

# Transferrin reverses the anti-invasive activity of human prostate cancer cells that overexpress sema3E

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**Abstract.** *In vitro* invasion and adhesion of stably sema3E-transfected PC-3 prostate cancer cells were determined in the presence and absence of transferrin. Invasion and adhesion decreased compared to untransfected cells; however, transferrin reversed the effects. Transferrin differentially regulated E-cadherin and  $\beta$ -catenin in these cells. Insulin growth factor 3 (IGFBP3) negated the invasive and adhesive effects of transferrin. Transferrin increased binding of insulin growth factor (IGF)-1 to the activated IGF-1 receptor, and IGF-1 mimicked the invasive and adhesive effects of transferrin. These data suggest that transferrin modulates sema3E-transfected cells through an IGFBP3/IGF-1-dependent pathway, in part, by regulation of adhesion proteins.

## Introduction

Transferrin is a potent mitogen for prostate cancer (1,2), where its levels are four times higher than in patients with benign prostatic hyperplasia and six times higher than in men with clinically normal prostates (3). Upregulated levels of transferrin often correlate with increased adhesion, invasion and metastasis (4). Semaphorins are developmental proteins that can modulate cancer invasion and metastasis (5,6), and sema3E is expressed in human prostate cancer cell lines (7). The significance of sema3E upregulation on prostate cancer growth and progression, and factors that regulate the effects of sema3E are largely unknown.

We found that sema3E transfection into human prostate cancer cell lines decreased invasion through and adhesion to Matrigel, and that exogenous transferrin reversed these effects. Insulin-like growth factor binding protein 3 (IGFBP3) and insulin-like growth factor 1 (IGF-1) modulated the effects of transferrin related to expression of the cell adhesion proteins, E-cadherin and  $\beta$ -catenin, in sema3E-transfected cells.

## Materials and methods

**Cell culture.** PC-3 prostate cancer cells were cultured in RPMI-1640 (Life Technologies, Grand Island, NY) containing 10% filter sterilized fetal bovine serum [(FBS), Equitech-Bio Inc, Kerrville, TX ] at 37°C in a tissue culture incubator at 5% CO<sub>2</sub>. In some experiments cells were cultured in medium containing 5  $\mu$ g/ml transferrin (Sigma, St. Louis, MO), IGF-1 (3 ng/ml), or IGFBP3 (0.15  $\mu$ g/ml) (R&D Biosystems, Minneapolis, MN). Cells were harvested at 50-70% confluency.

**RT-PCR.** Total RNA was extracted with Trizol (Invitrogen, Carlsbad, CA). First-strand cDNA synthesis was performed using a DT18 oligo primer from Invitrogen and Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI). The cDNA was amplified by PCR with sema3E gene specific primers (5'-TATAATAGCGGCCG CATGGCATCCGCGG-3' / 5'-GTCTGAATAGGATCCGGA GTCCAGCGTGT-3') with the addition of *Bam*HI and *Not*I restriction sites. The cDNA sequence was amplified by PCR. Hypoxanthine-guanine phosphoribosyltransferase (HPRT) (5'-GAGAGTTCAAGTTGAGTTT-3' / 5'-TTTTCTAACA CACGGTGGTA-3') was used as an internal control (7).

**Ligation and transfection.** Ligation and transfection were performed as described (8). The amplified sema3E PCR product was cleaved using *Not*I and *Bam*HI restriction enzymes (Promega) and were ligated into the pCMV-Tag1 vector (Stratagene, La Jolla, CA) according to the manufacturer's specifications. The vector was purified using the Concert nucleic acid purification system from Marligen (Ijamsville, MD) and transfected into cells using the Transit prostate transfection reagent from Mirus Bio Corporation (Madison, WI). The transfected cancer cells were selected and maintained in the presence of 0.5  $\mu$ g/ml G418 (9). Cells transfected with empty pCMV-Tag1 vector (mEV) were used as a control.

**RNA slot blot analysis.** Slot blot analysis was performed as described (10). mRNA was extracted using the Invitrogen Micro-FasTrack 2.0<sup>®</sup> system according to the manufacturer's specifications and then used in an RT-PCR reaction with biotin labeled CTP to create a sema3E gene-specific probe. Total RNA from cells was transferred to a nylon membrane (Osmonics Inc., Minnetonka, MN) using a slot blot manifold

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apparatus. The membrane was hybridized with an appropriate cell-specific cDNA probe and detected with alkaline-phosphatase-streptavidin and CDP-Star (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD). Differences in density of sema3E RNA were determined among untransfected, sema3E-transfected, and mEV cells and compared to HPRT (7,11).

