Endogenous Axon Guiding Chemorepulsant Semaphorin-3F Inhibits the Growth and Metastasis of Colorectal Carcinoma

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Abstract

Purpose: To elucidate the role of Semaphorin-3F (SEMA3F), originally described as an axon guiding chemorepulsant implicated in nerve development, in the progression of colorectal carcinoma.

Experimental Design: SEMA3F and its receptor NRP2 were examined in 72 cases of human colorectal carcinoma specimens and cell lines LoVo, SW480, and SW620 with immunohistochemistry and Western blotting. SEMA3F mRNA expression in the frozen tissue specimens and cell lines was examined with quantitative reverse transcriptase-PCR. Confocal laser scanning microscopy was used for detection of cellular localization of the proteins by immunofluorescent staining. MTT assay, flow cytometry, cell adhesion and migration, and xenografts were used to evaluate biological significance of SEMA3F.

Results: SEMA3F was significantly reduced in colorectal carcinoma tissues and cell lines. Overexpression of SEMA3F resulted in reduced proliferation, adhesion to fibronectin, and migratory capability as well as reduced S-phase population and integrin αvβ3 expression of SW480 colon cancer cells. In addition, SEMA3F-overexpressing cells exhibited diminished tumorigenesis when transplanted orthotopically in nude mice and reduced liver metastases. Moreover, transfection of siRNA targeting SEMA3F in colon cancer cells increased their tumorigenicity in vivo.

Conclusions: Endogenous SEMA3F acts as a suppressor of the growth and metastasis of human colorectal cancer cells. Clin Cancer Res; 17(9); 2702–11. ©2011 AACR.

Introduction

Metastasis is a crucial adverse factor determining the prognosis of cancer patients. The capabilities of proliferation and invasion of tumor cells have been identified as pro-metastasis factors in a variety of cancers, including colorectal carcinoma. Various types of growth-promoting proteins and peptides, for instance vascular endothelial growth factor (VEGF), are involved in the promotion of tumor cell proliferation and invasion. It has been reported that VEGF-C, one of VEGF protein family members, is overexpressed by neoplastic cells of advanced-stage cancer. VEGF-C, also known as VEGFR-3, is the receptor for Semaphorin-3F (SEMA3F) (5, 6). SEMA3F belongs to class 3 Semaphorin family, a large group of secreted axon guidance molecules involved in neuronal development (7–9). It has been reported that NRP2 was overexpressed in pancreatic, pulmonary, and colorectal carcinomas as well as melanoma, and the expression was correlated with advanced stage and grade of cancer (10–12). Moreover, blocking NRP2 function could inhibit lymphatic metastasis of cancer. Therefore, NRP2 might be a potential target of cancer therapy (13). However, the NRP2 ligand SEMA3F was found to perform an inhibitory role in tumorigenicity of lung, ovarian, and breast cancers (14–16). It was also found to inhibit the invasion and metastasis of melanoma cells expressing functional NRP2 and prevent blood vessel sprouting into the tumor mass (12), suggesting that inhibition of NRP2 activation and/or induction of SEMA3F production might be of significant therapeutic potential. The complicated relationship between SEMA3F/NRP2 pathway and the progression of colorectal cancer, particularly the metastasis, requires further clarification.

In this study, we examined the expression of endogenous SEMA3F and its receptor NRP2 in human colorectal carcinoma. We found that SEMA3F was expressed at low levels...
in most colorectal cancer cases, and overexpression of SEMA3F inhibited the proliferation and metastatic potential of cancer cells in vitro and in vivo. The reduced capabilities of cancer cell adhesion and migration by overexpression of SEMA3F might be due to the inhibition of integrin αvβ3 production by cancer cells. Our results suggest that endogenous SEMA3F, as a potent tumor suppressor, plays a critical role in the negative regulation of the growth and metastasis of colorectal cancer.

Materials and Methods

Tissue specimens and cell lines

Seventy-two cases of tumor tissues were from colorectal carcinoma patients who underwent colorectal lymph node dissection in the period from January to July, 2006 at Southwest Hospital of Third Military Medical University. The patients received neither chemotherapy nor radiotherapy before surgery. The diagnoses of colorectal carcinoma were made independently by at least 2 histopathologists.

