to levels comparable to that in the controls in all the cell lines. However, in HEC-1B SEMA3F expression levels did not go down to control levels in the presence of antagonists (Figure 2D). Mifepristone and telmisartan alone had no effect on SEMA3B and SEMA3F expression (not shown).

**P4 and 1,25(OH)_{2}D_{3}-induced cell growth inhibition of endometrial cells is attenuated by knockdown of SEMA3B and SEMA3F.** To demonstrate that P4 and 1,25(OH)_{2}D_{3} inhibit endometrial cancer cell growth by upregulating SEMA3B and SEMA3F, we knocked down the expression of these genes separately or conjointly using their respective siRNAs in two endometrial cancer cell lines, then treated them with P4 and 1,25(OH)_{2}D_{3} and analyzed the effect on cell growth. A significant loss of SEMA3B and SEMA3F protein expression was observed in cell cultures transfected with respective siRNAs (Figure 3A). No change in SEMA3B and SEMA3F expression was observed in control cultures compared to scrambled siRNA controls (Figure 3A). Mock transfected cells treated with P4 and 1,25(OH)_{2}D_{3} showed marked increase in SEMA3B and SEMA3F expression (Figure 3A). Treatment of cells with P4 and 1,25(OH)_{2}D_{3} for 72 h showed a significant decrease in cell viability in control cells compared to cells where
SEMA3B and SEMA3F were separately or conjointly silenced by siRNA (Figure 3B). After 72 h of 1,25(OH)₂D₃ exposure, 55-58% of cells were viable in the control group. However, the number of viable cells increased significantly when SEMA3B (72-80%), SEMA3F (70-75%) or both SEMAs (85-88%) were silenced (Figure 3B) indicating that SEMAs mediate the growth inhibitory effects of 1,25(OH)₂D₃. Progesterone treatment of cells resulted in 35-40% viable cells in the control group. Knockdown of SEMA3B (55-65%), SEMA3F (55-60%) or both SEMAs (83-87%) resulted in an increased number of viable cells and attenuation of P4 and 1,25(OH)₂D₃-induced growth (Figure 3B). SEMA3B and SEMA3F siRNAs have no effect on control cells (Figure 3B). To investigate whether P4 and 1,25-(OH)₂D₃ activate apoptosis, we measured the activation of caspase-3 in cells by a colorimetric substrate assay. Treatment of Ishikawa and HEC-1B cell cultures with 1,25(OH)₂D₃ and P4 induced significant decreases in cell growth and increases in caspase-3 activity (Figure 3B) when compared to vehicle-treated cultures. The proapoptotic effects of 1,25(OH)₂D₃ and P4 were attenuated in cells transfected with siRNAs directed against SEMA3B and SEMA3F (Figure 3B), suggesting that 1,25(OH)₂D₃ and P4 inhibit growth of endometrial cancer cells by upregulating SEMA3B and SEMA3F and inducing apoptosis.