**Growth and viability assay.** PC-3 cells were plated in 60x15-mm tissue culture dishes at  $2.5 \times 10^5$  cells. Cell numbers and viability were determined daily for 4 days using the Vi-Cell XR counter (Beckman Coulter, Fullerton, CA). Experiments were repeated twice with similar results.

**Chemoinvasion assay.** Chemoinvasion was determined in a microinvasion chamber (12) with cells cultured in the presence or absence of transferrin, IGF-1, or transferrin + IGFBP3 for 24 h. One hundred thousand cells suspended in serum-free medium supplemented with 0.1% bovine serum albumin (BSA) were transferred into the upper wells of the chamber. The lower wells contained medium with 10% FBS, which served as a chemoattractant to facilitate invasion. Transferrin, IGF-1, or transferrin + IGFBP3 were added to the upper and lower wells as appropriate. An 8- $\mu$ m porous Nucleopore Track Etch membrane (Whatman, Alameda, CA) coated with 50  $\mu$ g/ml of Matrigel (BD Biosciences, Bedford, MA) separated the upper and lower wells. The microinvasion chamber was incubated fluorescently for 20 h at 37°C. Cells were fixed in methanol and stained with DiFF Quik (Dade Behring Inc., Deerfield, IL). Invasion of cells to the underside of the membrane was measured by counting cells in three optical fields of duplicate wells at x400 magnification under a microscope. The results were averaged. Experiments were repeated three to six times and the results presented as the percentage of invasion  $\pm$  SE of the sema3E-transfected PC-3 cells compared to untransfected cells.

**Adhesion assay.** Adhesion was determined as described with slight modifications (12). Serum-free medium containing 0.1% BSA and  $2 \times 10^4$  cells cultured with and without transferrin, IGF-1 or transferrin + IGFBP3 were added to each well of a 24-well plate coated with 100  $\mu$ g/ml of Matrigel and incubated at 37°C for 20, 30, 45, and 60 min. Unattached cells were removed by washing with PBS, the plates fixed with methanol, stained with DiFF-Quik, five random fields were determined by light microscopy at x400 magnification in duplicate wells, and the results were averaged. Transfected cells were compared to untransfected cells. Experiments were repeated twice. The results at different time-points did not differ ( $p > 0.05$ ). Only data from 30 min are presented.

**Western blot analysis.** Anti-E-cadherin and anti- $\beta$ -catenin antibodies were from Cell Signaling Technology (Danvers, MA). Anti-transferrin and anti-glyceraldehyde 3-phosphate (GAPDH) were from Abcam (Cambridge, MA). E-cadherin,  $\beta$ -catenin and transferrin levels were determined by Western blotting as described, GAPDH was used as an internal control (12). Briefly, cells were lysed with an ice cold buffer (100 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate, 10% glycerol) supplemented with 10  $\mu$ l

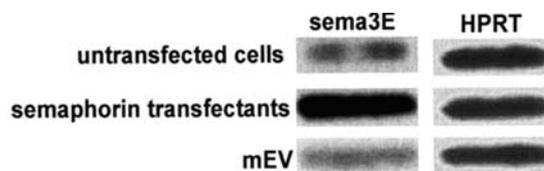


Figure 1. Sema3E overexpression measured by RNA slot blot analysis. Total RNA (5  $\mu$ g) extracted from sema3E-transfected, mEV and untransfected cells was transferred to a nitrocellulose membrane using an RNA slot blot manifold. The membrane was hybridized with biotin-linked with cell-specific PCR probes. HPRT is an internal loading control.

of Protease Inhibitor Cocktail, 10  $\mu$ l Phosphatase Inhibitor Cocktail I, and 10  $\mu$ l Phosphatase Inhibitor Cocktail II, all from Sigma. The supernatant was collected and the protein concentration determined using the Bradford assay (BioRad Laboratories, Hercules, CA). Equal protein concentration was added to a denaturing loading mix (125 mM Tris-HCl, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue) and boiled for 5 min. The samples were electrophoresed on an SDS PAGE gel and electro-transferred to a nitrocellulose membrane (Osmonics) and blotted with antibodies. The relative amount of protein in sema3E-transfectants was expressed as a percentage of protein from untransfected cells normalized to GAPDH.

