Angiogenesis, Metastasis, and the Cellular Microenvironment

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

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Abstract

Class 3 semaphorins (SEMA), SEMA3B and SEMA3F, are secreted proteins that regulate angiogenesis, tumor growth, and metastasis by binding to their transmembrane receptor complex consisting of plexins and neuropilins (NP). 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, NP1, and NP2 in normal versus endometrial cancer tissues. Endometrial cancer cells showed decreased expression of SEMA3B, SEMA3F, and plexin A3 compared with their normal counterparts. Treatment of cancer cells with progesterone (P⁴) and 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] for a period of 72 hours induced a significant upregulation of SEMA3B and SEMA3F as well as inhibited growth of cancer cells by increasing caspase-3 activity. Cotreatment of cell lines with P⁴ or 1,25(OH)₂D₃ 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)₂D₃-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 (MMP-2), MMP-9, integrin αvβ3, and proangiogenic genes and by upregulating antiangiogenic genes. Thus, we have identified two new P⁴ and 1,25(OH)₂D₃-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 reexpression of SEMAs by ectopic expression or with anticancer agents P⁴ or 1,25(OH)₂D₃ can be a promising therapeutic treatment against endometrial cancer. Mol Cancer Res; 9(11); 1479–92. ©2011 AACR.

Introduction

Endometrial cancer is the fourth most common gynecologic 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).

SEMA are glycoproteins that were originally described as mediators of neuronal guidance (10, 11). Class 3 SEMAs (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 SEMAs 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 SEMA3F, 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 (OC) for a period of at least 12 months confers a 30% to 50% reduction in the risk of endometrial cancer, a protective effect which lasts for 10 to 20 years after discontinuation of use (24, 25). In addition, progesterin-potent OCs have enhanced endometrial cancer protective effects compared with 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: (i) there is a significant inverse correlation between UVB exposure and endometrial cancer risk (30); (ii) the endometrium has been shown to express the 1-alpha hydroxylase enzyme (CYP27B1) and is thereby capable of synthesizing 1,25(OH)2D3 from circulating 25(OH)2D3; (iii) vitamin D has been shown to modulate the PTEN pathway and inhibit carcinogenesis in PTEN-related neoplasms (31); (iv) 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; ref. 32), and (v) 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-dihydroxyvitamin D3 [1,25(OH)2D3] has 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 progestogens 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 anticancer therapies for endometrial cancer patients.

Our results showed a decrease in the expression of SEMA3B and SEMA3F in endometrial tumors and in cancer cell lines as compared with 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 (MMP), integrins, αvβ3, and proangiogenic genes and increased expression of antianti-angiogenic genes, implicating a role of SEMAs in the inhibition of tumorigenesis.

### Materials and Methods

#### Immunohistochemistry

Endometrial tissue arrays, EM-C962, consisting of normal (12 cases per 24 cores) and cancerous (36 cases per 72 cores) tissues of the endometrium in duplicates were obtained from US Biomax. 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) in 10 mmol/L of sodium citrate buffer (pH 6). The staining kits used were Vectastain Elite ABC kit and DAB (Vector Laboratories Inc.). The primary antibodies were SEMA3B (1:100; Abcam Inc.), SEMA3F (1:50; Millipore), plexin-A1 (1:50; Cell Signaling Technology), plexin-A3 (1:50; Santa Cruz), NP1 (1:100; Abcam Inc.), and NP2 (1:50; Abcam Inc.). 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, and 3 = strong) × percentage cells stained. Staining intensity was quantified under high magnification (×20). 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 (EM-E6/E7/TERT) cells established and characterized by Kyo and colleagues (35) and endometrial cancer cell lines (Ishikawa, HEC-1B, and RL-95) from American Type Culture Collection were maintained and propagated in Dulbecco’s modified Eagle’s medium (DMEM; EM-E6/E7/TERT), DMEM:F12, (Ishikawa), Eagle MEM (HEC-1B), and DMEM:F12 (RL-95) supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin (Life Technologies), and 10 μg/mL insulin (Sigma, Chemical Co.) 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 × 104 per 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 72 hours. 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. Because 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.
For a set of experiments, HEC-1B, Ishikawa, and RL-95 cells were harvested when 80% confluent and washed with PBS. Then $2 \times 10^5$ cells were seeded per T-25 flask and allowed to attach for 24 hours. 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 hour prior to treatment with P4 (25 μmol/L) or 1,25(OH)2D3 (100 nmol/L), respectively, for 72 hours. 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 ($8 \times 10^4$) were seeded in 6-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). To study whether SEMA3B and SEMA3F have an 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 (5 nmol/L; Santa Cruz Biotechnology, Inc.) or nontargeting siRNA and treated 24 hours later with P4 or 1,25(OH)2D3 for 72 hours. To better understand the role of SEMA3B and SEMA3F in the inhibition of proliferation, cells were transfected with siRNAs directed against NP1 and NP2 (5 nmol/L; Santa Cruz Biotechnology, Inc.) or scrambled siRNA and treated 24 hours later with SEMA3F-CM or SEMA3B-CM for 72 hours. The overall transfection efficiency for endometrial cells assessed by X-Gal staining assay against pSV-β-galactosidase vector–transfected cells was 70% to 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 CellTiter 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 hours. 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 hours after plating, noninvading cells were removed, and invading cells were counted in 5 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 hours, transfected cells were cultured at 5,000 cells per 100-mm plate (4 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 4 weeks of culture.