Ectopic expression of SEMA3B and SEMA3F inhibit in vitro tumorigenicity. Consistent with the data from the human endometrial cancer specimens, SEMA3B and SEMA3F expression was lost/reduced in all endometrial cancer cell lines tested, although these proteins were abundantly expressed in normal endometrial cells. To gain insight into the functional role of SEMA3B and SEMA3F downregulation in endometrial cancer cells, SEMA3B and SEMA3F expression was restored in the cancer cell lines (HEC-1B and Ishikawa) by transfection with the
vectors containing SEMA3B and SEMA3F. An empty vector without the SEMA3 gene insert was used as a control (mock). Expression of SEMA3B and SEMA3F in the transfected cells was confirmed by Western blotting (Fig 4AB). Because the acquisition of cell migration and colony-forming ability positively correlates with tumorigenicity, we sought to determine whether SEMA3B and SEMA3F restoration might alter these characteristics of endometrial cancer cells. We investigated the effects of SEMA3B and SEMA3F reexpression on cell proliferation (Figure 4C) and migration (Figure 4D) using cell viability and cell invasion assays. Seventy-two hours after transfection, cell proliferation and migration was assessed. As shown in Figure 4C, a marked concentration-dependent decrease in cell proliferation was observed in SEMA3B and SEMA3F transfected cells compared to mock transfected cells. Of note, coexpression of SEMA3B and SEMAF inhibited cell proliferation in a synergistic manner (Figure 4C). The number of cells migrating to the other side of the Matrigel was significantly reduced when SEMA3B and SEMA3F expression was restored in cancer cells (Figure 4D). The effect of SEMA3B and SEMA3F restoration on the colony formation capacity of cancer cells was also analyzed (Fig. 4D). Upregulation of SEMA3B and SEMA3F in endometrial cancer cells showed a 30-40% and 60-70% respective decrease in colony-forming ability on culture plates as compared with mock-transfected cells. Taken together, these results demonstrate that SEMA3B and SEMA3F restoration inhibit cell proliferation, migration and colony forming ability, indicating a reduction of in vitro tumorigenicity. Similar results were obtained when SEMA3B and SEMA3F in the cells were upregulated by vitamin D or progesterone treatment (results not shown).
Silencing of neuropilin-2 in endometrial cells results in loss of responsiveness to SEMA3F but not to SEMA3B. SEMA3s act in a paracrine fashion by binding to either to NP-1 or NP-2 or both. NPs complex with the plexins to mediate downstream signaling. To better understand the requirement of NP-1 and NP-2 to mediate the effects of SEMA3B and SEMA3F respectively, Ishikawa and HEC-1B cells were transfected with either NP-1 or NP-2 specific siRNA or scrambled siRNA as a control. As shown in Figure 5A, NP-1 and NP-2 proteins were markedly reduced in both endometrial cancer cell lines with respective siRNA. Downregulation of NP-2 significantly reduced the ability of cells to respond to SEMA3F-CM and attenuated inhibition of tumor cell proliferation, whereas cells transfected with NP-1 or control siRNA (Fig. 5B) retained the responsiveness to SEMA3F and inhibited cell proliferation (Fig. 5B). Interestingly, silencing of NP-1 or NP-2 retained the responsiveness of cells to SEMA3B-CM, and a marked decrease in cell proliferation was seen compared to cells exposed to control-CM. However, when both NP-1 and NP-2 were silenced cells were not responsive to either SEMA3F-CM or to SEMA3B-CM (Figure 5B). Together, these studies indicate that treatment with soluble SEMA3F inhibits endometrial cell growth in a NP-2 dependent manner whereas soluble SEMA3B mediates it effect by employing both NP-1 and NP-2.

Upregulation of SEMA3B and SEMA3F inhibit matrix metalloproteinases, integrins and alter pro- and anti-angiogenic factors in endometrial cancer cells. Increased expression of MMPs is associated with cancer cell invasiveness and metastatic potential (39 40). To explore the mechanism by which SEMA-3 inhibits tumorogenesis, we evaluated the expression levels of MMPs in cells overexpressing SEMA3B and SEMA3F. Zymography done with CM from endometrial cancer HEC-1B and Ishikawa cells overexpressing SEMA3B or SEMA3F indicated
reduced MMPs activity in endometrial cancer cell lines (Fig. 5C). Since both SEMA3B and SEMA3F inhibited MMPs we explored the relationship between semaphorins and the specific receptors that transduce their signals. Expression of MMP-9 was significantly low in NP-1 downregulated endometrial cancer cells exposed to SEMA3F-CM or SEMA3B-CM compared to cells exposed to control-CM. No significant changes in the expression of MMP-9 and MMP-2 were detected in NP-2 silenced HEC-1B and Ishikawa cells treated with SEMA3F-CM and SEMA3B-CM compared to control cells (Figure 5D).