**Immunoprecipitation.** Equal amounts of lysate protein were immunoprecipitated overnight at 4°C. The immune complexes were collected with protein A sepharose beads (Santa Cruz Biotechnology, Santa Cruz, CA) for 4 h, washed with lysis buffer and then boiled in sample buffer as previously described (13). The samples were denatured at 95°C, electrophoresed into an SDS-PAGE gel, electro-transferred to a nitrocellulose membrane, and probed with antibodies.

**Statistics.** Data were analyzed using ANOVA followed by Bonferroni's multicomparison test.

## Results

**Sema3E expression, growth, and viability of transfected cells.** Sema3E mRNA was overexpressed two-fold in transfected compared to untransfected and mEV cells. Expression in mEV and untransfected cells was not different (Fig. 1). Antibodies to determine protein expression by Western blot analysis were not available. Cell viability of the stable sema3E and mEV transfectants was approximately 95%, and the cells did not show any significant differences in viability or growth rate compared to untransfected cells (not shown).

**Transferrin reversed invasion and adhesion of sema3E transfectants.** Sema3E-transfected cells exhibited a 33% decrease in chemoinvasion and a 28% decrease in adhesion compared to untransfected and mEV cells (Fig. 2A and B). Transferrin completely inhibited the anti-invasive and anti-adhesive effects of sema3E-transfected cells (Fig. 2A and B). Although exogenous transferrin increased cytosolic levels two-fold, levels were not different between sema3E-transfected and untransfected cells (Fig. 3).

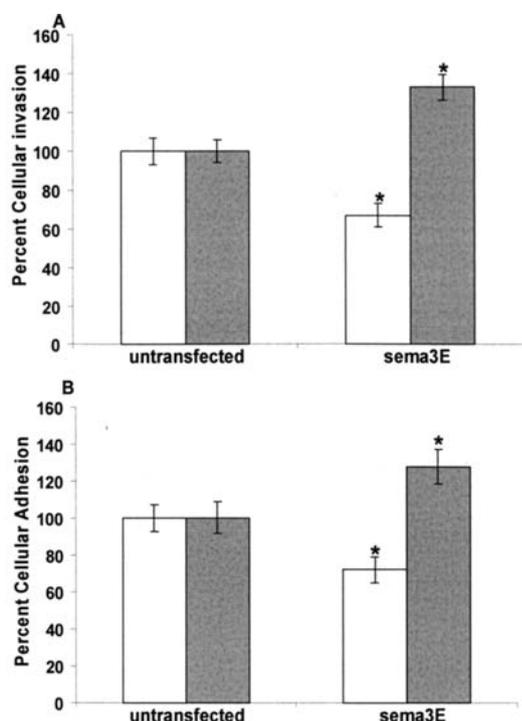


Figure 2. Transferrin (5 µg/ml) reversed the invasive and adhesive characteristics of sema3E-transfected cells. (A) Invasion assay. (B) Adhesion assay. The mean ± SE from three separate experiments is presented as the percentage of invasion or adhesion of sema3E transfectants compared to untransfected cells cultured without (□) or with (■) transferrin. \*Different from untransfected cells,  $p \leq 0.001$ .

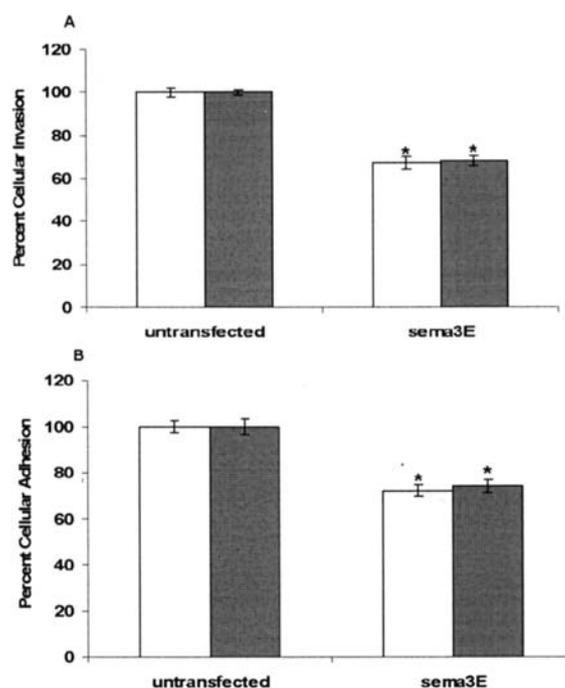


Figure 4. IGFBP3 rescued sema3E-transfected cells from invasive and adhesive changes induced by transferrin. (A) Invasion. (B) Adhesion. Cells were cultured alone or with 0.15 µg/ml IGFBP3 + 5 µg/ml transferrin. Three random fields of cells that invaded to the underside of the Matrigel-coated membrane were counted in duplicate and presented as percent invasion. The mean ± SE from three experiments is presented as the percentage of cellular invasion or cellular adhesion of sema3E-transfectants compared to untransfected cells without (□) or with (■) IGFBP3 + transferrin. \*Different from untransfected cells,  $p \leq 0.001$ .