The human colorectal carcinoma cell lines LoVo, SW480, and SW620 were obtained from the American Type Culture Collection (ATCC), and the highly metastatic human colorectal carcinoma cell line KM12SM was kindly provided by Dr. Isaiah J. Fidler (MD Anderson Cancer Center, The University of Texas, Houston, TX). The KM12SM cell line was isolated from a liver metastasis derived from the parental poorly metastatic KM12C cell line which was from a primary colon carcinoma classified as Dukes’ Stage B2 (17). All the cell lines were maintained in MEM (minimum essential medium; Invitrogen), supplemented with 10% FBS (PAA Corp.), 2 U/mL penicillin-streptomycin, vitamins, 1 mmol/L sodium pyruvate, 2 mmol/L L-glutamine, and nonessential amino acids (Sigma-Aldrich) at 37°C in 5% CO2. This study was carried out in accordance with the principles of the Helsinki Declaration and approved by the Ethical Committee of the Third Military Medical University.

Quantitative reverse transcriptase-PCR

Total RNA was extracted either from the frozen tissue specimens or cultured cells with Trizol Reagent (Roche) following the manufacturer’s instructions. The extracted RNA was examined by UV spectrophotometer and electrophoresis to determine the concentration and integrity. The relative mRNA levels of NRP2 and SEMA3F in cell lines were determined by reverse transcriptase (RT)-PCR in a Thermo PCR System (Thermo Fisher). SEMA3F mRNA in fresh cancer tissues was determined by real-time RT-PCR with C1000 real-time RT-PCR cycler (Bio-Rad). The High Fidelity RNA PCR Kit, SYBR Primerscript RT-PCR Kit, and DNA ladders were purchased from Takara Biotech. PCR and quantitative PCR primers were designed by Primer Premier 5.0 software based on the sequence data in the GenBank. The reaction conditions and primer information are provided in the Supplementary Data. For conventional PCR, products were examined by electrophoresis in 1.5% agarose gel and analyzed with a Gel Doc XR system (Bio-Rad). For quantitative PCR, the average intensity of fluorescence in 10% previous cycle time was recorded automatically after each cycle. After the reaction, the reaction curve was plotted and data were analyzed. Baseline was adjusted and threshold cycle (Ct) was calculated. RNase-free water was used for negative reaction control. The quantitative data were used for calculation of the relative quantity using reported methods (18).

Western blotting

Total proteins of the cultured cells were extracted with M-PER Mammalian Protein Extraction Reagent (PIERC). Proteins (100 μg) were denatured and electrophoresed with SDS-PAGE. The proteins were then transferred onto Hybond ECL nitrocellulose membranes (Amerham Pharmacia Biosciences). Following incubation with 5% nonfat milk in PBS, the membranes were incubated overnight with a long form (endogenous) SEMA3F antibody (AB5471P, Chemicon), and antibodies against NRP2 (sc-13117, Santa Cruz), integrin subunit αvβ3 (sc-6595, sc-13579, Santa Cruz), β-actin (ab75186, Abcam), and glyceraldehyde 3 phosphate dehydrogenase (GAPDH; sc-5940, Santa Cruz) respectively. The protein expression was revealed by using HRP-conjugated secondary antibodies and visualized with an enhanced chemiluminescence (ECL) detection system (Amerham Pharmacia Biosciences, NJ).

Confocal laser scanning microscopy and immunohistochemical studies

Immunofluorescent staining was conducted to localize the expression of SEMA3F and NRP2. Colorectal cancer cells grown on glass coverslips were fixed for 20 minutes in 4% paraformaldehyde. The cells were then rinsed 3 times with PBS for 5 minutes each time, and incubated in a protein-blocking solution for 20 minutes at room temperature. After incubation with the primary antibody against
either human SEMA3F (1:100; Chemicon) or human NRP2 (1:100; Santa Cruz Biotechnology) overnight at 4°C, followed by incubation with the secondary antibody (either FITC-conjugated IgG, 1:50, Santa Cruz; or Cy3-conjugated IgG, 1:500, Beyotime) at 37°C for 1 hour, the cells were counterstained with Hoechst 33342 to reveal the nuclei. Cells or slides without incubation primary antibodies were served as negative controls. The cells or slides were then analyzed by confocal laser scanning microscopy.