Western blotting

Cellular extracts were prepared as previously described (37). Briefly, cell lysates were prepared in radiolmmunoprecipitation 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 (1:5,000; Abcam Inc.), SEMA3F (1:5,000; Millipore), NP1 (1:1,000; Abcam Inc.), NP2 (1:1000; Abcam Inc.), plexin-A1 (1:1,000; Cell Signaling Technology), plexin-A3 (1:250; Novus Biologicals), MMP-2 (1:200; Abcam Inc.), MMP-9 (1:1,000; Cell Signaling), αβ3 (1:1,000; Cell Signaling), or β-actin antibody (1:25,000; Sigma-Aldrich). After washing with PBS Tween-20 (PBST), the membranes were then incubated with the respective secondary antibodies at a 1:3,000 dilution in 5% nonfat dry milk in PBS Tween-20 for 2 hours 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 5-fold using Centricon centrifugal filters (Millipore). CM, equivalent to 200 μg of endometrial cancer cells, were analyzed with 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 hours, 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.

**Caspase-3 activity assay**

Endometrial cells were treated with P4 or 1,25(OH)₂D₃ or transfected with siRNA of SEMAs. After 72 hours 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). Following sonification, the cell lysates were centrifuged for 20 minutes at 14,000 × g 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 done 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 (SABiosciences) per the company’s instructions. Gene expression profiling using the Angiogenesis RT² Profiler PCR Array (SA Biosciences) 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). Quantitative reverse transcriptase PCR (RT-PCR) was conducted using the 7500 RT-PCR System (AB Applied Biosciences) following 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).

**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 5 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 log₂ 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 ΔΔCₜ calculation when the signal was below detectable limits. A list of differentially expressed genes was identified using a 2-tailed student t test. Changes in gene expression between SEMA-transfected 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 2-fold change in expression levels after treatment. The statistical calculation was based on the web-based program forProfiler TM PCR Array Data Analysis. Alterations in mRNA levels that fitted the criteria above were considered to be up- or downregulated. The experiments were repeated 3 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 less than 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.). 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 clinicopathologic parameters of patients (Table 1). Stained TMAs were examined by 3 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 (Fig. 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 (Fig. 1, panels 5 and 10). SEMA receptors, plexin A1 and plexin A3, were expressed in the gland and stromal cells of normal endometrial tissues (Fig. 1, panels 12 and 17). Gradual decrease in plexin A3 expression was seen in cancer specimens (Fig. 1, panels 18–20). Complete loss of plexin A3 was evident in grade III endometrial cancer (Fig. 1, panel 20). No changes in expression of NP1 and NP2 were noticed between normal and different grades of endometrial cancer tissues. Taken together, these 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. Because SEMAs are secretory proteins,
the expression of SEMA3B and SEMA3F was examined in CM and cellular extracts of a panel of normal and malignant endometrial cell lines. Analysis of endogenous SEMA3B and SEMA3F protein expression in 1 normal endometrial (EM-E6/E7/TERT) and 3 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