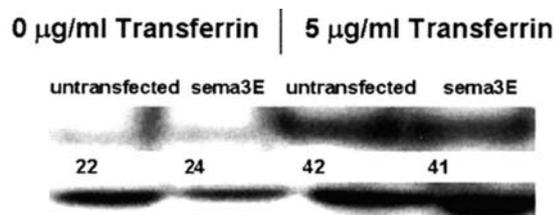


Figure 3. Sema3E did not regulate transferrin levels. Transferrin was measured by Western blot analysis. GAPDH is an internal loading control. The numbers indicate relative density of protein expression in sema3E-transfected cells compared to untransfected cells.

*IGFBP3 abolished and IGF-1 mimicked the effects of transferrin.* IGFBP3 reversed the pro-invasive and pro-adhesive effects of exogenous transferrin on sema3E-transfected cells (Fig. 4A and B). Fig. 5 shows that binding of IGF-1 and IGF-1 receptor (IGF-1R) are decreased in sema3E-transfected compared to untransfected cells cultured without transferrin. IGF-1 binding to the phosphorylated receptor in sema3E-transfectants is strongly upregulated by transferrin compared to untransfected and to sema3E-transfected cells cultured without transferrin. Fig. 6A and B shows that IGF-1 added to sema3E-transfected cells mimics the effects of transferrin by similarly increasing invasion and adhesion.

*Transferrin differentially regulated E-cadherin and β-catenin expression in sema3E-transfected cells.* Sema3E-transfected

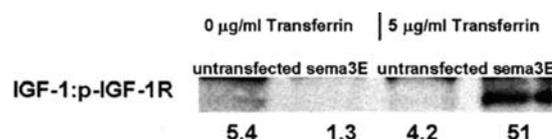


Figure 5. The effect of transferrin on binding and activation of IGF-1R receptor by IGF-1. Lysates were obtained from sema3E-transfected cells cultured for 40 h with and without 5 µg/ml transferrin, and then immunoprecipitated overnight at 4°C with anti-phospho-IGF-1R. The membrane was probed with primary IGF-1 antibodies.

cells expressed one-half of the amount of E-cadherin found in untransfected cells (Fig. 7A). E-cadherin levels were one-fourth lower in sema3E-transfected cells cultured with exogenous transferrin compared to similarly exposed untransfected cells (Fig. 7A). β-catenin levels were not different in sema3E transfectants compared to untransfected cells cultured without transferrin (Fig. 7B). β-catenin was four-fold lower in sema3E-transfected as compared to untransfected cells cultured with transferrin (Fig. 7B). β-catenin bound to E-cadherin was two-fold higher in sema3E transfectants than in untransfected cells; the bound proteins were not different in the cells cultured with transferrin (Fig. 7C).

## Discussion

Sema3E, like other guidance semaphorins, naturally exhibits bipolar activity and this also appears to be important in