We then determined whether the expression of integrin αvβ3, which has been shown as a key mediator of cell-matrix attachment and motility (12-14) was regulated in the cancer cells by SEMA3 alteration. We found that SEMA3B and SEMA3F restoration by ectopic expression resulted in the reduction of integrin αvβ3 (Figure 5E). Next, we sought to evaluate the effect of SEMA3B and SEMA3F upregulation on the expression of angiogenic factors in vitro. Tumor-associated angiogenesis is a complex process, involving many pro-angiogenic and anti-angiogenic factors. RNAs extracted from control, SEMA3B and SEMA3F overexpressing cells were subjected to an angiogenesis RT2 Profiler RT-PCR array. The changes in mRNA levels of angiogenesis related genes in SEMA3B and SEMA3F upregulated endometrial cancer cells are shown in Table 2. A two-fold change in mRNA expression was considered as significant when compared with the mock transfected control. It is evident from the results that SEMA3 overexpression led to decreased expression of pro-angiogenic factors (e.g., IL-8, VEGF, FGFR, ECGF and MMP-9; Table 2), and increased expression of anti-angiogenic factors (e.g., TIMP-3 and transforming growth factor-β, CXCL10) (Table 2).
DISCUSSION

Endometrial cancer is a common and invasive gynecologic cancer in women, and the incidence and mortality is moderately increasing in the United States while rapidly increasing in Asia. It would therefore be beneficial to develop molecular biomarkers, which could be used to detect endometrial cancer, predict disease progression, monitor therapeutic response and perhaps be used as therapeutic targets.

Recently, the role of SEMA3s in the pathogenesis of multiple types of cancer has been investigated in preclinical studies, and SEMA3B and SEMA3F have been implicated in the inhibition of tumor cell growth, chemotaxis and angiogenesis in breast, ovarian and lung cancer cells (20-22, 41). In the present study we investigated the status, functional role and regulation of SEMA3B and SEMA3F in human endometrial carcinogenesis either by ectopic expression or following exposure of cells to P4 and 1,25(OH)2D3.

Our study demonstrated a significant reduction in the expression of both SEMAs and their receptor plexin A3 in grade III endometrial cancer compared with grade I or normal endometrial tissues. To our knowledge, this is the first report revealing the inverse correlation of SEMAs and plexin A3 levels with tumor aggressiveness. Our results are consistent with the pattern previously shown of loss of SEMA3B and SEMA3F expression in ovarian carcinomas compared with benign and borderline tumors (20, 42). We also investigated the expression of both SEMAs and their receptors in human endometrial cancer cell lines and found higher levels of SEMA3B and SEMA3F in the cell lysates and conditioned media of normal cells than in cancer cells, indicating a potential role for class 3 semaphorins in endometrial cancer. Coexpression of semaphorins and their receptors by tumor cells suggests that semaphorins may
act through an autocrine or paracrine mechanism in these cells. Semaphorins secreted by endometrial tumor cells may therefore have a direct effect on tumor growth, as seen in breast cancer cells (43).

We are the first to report that SEMA3B and SEMA3F are strongly induced by P4 and 1,25(OH)₂D₃ in endometrial cancer cells. Although the precise regulatory mechanisms remain unclear, upregulation of SEMAs was blocked by the antagonists mifepristone or telmisartan, providing evidence for the specificity of P4 and 1,25(OH)₂D₃ action in cancer cells. Telmisartan was shown to strongly antagonize VDR (44). Our findings are consistent with the previous study revealing strong induction of SEMA3B mRNA by 1,25(OH)₂D₃ in multiple osteoblastic cell lines (MG-63, ST-2, MC3T3) and primary osteoblastic cells, suggesting a potential role for SEMA3B in modulating osteoclastogenesis and bone resorption (45).