All tumor and adjacent normal tissue sections were routinely dewaxed, rehydrated, and prepared for immunohistochemistry. The sections were incubated in 0.3% H2O2 in methanol for 30 minutes to block endogenous peroxidase. Antigens were retrieved with 10 mmol/L sodium citrate (pH 6) for 15 minutes in a microwave oven. The anti-SEMA3F or anti-NRP2 antibody was added and the sections were incubated at 37°C for 1 hour and 4°C overnight. The reactions were developed with an EnVision method (DAKO). The sections were visualized using the diaminobenzidine solution (DAKO), and then lightly counterstained with hematoxylin. To detect micro-metastasis in the liver and lungs in mouse experiments, staining for human CK20 (cytokeratin 20) was conducted on xenograft tissues. Sections without treatment with primary antibody served as negative controls. Rat and human brain tissues were chosen for positive controls. The anti-SEMA3F antibody recognized neurons of normal rat and human brain tissues (data not shown). The results were verified by 2 histopathologists in a blinded manner.

RNA interference

The SEMA3F expression vector pSectag-SEMA3F was kindly provided by Dr. David Ginty. The vector (1 ng) was transfected into SW480 cells using FuGene HD transfection reagent (Roche) to generate overexpressing cells (S cells). The transfected cells were cultured for 12 hours and then fresh medium containing 10% FBS and 800 μg/mL zeocin (Invitrogen) was used to continue culture for 2 weeks before individual clones were isolated.

RNAi vectors were constructed with the use of pSilencer 3.1-Hygro (Invitrogen). Based on the published sequence of SEMA3F (GI: 31377801) and Ambion RNAi web design tool (http://www.ambicon/simaca), we designed 2 SEMA3F-specific targeting sequences (SEMA3F-#1: 5’-AACTTCCGTCTCAACACAACC-3’ and SEMA3F-#2: 5’-AATCITGTCAAGGACGAGA-3’). Two annealed oligonucleotides, each encoding one of the target sequences followed by a 9-bp hairpin sequence (tctcaagaga) and flanked by 5’ BamHI and 3’ HindIII overhangs as follows, were ligated into the pSilencer 3.1-Hygro expression plasmid. The sequences were SEMA3F-#1 forward: 5’-GATCCGTCITCGTCITCAACACAACC-tctcaagaga GTGTTGTTGTACG-CAGCAAGATTHTTGGAAA-3’, reverse: 5’-AGCTTTTCCTCAA-AAA CTCTCCTCGCICAAACAGGAGCCTgtcttgaa GTGTTGTTG-TCAGCAGCAAGGC-3’ and SEMA3F-#2 forward: 5’-GATCCGTCITCGTCITCAACACAACC-tctcaagaga GTGTTGTTG-TCAGCAGCAAGGC-3’ and SEMA3F-#2 reverse: 5’-AGCTTTTCCTCAA-AAA CTCTCCTCGCICAAACAGGAGCCTgtcttgaa GTGTTGTTG-TCAGCAGCAAGGC-3’. The 2 resulting RNAi expression plasmids, pSilencer-SEMA3F-#1 and pSilencer-SEMA3F-#2, were confirmed by sequencing. Using NIH BLAST analysis to ensure no substantial homology to sequences in other vertebrate genes. For negative controls, RNAi-Mock vectors were constructed using a scrambled sequence (Mock: 5’-GACTTCATAGGCGCATGC-3’). Both targeting vectors and control vectors at 1 ng were transfected into SW480 cells using FuGene HD transfection reagent (Roche) to generate RNAi (Si cells) and Mock-RNAi cells (Mock). The transfected cells were cultured for 12 hours then in fresh media containing 10% FBS and 250 μg/mL hygromycin (Sigma-Aldrich) for 2 weeks. SEMA3F expression level in isolated clones was determined by Western blot.

The pBMN-COS-NRP2 vector (Invitrogen Corp) was also kindly provided by David Ginty. For generation of pan- tropic retroviruses, the vector was transfected into COS-7 cells with FuGene HD Transfection Reagent (Roche). Stable transfectants were selected under 800 μg/mL G418 (Sigma-Aldrich). S-cells and Si-cells were infected for 2 cycles with 2 μl retrovirus containing supernatant from the COS-7 cells for 6 hours each. Stable NRP2 expressing SW480 clones (SN-cells and SiN-cells) were expanded in media containing 200 μg/mL G418. pBMN-COS empty vector was used to generate control cells.