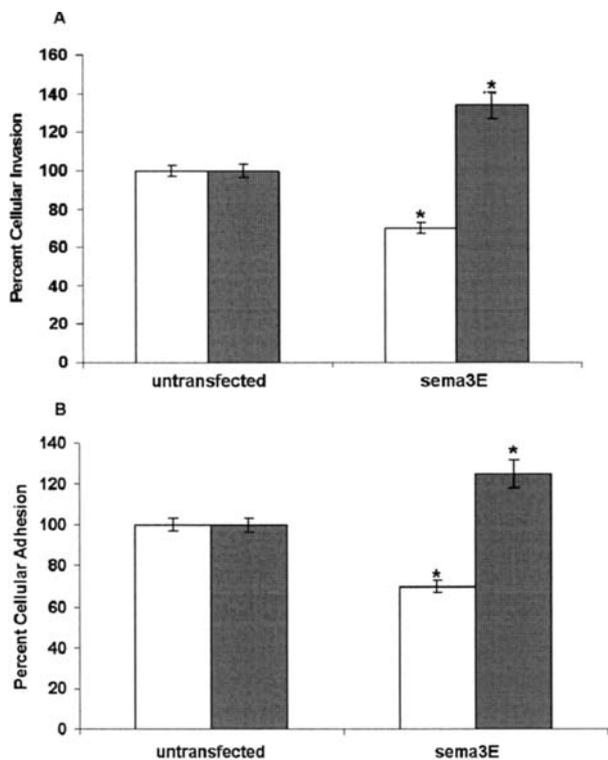


Figure 6. IGF-1 (3 ng/ml) mimicked the invasion and adhesion effects of transferrin on sema3E transfectants. (A) Invasion (B) Adhesion. Three random fields of cells that invaded to the underside of the Matrigel-coated membrane were counted in duplicate. The mean  $\pm$  SE of separate experiments is presented as the percentage of cellular invasion of sema3E-transfected compared to untransfected cells without (□) or with (■) IGF-1. \*Different from untransfected cells,  $p < 0.001$ .

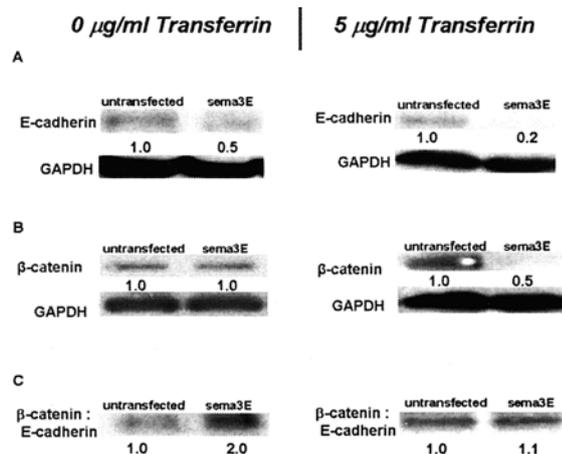


Figure 7. Total E-cadherin and  $\beta$ -catenin levels and binding was differently expressed and regulated in sema3E-transfected cells by transferrin. Protein was collected from sema3E-transfected and untransfected cells cultured with and without 5  $\mu$ g/ml transferrin. (A) E-cadherin and (B)  $\beta$ -catenin were measured by Western blot analysis. GAPDH was used as an internal control. (C) Levels of bound  $\beta$ -catenin and E-cadherin as determined by immunoprecipitation. Numbers indicate the relative density of protein expression in sema3E-transfected cells compared to untransfected cells.

cancer (6). We found in this initial study that sema3E-transfected cells cultured in medium without exogenous transferrin supplementation exhibit decreased invasion and

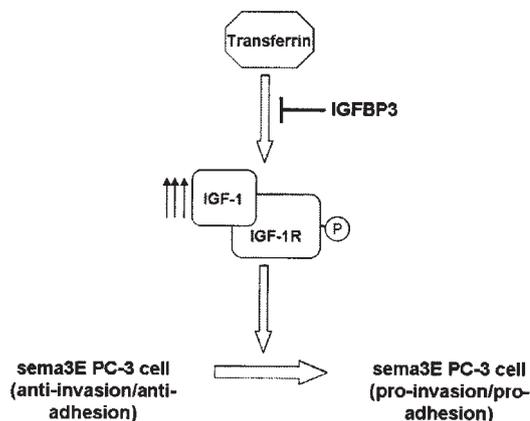


Figure 8. Transferrin regulated invasion and adhesion of sema3E-transfected cells by an IGF-1-dependent pathway. Transferrin through the upregulation of IGF-1 facilitated the invasive and adhesive switch of sema3E-transfected PC-3 cells. By increasing IGF-1R activity through increased IGF-1 binding, transferrin caused the anti-invasive and anti-adhesive sema3E-transfected cells to become pro-invasive and pro-adhesive. IGFBP3 inhibited transferrin and blocked this effect.

adhesion while cells cultured in medium with transferrin exhibited enhanced invasion and adhesion. Elevated levels of transferrin often correlate with increased adhesion, invasion and metastasis (4,14,15). Additionally, increased sema3E levels correlate with highly malignant invasive and metastatic murine breast adenocarcinoma cell lines (5), and sema3E overexpression in human breast cancer is associated with lung metastasis formation (6). These findings support the findings of the present investigation in human prostate cancer.