Our results demonstrate that siRNA-mediated downregulation of SEMA3B and SEMA3F expression attenuated progesterone and 1,25(OH)₂D₃-induced cell growth, strongly suggesting that SEMA3B and SEMA3F expression is required to mediate growth inhibitory function induced by progesterone and 1,25(OH)₂D₃ in endometrial cancer cells. Interestingly, the combined suppression of SEMA3B and SEMA3F expression in cancer cells resulted in a greater attenuation of progesterone and 1,25(OH)₂D₃-induced cell growth inhibition compared to SEMA3B and SEMA3F alone, implicating that combined progesterone and 1,25(OH)₂D₃ treatment of cells may have a cumulative growth inhibitory effect on cancer.
Studies reported herein highlight the roles of SEMA3B and SEMA3F as negative regulators of tumor growth in endometrial cancer. SEMA3F binds to NP2 and SEMA3B binds to both NP1 and NP2, leading to growth suppression of tumor cells in vitro. We found that SEMA3B and SEMA3F expression was low in endometrial cancer cell lines. Constitutive activation of SEMA3B and SEMA3F expression in endometrial cancer cell lines, HEC-1B and Ishikawa, changes their in vitro attributes of malignancy. Ectopic expression of SEMA3B and SEMA3F reduced cell proliferation in a synergistic manner and attenuated invasion and anchorage-independent growth. These observations strongly demonstrate the negative influences of SEMA3B and SEMA3F on endometrial tumor progression. Our results are in agreement with earlier studies proposing that SEMA3B and SEMA3F act as tumor suppressors (20-22). Downregulation of SEMA expression in human tumors is attributed to loss of heterozygosity and promoter hypermethylation (46). Consistently, SEMA3B and SEMA3F overexpression in tumor cell lines induced apoptosis, inhibited cell proliferation and colony formation in soft agar (15, 20, 22). In general, the tumor suppressing role of SEMA3B and SEMA3F is supported by in vitro and in vivo data; however, Rolny et al., (47) demonstrated that SEMA3B-induced IL-8 secretion from tumor cells promotes cancer progression and metastasis.

To search for the underlying mechanisms by which SEMAs upregulation enacts their anti-tumor effects we used Human Angiogenesis RT2 Profiler™ PCR Array and found a significant downregulation of many pivotal angiogenic genes in SEMA3B and SEMA3F overexpressing Ishikawa and HEC-1B cells compared to their receptive control cells. It is of interest to note that the two different cell cultures responded differently in terms of the regulation of angiogenesis-related molecules, probably because of the differences in their genetic profiles. In many types of cancers, several genes such as LAMA-5, ID, NOTCH4 and MMP-9 have been
observed to be highly expressed and are shown to play a key role in promoting angiogenesis (48, 49). Our results demonstrated downregulation of several pro-angiogenic genes (FGF2, FGFR3, MDK, MMP-2, MMP-9) and upregulation of anti-angiogenesis genes (TIMP1, TIMP2, TIMP3) in cancer cells ectopically expressing SEMA3B and SEMA3F. These results suggest that the reexpression of SEMA3B and SEMA3F affect angiogenesis. In our previous study (20), we have shown that the reexpression of SEMA3F, but not SEMA3B, in ovarian cancer cells inhibited tube formation of endothelial cells, implicating that SEMA3F plays a role in tumor angiogenesis.

The mechanism of tumor suppression by SEMAs is not fully understood. The data presented here shows that restoration of SEMA3B and SEMA3F in endometrial cancer cells reduces their invasive properties by inhibiting MMP-2, MMP-9, αvβ3 and pro-angiogenic genes and by upregulating anti-angiogenic genes. The results of the studies reported here showed that exogenously added SEMA3F and SEMA3B inhibit MMPs by acting through NP-2 receptors in cancer cells, leading to decreased cell survival. Matrix metalloproteinases (MMPs) are key players in the degradation of ECM and basement membranes and are thus important in tumor invasion. Gelatinases, in particular, are prognostic factors in many solid tumors (50). Previous reports showed that MMP-2 and MMP-9 were expressed in a high percentage of primary endometrial carcinomas and that their expression was associated closely with tumor aggressiveness (50, 51). In our study, the downregulation of MMP-2 and MMP-9 may contribute to reduced invasiveness in SEMA3-restored cells. Interestingly, MMP’s inhibitor TIMP3, which is also a key player in tumor cell invasion, angiogenesis and cell growth processes (51), has been found upregulated upon SEMA restoration. This data supports our observation that SEMA can regulate both active gelatinases and their inhibitor in the cellular invasion pathway.
In conclusion, this is the first report to show that SEMA3B and SEMA3F expression is downregulated in endometrial cancer tissues and that SEMA upregulation inhibited cell growth and invasion in endometrial cancer cells through downregulation of MMP-9, MMP-2, αvβ3 and pro-angiogenic genes and by upregulation of antiangiogenic genes. These findings also suggest that the differential expression levels of SEMAs in various stages of endometrial cancer may serve as endometrial tumor markers, and that the restoration of SEMAs may be beneficial as a therapeutic tool for endometrial cancer.