### Table 1. Correlation between clinicopathologic features of patients and staining intensity of SEMA3B, SEMA3F, plexins, and NPs

<table>
<thead>
<tr>
<th>Clinical parameters</th>
<th>Antigens examined</th>
<th>Cores (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue</td>
<td>SEMA3B</td>
<td>SEMA3F</td>
</tr>
<tr>
<td></td>
<td>Low (0–1)</td>
<td>High (2–3)</td>
</tr>
<tr>
<td>Normal</td>
<td>8.0 ± 0.8</td>
<td>37.5 ± 2.8</td>
</tr>
<tr>
<td>Malignant</td>
<td>92.0 ± 8.8</td>
<td>62.5 ± 4.0</td>
</tr>
<tr>
<td>Gr. I</td>
<td>55.0 ± 3.4</td>
<td>13.6 ± 0.6</td>
</tr>
<tr>
<td>Gr. II</td>
<td>54.0 ± 4.8</td>
<td>92.3 ± 4.6</td>
</tr>
<tr>
<td>Gr. III</td>
<td>46.0 ± 4.1</td>
<td>3.8 ± 0.3</td>
</tr>
<tr>
<td>Normal</td>
<td>81.2 ± 5.1</td>
<td>93.7 ± 6.9</td>
</tr>
<tr>
<td>Gr. I</td>
<td>18.8 ± 1.7</td>
<td>6.2 ± 0.4</td>
</tr>
<tr>
<td>Gr. II</td>
<td>12.3 ± 1.1</td>
<td>9.7 ± 0.3</td>
</tr>
<tr>
<td>Gr. III</td>
<td>9.1 ± 0.7</td>
<td>7.6 ± 0.2</td>
</tr>
</tbody>
</table>

NOTE: Grade I, indicates well differentiated; Grade II, moderately differentiated; Grade III, poorly differentiated; T1, indicates tumor invades submucosa; T2, tumor invades muscularis propria; T3, tumor invades through muscularis propria into subserosa or into nonperitonealized pericolic or perirectal tissues. Staining intensity: 0, no staining; 1, weak; 2, moderate; 3, strong. Statistically significant (P < 0.05) values are given in bold.
cells compared with the normal cell line (Fig. 2A). SEMA3 family members act through a membrane-associated receptor complex composed of plexin A1 or plexin A3, in addition to NP1 and NP2 receptors. Therefore, we also analyzed the expression of these receptors in normal and malignant endometrial cells. Normal endometrial epithelial cells (EM-E6/E7/TERT) showed higher expression of plexin A3 (220 kDa) compared with Ishikawa, HEC-1B, and RL-95 cells, whereas the expression of plexin A1 (211 kDa) was not altered (Fig. 2A). No marked changes were observed

![Figure 2](image-url)

**Figure 2.** Upregulation of SEMA3B and SEMA3F in endometrial cancer cells by P4 and 1,25(OH)2D3. A, normal immortalized EM-E6/E7/TERT 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)2D3 on endometrial cancer cell (Ishikawa, HEC-1B, and RL-95) growth and apoptosis. The cells were treated with P4 (12.5–200 μmol/L) or 1,25(OH)2D3 (50–400 nmol/L) for 72 hours, and the viability of cells was determined by MTT assay. Data shown are mean ± SD of 3 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)2D3 for 72 hours, harvested, and cell lysates were prepared for caspase-3 enzyme activity. The data are representative of 3 independent experiments with similar results. *statistically significant changes in cell viability/apoptosis, compared with those seen in control cells (*P < 0.05). D, the effect of P4 (25 μmol/L) and 1,25(OH)2D3 (100 nmol/L) on the expression of SEMA3B and SEMA3F was studied in endometrial cancer cell lines. Cells were cultured with P4 or 1,25(OH)2D3 in the presence or absence of antagonists (mifepristone for P4 and telmisartan for 1,25(OH)2D3) for 72 hours. 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.
in the expression of NP1 (130 kDa) and NP2 (116 kDa) in normal and malignant cells (Fig. 2A). Mouse E16 cerebellum and rat brain lysates were used as positive controls for SEMA3B and SEMA3F, respectively (Fig. 2B).