Although transferrin is present in FBS at a level of 1.8-2.2  $\mu$ g/ml (1), the concentration in culture media is too low to alter the invasive and adhesive characteristics of the sema3E-transfected cells. We found in preliminary dose-response studies that 5  $\mu$ g/ml of transferrin added to culture medium was the lowest concentration that could modulate invasion and adhesion and that increasing the concentration to 100  $\mu$ g/ml had no additional affect.

IGF-1 is an important mitogen for prostate cancer that regulates migration, invasion, apoptosis and adhesion (4,16). IGF-1 added to sema3E-transfected cells paralleled the effects of transferrin on invasion, and increased transferrin levels correlated with increased IGF-1 levels (17). Sema3E-transfected cells exhibit lower levels of IGF-1 binding to the active phosphorylated form of IGF-1R compared to untransfected cells, and decreased IGF-1 activity correlates with decreased prostate cancer invasion (6). Transferrin strongly upregulated IGF-1 binding to phosphorylated IGF-1R in sema3E-transfected cells compared to untransfected cells. Elevated IGF-1 activities are associated with increased prostate cancer progression (18-20). Additionally, IGF-1 regulates numerous pathways leading to changes in adhesion and upregulation of invasion and metastasis (18,19). Thus, we propose that transferrin increases the invasive and adhesive properties of sema3E-transfected cells by increasing the binding and activity of IGF-1 through IGF-1R (Fig. 8). Further support for this mechanism is the demonstrated ability of IGFBP3 to inhibit the effects of transferrin on sema3E-

transfected cells. IGFBP3 is a direct inhibitor of IGF-1 (4), and it also inhibits transferrin activity (21). Deregulation of the normal balance between IGFBP3 and IGF-1 is associated with an increased risk of prostate cancer, although these results are controversial (19). Nevertheless, as IGF-1 becomes elevated compared to IGFBP3, this leads to increased IGF-1 activity that is associated with an increased risk of prostate cancer development, progression, and increased invasion (18,19,22-24).

Expression of adhesion proteins such as  $\beta$ -catenin and E-cadherin also controls tumor cell invasion (25). Lower expression of functional E-cadherin generally correlates with increased invasion and is a prognostic marker for decreased survivability of patients with prostate cancer (26). Binding between E-cadherin and  $\beta$ -catenin must occur to form a functional adhesion complex (27,28). Although the sema3E-transfectants cultured without transferrin exhibited decreased E-cadherin levels and no difference in  $\beta$ -catenin levels compared to untransfected cells, the transfected cells also expressed increased binding of E-cadherin to  $\beta$ -catenin reflecting the presence of stable E-cadherin/ $\beta$ -catenin adhesion complexes and greater cell-cell adhesion - a characteristic associated with decreased invasion in prostate cancer (28-30). However, in the presence of transferrin E-cadherin expression decreased further and  $\beta$ -catenin expression decreased nearly two-fold in sema3E-transfected cells compared to untransfected cells. E-cadherin/ $\beta$ -catenin complexes were also decreased two-fold in the sema3E-transfected cells cultured with transferrin compared to sema3E cells cultured without transferrin. These findings are consistent with the increased invasion and adhesion to Matrigel observed in the sema3E-transfected cells. Transferrin regulates endocytosis of E-cadherin and  $\beta$ -catenin (31), and this could explain, in part, the decreased levels of these proteins in sema3E-transfected cells. Also, increased IGF-1 activity and activation of IGF-1R leads to a downregulation of E-cadherin and  $\beta$ -catenin activity and this correlates with increased adhesion and invasion (32).

Herein we show a novel mechanism by which transferrin and IGF-1 regulate the invasive and adhesive characteristics of PC-3 prostate cancer cells overexpressing sema3E. This is the first study to show that sema3E up-regulation decreases the invasive and adhesive characteristics of PC-3 prostate cancer cells and that elevated transferrin levels abrogate this anti-invasive activity. Since men with prostate cancer often have elevated transferrin levels, these findings suggest one mechanism whereby transferrin promotes the invasive characteristics of prostate cancer cells that could lead to increased metastasis and a poor prognosis for survival. The findings also suggest that transferrin is a viable target for therapeutic intervention in prostate cancer patients that exhibit elevated levels of sema3E. Furthermore, these data link transferrin and the activity of IGF-1 to the invasive properties of prostate cancer cells overexpressing sema3E, and this is important since the IGF axis is involved in bone metastasis arising from invasive prostate cancer (22,23).

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