MTT assay

To investigate the role of SEMA3F in the proliferation of tumor cells in vitro, Si-cells, SiN-cells, SN-cells, and parental cells were plated in 96-well plates (Invitrogen) at a density of 2,000 cells per well in 10% FBS in MEM. Cell number was assessed by measuring the mitochondrial reduction of MTT (Sigma) to formazan. MTT was added every 24 hours after plating at a final concentration of 2.5 mg/mL. After incubation for 180 minutes, the medium and MTT were removed and dimethyl sulfoxide was added for 1 minute. Optical absorbance was measured at 490 nm.

Flow cytometry

For analysis of cellular DNA diploids, cancer cells were harvested and fixed in 75% ethanol at 4°C for 24 hours. After incubation with RNase at 37°C, the cell suspensions were stained with propidium iodide (PI) for 15 minutes. Optical absorbance was determined by flow cytometry (Calibur).

Cell adhesion and migration

Cancer cells were plated at 4 × 10^4/mL onto fibronectin-coated culture dishes and maintained in complete medium. After seeding for 30, 60, and 90 minutes, the attached cells were harvested and quantified. Adhesion percent = attached cell number/total cell number. Cancer cell migration was evaluated in transwells as described previously with modifications (12). The cells (1 × 10^5/well) were added in the upper chambers and pure fetal calf serum was added to the lower chambers as chemoattractants. After incubation for 12 hours at 37°C, the remaining cells left in the upper chamber were removed and the cells migrated to
the lower chambers were fixed and stained with hematoxylin. The number of migrated cells was counted in 5 distinct areas at ×400 magnification. The results represent the average cell number in 4 wells per cell line. The experiments were repeated 3 times with similar results.

Xenografts

Eight-week-old male nu/nu mice (Production license: SCXK-Military-2002007, from the Experimental Animal Center, Third Military Medical University) were acclimated for a week while caged in groups of 4. Animal care was provided in accordance with the Guidelines for the Care and Use of Laboratory Animals. Mice were fed a diet of animal chow and water ad libitum throughout the experiments and were randomly assigned to 1 of 3 groups (15 mice per group). Mouse body weight at the assignment was similar among the groups. To establish orthotopic xenografts, after cell viability was verified as being 80% or greater by trypan blue exclusion, control and cancer cells (1 × 10^7 cells in 200 μL of PBS) transfected with various vectors were injected into the subserosa of the mouse colon. Stool shape and abdominal pertrusion in the animals were observed every other day. Subsequently, 5 mice of each group were sacrificed at 4, 8, and 12 weeks after the cancer cell injection. Xenografts, regional lymph nodes, the liver and lung were harvested and placed in 10% formalin for section preparation. Xenograft volume was calculated as follows: 

\[ V_T = \frac{l \times w^2 \times 0.52}{C^2} \]

Statistical analysis

To compare metastasis rate among different groups, independent samples comparisons and 1-way analyses of variance followed by the post hoc Fisher’s test were carried out using the SPSS software (Version 13.0, LEAD Technologies). Other data were expressed as 3 to 5 independent experiments. \( P < 0.05 \) was considered statistically significant.

Results

SEMA3F expression was reduced in more malignant human colorectal cancer specimens

For investigation of the possible relationship between the SEMA3F/NRP2 pathway and the malignancy degree of colorectal carcinoma, we examined the expression of both SEMA3F and the receptor NRP2 in human colorectal carcinoma specimens. NRP2 protein was detected in 38% (19/50) of cases without metastasis, whereas it was found in 91% (20/22) of cases with metastasis. NRP2 mRNA was expressed in 39.1% (9/23) cases without metastasis and in 100% (11/11) cases with metastasis (Fig. 1A). Our results are consistent with the previous studies that NRP2 might be a prometastatic factor (19).