P4 and 1,25(OH)2D3-induced growth inhibition of endometrial cancer cells is associated with enhanced expression of SEMA3B and SEMA3F

The effects of P4 and 1,25(OH)2D3 on cell proliferation in 2 endometrial cancer lines (HEC-1B and Ishikawa) were investigated over a wide concentration range. Cells were treated with either P4 (12.5–200 μmol/L) or 1,25(OH)2D3 (50–400 nmol/L) for 72 hours and cell proliferation was assessed by MTS assay. Although both P4 and 1,25(OH)2D3 showed dose-dependent decreases in cell viability, P4 illustrated a more pronounced effect on cell growth compared with 1,25(OH)2D3 (Fig. 2C). To assess whether P4 or 1,25(OH)2D3-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)2D3 treatments (Fig. 2C).

As all 3 endometrial cancer cell lines showed low expression of SEMA3B and SEMA3F compared with the normal cell line, we next sought to determine whether exposure of cells to endometrial cancer preventive agents P4 or 1,25(OH)2D3 could restore the expression of SEMA3B and SEMA3F in cells. Treatment with P4 (25 μmol/L) or 1,25(OH)2D3 (100 nmol/L) for 72 hours 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 (Fig. 2D). To ascertain whether the observed P4 or 1,25(OH)2D3-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 hour prior to addition of P4 or 1,25(OH)2D3. Addition of mifepristone or telmisartan blocked P4- and 1,25(OH)2D3-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 (Fig. 2D). Mifepristone and telmisartan alone had no effect on SEMA3B and SEMA3F expression (not shown).

P4- and 1,25(OH)2D3-induced cell growth inhibition of endometrial cells is attenuated by knockdown of SEMA3B and SEMA3F

To show that P4 and 1,25(OH)2D3 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 2 endometrial cancer cell lines, then treated them with P4 and 1,25(OH)2D3 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 (Fig. 3A). No change in SEMA3B and SEMA3F expression was observed in control cultures compared with scrambled siRNA controls (Fig. 3A). Mock-transfected cells treated with P4 and 1,25(OH)2D3 showed a marked increase in SEMA3B and SEMA3F expression (Fig. 3A). Treatment of cells with P4 and 1,25(OH)2D3 for 72 hours showed a significant decrease in cell viability in control cells compared with cells in which SEMA3B and SEMA3F were separately or conjointly silenced by siRNA (Fig. 3B). After 72 hours of 1,25(OH)2D3 exposure, 55% to 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 (Fig. 3B), indicating that SEMAs mediate the growth inhibitory effects of 1,25(OH)2D3. Progesterone treatment of cells resulted in 35% to 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 (Fig. 3B). SEMA3B and SEMA3F siRNAs have no effect on control cells (Fig. 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 (Fig. 3B), when compared with 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 (Fig. 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 4A and B). 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 (Fig. 4C) and migration (Fig. 4D) using cell viability and cell invasion assays. Seventy-two hours after transfection, cell proliferation and migration was assessed. As shown in Fig. 4C, a
marked concentration-dependent decrease in cell proliferation was observed in SEMA3B- and SEMA3F-transfected cells compared with mock transfected cells. Of note, coexpression of SEMA3B and SEMA3F inhibited cell proliferation in a synergistic manner (Fig. 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 (Fig. 4D). Upregulation of SEMA3B and SEMA3F in endometrial cancer cells showed a 30% to 40% and 60% to 70% respective decrease in colony-forming ability on culture plates as compared with mock-transfected cells. Taken together, these results show 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 NP1 or NP2, or both. NPs complex with the plexins to mediate downstream signaling. To better understand the requirement of NP1 and NP2 to mediate the effects of SEMA3B and SEMA3F, respectively, Ishikawa and HEC-1B cells were transfected with either NP1- or NP2-specific siRNA or scrambled siRNA as a control. As shown in Fig. 5A, NP1 and NP2 proteins were markedly reduced in both endometrial cancer cell lines with respective siRNA. Downregulation of NP2 significantly reduced the ability of cells to respond to SEMA3F-CM and attenuated inhibition of tumor cell proliferation, whereas cells transfected with NP1 or control siRNA (Fig. 5B) retained the responsiveness to SEMA3F and inhibited cell proliferation (Fig. 5B). Interestingly, silencing NP1 or NP2 retained the responsiveness of cells to SEMA3B-CM, and a marked decrease in cell proliferation was seen compared with cells exposed to control-CM. However, when both NP1 and NP2 were silenced, cells were not responsive to either SEMA3F-CM or to SEMA3B-CM (Fig. 5B). Together, these studies