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**Figure Legends**

**Figure 1.** Expression of SEMA3B, SEMA3F, Plexin A1, Plexin A3, NP1 and NP2 in human endometrial tumors. Immunohistochemical analysis of endometrial tumors from TMA using anti-SEMA3B, SEMA3F, Plexin A1, Plexin A3, NP1 and NP2 antibodies was performed. Expression of SEMA3B (2) and SEMA3F (7) was significantly high in normal endometrial tissues. Gradual decrease of SEMA3B (3-4) and SEMA3F (8-9) expression was seen in grades I-II. Grade III (5 and 10) showed no to weak expression of SEMA3B and SEMA3F. Negative controls for SEMA3B (1) and SEMA3F (6) are shown. Expression of plexin A1 (12) and plexin A3 (17) was significantly high in normal endometrial tissues. No change in plexin A1 expression was noticed in different grades of endometrial cancer. Gradual decrease of plexin A3 expression was seen in grades I-III (18-20). Negative control for plexin A1 (11) and plexin A3 (16) are shown. No alterations of NP1 (22) and NP2 (27) in normal and different grades of endometrial cancer tissues were seen. Original magnification, 400x.

**Figure 2.** Upregulation of SEMA3B and SEMA3F in endometrial cancer cells by P4 and 1,25(OH)₂D₃. (A) Normal immortalized EC-E6/E7/HERT and endometrial cancer cells (Ishikawa, HEC-1B and RL-95) were evaluated by Western blot for basal expression of SEMA3B, SEMA3F, plexin A1, plexin A3, NP1 and NP2. 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 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. (C)
Effect of P4 and 1,25(OH)\textsubscript{2}D\textsubscript{3} on endometrial cancer cell (Ishikawa, HEC-1B and RL-95) growth and apoptosis. The cells were treated with P4 (12.5-200 \textmu M) or 1,25(OH)\textsubscript{2}D\textsubscript{3} (50-400 nM) for 72 h, and the viability of cells was determined by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide assay. Data shown are mean ± SD of three separate experiments in which each treatment was repeated in 8 wells. The cells were treated with vehicle only or specified concentrations of P4 and 1,25(OH)\textsubscript{2}D\textsubscript{3} for 72 h, harvested, and cell lysates were prepared for caspase-3 enzyme activity. The data is representative of three independent experiments with similar results. (D) The effect of P4 (25 \textmu M) and 1,25(OH)\textsubscript{2}D\textsubscript{3} (100nM) on the expression of SEMA3B and SEMA3F was studied in endometrial cancer cell lines. Cells were cultured with P4 or 1,25(OH)\textsubscript{2}D\textsubscript{3} in the presence or absence antagonists (mifepristone for P4 and telmisartan for 1,25(OH)\textsubscript{2}D\textsubscript{3}) for 72 h. Protein was extracted from cell cultures and loaded in each lane and blots were probed with SEMA3B and SEMA3F. β-Actin was used as a loading control.