We then examined the expression of SEMA3F, a ligand of NRP2, in the human specimens of both cancers and benign mucosa. We detected SEMA3F protein in the cytoplasm of gland epithelial cells near the basement membrane in 83.3% (60/72) of benign mucosa but in 44.4% (32/72) of adenocarcinomas (Fig. 1B). We also examined SEMA3F mRNA in fresh tissues of human colon cancer and benign colon mucosa by real-time RT-PCR. Our results showed that SEMA3F was significantly reduced in tumor tissues, as compared with normal mucosa (\( P < 0.05 \)). The reduced expression of SEMA3F was correlated with high rate of metastasis.
metastasis, poor differentiation, and advanced Duke stage. The patient gender and age were not correlated with SEMA3F expression (Table 1).

**Highly metastatic cancer cells expressed less SEMA3F**

We further studied SEMA3F and NRP2 expression in 4 colorectal carcinoma cell lines, 3 of which were isolated from metastatic sites (KM12SM, LoVo, and SW620) and 1 was from a primary tumor (SW480). SEMA3F expression level was higher in SW480 than in SW620, while NRP2 expression was lower in SW480 than in KM12SM, LoVo, and SW620 cells. We also found that VEGF-C was expressed in LoVo but not in SW480 cells (Fig. 2A–C). Confocal laser scanning microscopy revealed that SEMA3F and NRP2 were expressed in the cytoplasm of cancer cells, except in SW480 cells in which NRP2 was detected on cell membrane.

**SEMA3F negatively modulated cell proliferation**

SEMA3F expression was significantly reduced in Si-cells and SiN-cells stably transfected with SEMA3F siRNA, and elevated in S-cells and SN-cells stably transfected with SEMA3F. The expression of NRP2 was significantly upregulated in SN-cells and SiN-cells transduced with NRP2 gene (Fig. 3A). The proliferation of the cells transfected with SEMA3F siRNA was increased. In contrast, proliferation of the cells overexpressing SEMA3F was inhibited (Fig. 3B). However, the ratio of apoptosis in all groups was not significantly changed. To verify the results, cell cycle was analyzed by DNA diploid analysis with flow cytometry. The percentages of S-phase cells were approximately 31.53% in Si-cells and 11.19% and 22.3% in S-cells and parental SW480 cells (Par cells), respectively. There were fewer cells in S phase when SEMA3F was increased by transfection. When SEMA3F expression was depleted with siRNA, the percentage of cells at S phase was increased (Fig. 3C).

**SEMA3F inhibited cancer cell adhesion and migration by reducing the level of integrin αvβ3**

Since tumor cell adhesion and migration are 2 crucial events for metastasis, we assessed the effect of SEMA3F on cancer cell adhesion by fibronectin-coated plates. SEMA3F transfected cells exhibited significantly lower adhesion capability as compared with control cells at 60 minutes (Fig. 4A). In transwell migration assays, the cells with reduced SEMA3F levels showed higher cell migration capability than controls and SEMA3F-overexpressing cells.

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**Table 1. Relationship between clinicopathological features of colon cancer cases and expression of endogenous SEMA3F and NRP2**

<table>
<thead>
<tr>
<th>Clinicopathologic features</th>
<th>N</th>
<th>IHC⁺</th>
<th>P</th>
<th>SEMA3F</th>
<th>qRT-PCR⁺</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>Gender</td>
<td></td>
<td>IHC⁺</td>
<td>P</td>
<td></td>
<td>qRT-PCR⁺</td>
<td>P</td>
</tr>
<tr>
<td>Female</td>
<td>42</td>
<td>26</td>
<td>0.119</td>
<td>18</td>
<td>0.939</td>
<td>0.176 ± 0.156</td>
</tr>
<tr>
<td>Male</td>
<td>30</td>
<td>13</td>
<td></td>
<td>14</td>
<td></td>
<td>0.163 ± 0.156</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤51</td>
<td>22</td>
<td>16</td>
<td>0.036</td>
<td>12</td>
<td>0.253</td>
<td>0.148 ± 0.137</td>
</tr>
<tr>
<td>&gt;51</td>
<td>50</td>
<td>23</td>
<td></td>
<td>20</td>
<td></td>
<td>0.182 ± 0.163</td>
</tr>
<tr>
<td>Differentiation</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Well</td>
<td>9</td>
<td>6</td>
<td>0.264</td>
<td>8</td>
<td>0.006</td>
<td>0.281 ± 0.169</td>
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<tr>
<td>Moderate</td>
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<td>28</td>
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<td>21</td>
<td></td>
<td>0.258 ± 0.233</td>
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<tr>
<td>Poor</td>
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<td>5</td>
<td></td>
<td>3</td>
<td></td>
<td>0.119 ± 0.095</td>
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<tr>
<td>Positive</td>
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<td>0.026</td>
<td>6</td>
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<td>0.226 ± 0.275</td>
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<tr>
<td>Negative</td>
<td>60</td>
<td>29</td>
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<td>26</td>
<td></td>
<td>0.162 ± 0.135</td>
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<td>RLN metastasis</td>
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<tr>
<td>Positive</td>
<td>22</td>
<td>20</td>
<td>0.000</td>
<td>5</td>
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<td>0.041 ± 0.027</td>
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<td>Negative</td>
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<td>19</td>
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<td>27</td>
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<td>0.238 ± 0.149</td>
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<tr>
<td>Dukes’ stage</td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>A</td>
<td>4</td>
<td>2</td>
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<td>4</td>
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<tr>
<td>B</td>
<td>46</td>
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<td></td>
<td>21</td>
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<tr>
<td>C</td>
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<td>18</td>
<td></td>
<td>7</td>
<td></td>
<td>0.084 ± 0.000</td>
</tr>
<tr>
<td>D</td>
<td>4</td>
<td>4</td>
<td></td>
<td>0</td>
<td></td>
<td>0.035 ± 0.023</td>
</tr>
</tbody>
</table>