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 2 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 days 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 3 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 3 independent experiments; bars, ± SD. **, Statistically significant changes in colony formation, compared with those seen in control cells (P < 0.05). Endometrial cancer cells transfected with SEMA3B and SEMA3F were plated on Matrigel to assess cell migration. After 22 hours, cells that migrated through the Matrigel were counted. Columns, mean of 3 independent experiments; bars, ± SD. **, Statistically significant changes in cell invasion, compared with those seen in control cells (P < 0.05).
indicate that treatment with soluble SEMA3F inhibits endometrial cell growth in a NP2–dependent manner, whereas soluble SEMA3B mediates its effect by employing both NP1 and NP2.

**Upregulation of SEMA3B and SEMA3F inhibit matrix metalloproteinases, integrins, and alter pro- and antiangiogenic 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 SEMA3 inhibits tumorigenesis, 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). Because both SEMA3B and SEMA3F inhibited MMPs, we explored the relationship between SEMAs and the specific receptors that transduce their signals. Expression of MMP-9 was significantly lower in NP1–downregulated

**Figure 5.** Expression of NP2 is required for SEMA3F and SEMA3B inhibition of MMPs. Endometrial cancer cells (HEC-1B and Ishikawa) were transfected with a scrambled siRNA, siRNA for NP1, siRNA for NP2 or both. Transfected cells were exposed to SEMA3F-CM, or SEMA3B-CM or control-CM for 72 hours. 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 hours and then incubated in the serum-free culture medium for 24 hours. CM were collected and then subjected to gelatin zymography. Representative picture from 3 independent experiments is shown. D, Western blot for MMP-9 and MMP-2 in HEC-1B and Ishikawa cells after NP1 siRNA, NP2 siRNA, both NP1 and NP2 siRNAs, or control siRNA knockdown followed by treatment with SEMA3F-CM or SEMA3B-CM for 72 hours. E, total cellular protein extracts were analyzed for αVβ3 integrin using specific antibodies. β-Actin was used as a loading control.
endometrial cancer cells exposed to SEMA3F-CM or SEMA3B-CM compared with cells exposed to control-CM. No significant changes in the expression of MMP-9 and MMP-2 were detected in NP2-silenced HEC-1B and Ishikawa cells treated with SEMA3F-CM and SEMA3B-CM compared with control cells (Fig. 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 (Fig. 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 proangiogenic and antiangiogenic 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 2-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 proangiogenic factors (e.g., IL-8, VEGF, FGFR, ECGF, and MMP-9; Table 2); and increased expression of antiangiogenic factors (e.g., TIMP-3 and TGF-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, whereas 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 this 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 showed a significant reduction in the expression of both SEMAs and their receptor plexin A3 in grade III endometrial cancer compared with grade 1 or normal endometrial tissues. To our knowledge, this is the first article 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 CM of normal cells than in cancer cells, indicating a potential role for class 3 SEMAs in endometrial cancer. Coexpression of SEMAs and their receptors by tumor cells suggests that SEMAs may act through an autocrine or paracrine mechanism in these cells. SEMAs 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 show 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 with 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 show 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 LOH 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 and colleagues (47) showed that SEMA3B-induced
IL-8 secretion from tumor cells promotes cancer progression and metastasis.

To search for the underlying mechanisms by which SEMAs upregulation enact their antitumor 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 with their receptive control cells. It is of interest to note that the 2 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 showed downregulation of several proangiogenic genes (FGF2, FGF3, MDK, MMP-2, and MMP-9) and upregulation of antiangiogenesis genes (TIMP1, TIMP2, and 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 proangiogenic genes and by upregulating antiangiogenic genes. The results of the studies reported here showed that exogenously added SEMA3F and SEMA3B inhibit MMPs by acting through NP2 receptors in cancer cells, leading to decreased cell survival. 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, inhibitor of MMP, that is, 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 article 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 proangiogenic 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.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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

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