**Figure 3.** Silencing of SEMA3B and SEMA3F attenuate progesterone and 1,25(OH)\textsubscript{2}D\textsubscript{3}-induced cell growth inhibition. The cell lines (HEC-1B and Ishikawa) were transfected with vector containing SEMA3B or SEMA3F alone, in combination or with the respective empty vectors. Transfected and mock transfected cells were treated with P4 (25 mM) or 1,25(OH)\textsubscript{2}D\textsubscript{3} (100nM) for 72 h. (A) Protein was extracted from cell cultures for analyses of SEMA3B and SEMA3F expression by Western blotting. (B) Cell viability was assessed by MTS assay and (C) Caspase-3 activity was determined by enzyme activity assay. Experiment was repeated 3 times. *Statistically significant changes in cell viability/apoposis, compared with those seen in control cells (p < 0.05).
Figure 4. The effect of SEMA3B and SEMA3F overexpression on endometrial cancer cell malignant phenotypes. The effect of SEMA3B and SEMA3F overexpression on the viability, colony formation and migration was investigated in two endometrial cancer cell lines. The cell lines (HEC-1B and Ishikawa) were transfected with a vector containing SEMA3B or SEMA3F. Control cells were transfected with the respective empty vectors. The second control was cells without transfection. Protein from control and transfected HEC-1B (A) and Ishikawa (B) was extracted and analyzed for the expression of SEMA3B and SEMA3F by Western blot. A significant difference between the protein levels in transfected cell lines and those observed in control cell cultures is shown in the figure. (C) 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, ±SD. Statistically significant decreases in cell growth compared with those seen in control cells are indicated by *, p < 0.05. (D) SEMA3B and SEMA3F-transfected and control cells were cultured on soft agar, and 4 weeks 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, ±SD. *, Statistically significant changes in colony formation, compared with those seen in control cells (p < 0.05). (E) Endometrial 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, ±SD. *, Statistically significant changes in cell invasion, compared with those seen in control cells (p < 0.05).

Figure 5. Expression of NP-2 is required for SEMA3F and SEMA3B inhibition of MMPs. Endometrial cancer cells (HEC-1B and Ishikawa) were transfected with a scrambled siRNA,
siRNA for NP-1, siRNA for NP-2 or both. Transfected cells were exposed to SEMA3F-CM, or SEMA3B-CM or Control-CM for 72 hr. (A) Cells extracts were analyzed by Western blotting for the expression of MMP-9 and MMP-2. β-Actin was used as a loading control. (B) Cell viability was assessed by MTS assay. Columns, mean; bars, SD. *, p < 0.05, significant comparisons between the different treatments shown. (C) The gelatinolytic activity of MMP-2 and MMP-9 in CM of endometrial cancer cells. Endometrial 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. Representative picture from three independent experiments is shown. (D) Western blot for MMP-9 and MMP-2 in HEC-1B and Ishikawa cells after NP-1 siRNA, NP-2 siRNA, both NP-1 NP-2 siRNAs or control siRNA knockdown followed by treatment with SEMA3F-CM or SEMA3B-CM for 72 hr. (E) Total cellular protein extracts were analyzed for αVβ3 integrin using specific antibodies. β-Actin was used as a loading control.

Table 1. Correlation between clinicopathological features of patients and staining intensity of SEMA3B, SEMA3F, plexins and neuropilins.

Table 2. Fold-change in angiogenesis-related genes in endometrial cancer cells overexpressing SEMA3B and SEMA3F.
REFERENCES


17. He Z, Tessier-Lavigne M. Neuropilin is a receptor for the axonal chemorepellent semaphorin III. Cell 1997;90:739-51.


Figure 2
Figure 3
Figure 4
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<td>5.38 ± 1.57</td>
<td>4.31 ± 1.07</td>
<td>3.98 ± 0.75</td>
</tr>
</tbody>
</table>
Molecular Cancer Research

Progesterone and 1,25-Dihydroxyvitamin D3 Inhibit Endometrial Cancer Cell Growth by Upregulating Semaphorin 3B and Semaphorin 3F

Huyen Nguyen, Vessela S Ivanova, Leyla Kavandi, et al.

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