*qRT-PCR data from 34 random cases, relative to normal mucosa.
Abbreviations: BV: blood vessel; RLN: regional lymph nodes.
We then determined whether the expression of integrin αvβ3, which has been shown as a key mediator of cell–matrix attachment and motility (20–22), was regulated in the cancer cells by SEMA3F alteration. We found that SEMA3F expression resulted in the reduction of integrin αvβ3, and knockdown SEMA3F by RNA interference increased the levels of integrin αvβ3 (Fig. 4C). However, changes in NRP2 expression did not significantly affect cancer cell adhesion (Fig. 4A and B).

**SEMA3F overexpression inhibited tumor growth and metastasis in vivo**

S-cells, SN-cells, SiN-cells, and Par cells were implanted orthotopically into nude mice to analyze their tumorigenic potential. All cell lines formed tumors 4 weeks after implantation. Tumors formed by SN-cells were the smallest and grew more slowly, whereas tumors formed by SiN-cells grew most rapidly measured at 4, 8, and 12 weeks after implantation (Fig. 5A). Proliferative cell nuclear antigen (PCNA) immunostaining showed that SEMA3F and NRP2 overexpressing tumor xenografts exhibited lower proliferative activity as compared with control xenografts (P < 0.05; Fig. 5B). The result was support of our flow cytometry data outcome and suggests that SEMA3F might serve as a tumor suppressor by reducing tumor cell proliferation.

We further found that cancer cells overexpressing SEMA3F were less prone to metastasize to the lung and liver (Table 2). Various organs were examined for micro-metastasis with immunostaining for CK20, a biomarker of colorectal carcinoma. Metastatic nodules or cells were found in the liver, lung, and mesentery lymph nodes after implantation in both control mice receiving Par cells and mice receiving SiN-cells. Few metastases in the liver were found in mice implanted with SEMA3F-overexpressing S-cells and SN-cells. Mice bearing Par-cell–derived tumors developed lung and liver metastases. Therefore, overexpression of SEMA3F reduced metastasis of colorectal cancer cells, in particular when in combination with overexpression of NRP2 (Fig. 5C).

**Discussion**

The **SEMA3F** gene was originally isolated from 3 smallcell lung cancer cell lines with a homozygous deletion on chromosome 3p21.3, suggesting that **SEMA3F** might be a candidate tumor suppressor gene (23–25). Recent studies suggest that **SEMA3F** might play an important repressive role in the growth, angiogenesis, invasion, and metastasis of colorectal cancer.

### Table 2. Metastasis incidence of each cell line for each metastatic site

<table>
<thead>
<tr>
<th>Cell</th>
<th>Incidence of lymph node metastasis</th>
<th>Incidence of lung metastasis</th>
<th>Incidence of liver metastasis</th>
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<tr>
<td>Par</td>
<td>7/15</td>
<td>10/15</td>
<td>12/15</td>
</tr>
<tr>
<td>S</td>
<td>2/15</td>
<td>2/15</td>
<td>0/15</td>
</tr>
<tr>
<td>SN</td>
<td>0/15</td>
<td>0/15</td>
<td>0/15</td>
</tr>
<tr>
<td>SiN</td>
<td>15/15</td>
<td>10/15</td>
<td>10/15</td>
</tr>
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</table>
of several cancers (26–29). Nasarre and colleagues (27) found that SEMA3F had an opposing effect against VEGF and inhibited cell attachment. When SEMA3F was over-expressed in the breast cancer cell line MCF7 by transfection, cell spreading was reduced, which was attributed to the loss of E-cadherin and cell contact (27). Similar results were reported in melanoma and lung cancer. Xenografts derived from SEMA3F transfected melanoma cells showed a poorly vascularized, encapsulated, and nonmetastatic phenotype (12). The human ovarian cancer cell line HEY transfected with SEMA3F exhibited a diminished tumorigenicity in nude mice (28). Meanwhile, highly metastatic lung and breast cancer as well as melanoma cell lines showed a reduced SEMA3F level. However, some studies did not observe the inhibitory effect of SEMA3F on the growth of tumor cells in vitro (12, 29, 30). In addition, the function of endogenous SEMA3F in growth and/or metastasis of cancers including colorectal cancer is not clear.

In the present study, we found dramatically reduced proliferation and diminished metastasis in SEMA3F transfected tumor cells and the resultant xenografts. We found that SEMA3F was expressed at low levels in highly metastatic human colon cancer cell lines. Interestingly, SW480 cell line, established from a primary colon adenocarcinoma of a 51-year-old Caucasian male, expressed lower levels of NRP2 but higher levels of SEMA3F. In contrast, SW620 cells, which were derived from a lymph node metastasis of the same patient 1 year later expressed higher levels of NRP2, but lower levels of SEMA3F, indicating a significant alteration in the expression profile of SEMA3F/NRP2 genes associated with tumor metastasis. These results suggest that a correlation between the acquisition of more aggressive properties and alteration of SEMA3F/NRP2 expression profiles is crucial in the process in which tumor cells become metastatic. Our data showed that SEMA3F/NRP2 exist in the cytoplasm of tumor cells. A previous report indicated that NRP1 could be internalized through binding 2 optional ligands SEMA3A and VEGF165 (31, 32). However, to our knowledge, there is no similar effect of SEMA3F on NRP2 internalization. We suggest that colocalization of SEMA3F and NRP2 in the cytoplasm may be evidence for NRP2 internalization.

It has been reported that Sema3B inhibited cell proliferation by inducing apoptosis in ovarian cancer cells (28). Surprisingly, our data showed that transfection either with SEMA3F or siRNA against SEMA3F did not alter the ratio of apoptosis in colon cancer. We found that alteration of SEMA3F expression resulted in a marked change in cell cycle at S phase. The level of SEMA3F was inversely correlated with the percentage of S phase in CRC cell lines. Moreover, SEMA3F blocks apoptosis blockage of transfected A9 cells treated with Taxol or Adriamycin (33). When Sema3A, another protein homologous to SEMA3F, was transfected into malignant mesothelial cells, VEGF-induced cyclin D1 upregulation and cell proliferation were inhibited (34). Thus the antiproliferative effect of SEMA3F is not due to the induction of cell apoptosis but may be attributed to its regulation of cell cycle.

In our study, colon cancer cells exhibit reduced migration when endogenous SEMA3F was increased by transfection. Reduced integrin αvβ3 was found in SEMA3F transfected colon cancer cells, along with reduced adhesion to fibronectin. Current knowledge of the molecular mechanisms comes from investigations of Sema3 members on repulsing and attracting growth cones of axons in nerve development. NRPs can form complexes with type A plexins, which increases the affinity of Sema3 to NRPs (35, 36). This subsequently induces a conformational change that relieves the autoinhibition of spontaneous activation of plexinA1 cascades, leading to further repression of integrin function and disassembly of focal adhesion and migrations (37–39). Colon cancer cells were reported to express several type A plexins (40). Our results suggest that this may also be the

Figure 3. Antiproliferative effect of overexpressed endogenous SEMA3F in selected SW480 transfectants. A, establishment of SEMA3F and NRP2 transfectants. Gene expression specificity was determined by immunoblot. β-Actin was a loading control. B, cells were plated onto 96-well plates and proliferation was determined by MTT assay. *, P < 0.05 versus Par. C, cell cycle was determined and cellular DNA diploid was detected using PI staining and flow cytometry.
reason for the antitumorigenic and antimetastatic effects of SEMA3F.

NRPs, known as the regulators of neuronal patterning through semaphorins, have been reported to show pro-metastatic roles in malignancies. In non–small cell lung cancer, NRP1 and NRP2 are coexpressed and correlated with tumor progression via promoting neovascularization (11). However, when SEMA3F was transfected into SEMA3F/NRP2+ melanoma cells, the antimetastatic effect of SEMA3F was observed in animals, suggesting that SEMA3F acts as a metastasis inhibitor through NRP2 (12). In our study, although no differences of NRP2 expression were found between cancer and peri-tumoral mucosa, NRP2 expression was significantly higher in highly metastatic cell lines. The result was confirmed by findings in clinical specimens. The orthotopic model of colon cancer also showed fewer metastasis of tumor xenografts formed by NRP2/SEMA3F overexpressing cells. The results suggest that SEMA3F may attenuate the pro-metastatic effect of NRP2 in vivo, and the antimetastasis effect of SEMA3F might be mediated through a NRP2-dependent pathway.

The fact that NRP2 overexpression promotes the growth and spreading of cancer suggests that NRP2 might be target for cancer treatment (13, 19). However, NRP2 is constitutively expressed in a variety of normal cells including lymphatic endothelial cells and is crucial for lymphangiogenesis (41, 42). Thus, it is important to target NRP2 specifically in tumors without affecting its normal function. Interestingly, our results showed that the tumor suppressive activity of endogenous SEMA3F could be directly mediated through the receptor NRP2. Overexpression of endogenous SEMA3F in tumor cells inhibited their proliferation and mobility which is likely via the SEMA3F/NRP2/plexinA complex signaling pathway. It is plausible that once NRP2 forms complex with plexins and binds with SEMA3F, it may be devoid of its coreceptor function for VEGF/VEGFR resulting in the impairment of tumor lymphangiogenesis. Thus, our results suggest that SEMA3F may directly suppress tumor progression through NRP2 in tumor cells.

In conclusion, we have showed for the first time that SEMA3F expression was expressed at low levels in most human colorectal carcinomas, and overexpression of this

Figure 4. Antiadhesion and migration effect of overexpressed endogenous SEMA3F in selected SW480 transfectants. A, cells were plated into fibronectin-coated plates and counted for the number of adherent cells. *, P < 0.05 versus Par. There were significant differences in the number of adherent cells at each time point. Representative images of adherent cells at 60 minutes were presented (top). B, cell migration was determined using a transwell assay. Cells that migrated into the lower chambers were counted and a migration graph was plotted for different cell transfectants. C, protein levels of integrin subunits αv and β3 were determined by immunoblot. β-Actin was a loading control.
protein suppressed the proliferation and invasion of cancer cells in vitro, in association with inhibition of growth and metastasis of the xenografted tumors in vivo. Therefore, SEMA3F may act as a tumor suppressor and is a candidate for cancer therapy.

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Figure 5. Orthotopic xenografts derived from different SW480 transfectants. Four SW480 transfectants were implanted orthotopically into the colon of nude mice, which were sacrificed after 4, 8, and 12 weeks. A, the volume of xenografts was calculated and growth curves were plotted. B, images of xenografts derived from SN-cells and parental control cells with PCNA immunolabeling indicate reduced proliferative activity in SN tumors. C, representative images of xenografts and tumor metastases. Top: orthotopic images of xenograft tumors derived from 4 transfectant cell lines, scale bar = 5 mm. Bottom: lung metastasis from Par group, a image was given for illustration, scale bar = 1 cm; The CK20 immunolabeling was carried out on paraffin-embedded sections to reveal a metastasis occurred in SIN-cells derived tumor, scale bar = 1 μm, an amplified image of liver metastasis.

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## Endogenous Axon Guiding Chemorepulsant Semaphorin-3F Inhibits the Growth and Metastasis of Colorectal Carcinoma

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