

High gene expression of semaphorin 5A in pancreatic cancer is associated with tumor growth, invasion and metastasis

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Semaphorin 5A (SEMA5A) is an axonal regulator molecule, which belongs to the Semaphorin family of proteins. Previously, we identified SEMA5A as a putative marker for aggressive pancreatic tumors. However, the expression, localization and functional significance of SEMA5A in pancreatic tumors remain unclear. In our study, we hypothesized that SEMA5A expression modulates pancreatic tumor growth and metastasis. We analyzed the constitutive expression and localization of SEMA5A in patient pancreatic tumors ($n = 33$) and unmatched normal pancreatic ($n = 8$) tissues and human pancreatic cancer cell lines ($n = 16$) with different histopathological characteristics. We observed significantly higher expression of SEMA5A protein expression ($p < 0.05$) in human pancreatic tumor tissue samples compared to normal pancreatic tissues. Similarly, the pancreatic cancer cell lines with higher tumorigenic and metastatic potentials as xenografts in nude mice expressed higher levels of SEMA5A mRNA compared to those with lower tumorigenic and metastatic potentials. Furthermore, we examined the functional role of SEMA5A in pancreatic tumor growth and invasion. Ectopic expression of mouse full-length Sema5A in Panc1 (SEMA5A negative) cells significantly ($p < 0.05$) enhanced tumorigenesis, growth and metastasis *in vivo* as well as proliferation, invasiveness and homotypic aggregation *in vitro*. Together, these data demonstrate that the expression of SEMA5A in pancreatic cancer cells regulates tumorigenesis, growth, invasion and metastasis, and it also suggests a novel target for diagnosis and treatment of pancreatic cancer.

Key words: pancreatic cancer, SEMA5A, tumor growth and metastasis, semaphorin

Additional Supporting Information may be found in the online version of this article

Abbreviations: Ct: threshold cycle; FCS: fetal calf serum; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; IACUC: Institution Animal Care and Use Committee; IHC: immunohistochemistry; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PCNA: proliferating cell nuclear antigen; PEI: protein expression index; RT-PCR: reverse-transcriptase polymerase chain reaction; SD: standard deviation; SEMA5A: human semaphorin 5A; Sema5A: mouse semaphorin 5A; TMA: tissue microarray; UNMC: University of Nebraska Medical Center

Grant sponsor: Cancer Glycobiology Program, Nebraska Research Initiative; **Grant numbers:** CA72781, CA78590; **Grant sponsor:** National Cancer Institute, National Institutes of Health (Cancer Center Support Grant); **Grant number:** P30CA036727

DOI: 10.1002/ijc.25166

History: Received 26 May 2009; Accepted 30 Dec 2009; Online 13 Jan 2010

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Pancreatic adenocarcinoma is the fourth leading cause of cancer-related deaths in the United States, and current treatment options have made little impact on the overall survival rate.^{1,2} The incidence and mortality of pancreatic adenocarcinoma are almost identical.³ Most pancreatic cancer patients have metastatic disease at the time of diagnosis with an extremely poor prognosis.^{1,2} Understanding the molecular mechanism(s) underlying progression and metastasis may provide an insight into the development of novel diagnostics and targeted therapeutics.

Recently, we identified Semaphorin 5A (SEMA5A) as a putative molecule expressed in pancreatic cancer, by using phage display peptide library assisted with bioinformatics analyses.⁴ SEMA5A belongs to the class V of the semaphorin/collapsin family of proteins, characterized by a conserved sema domain of ~500 amino acids.⁵⁻⁷ There are more than 20 secreted and membrane-bound semaphorins belonging to 7 subclasses classified according to their protein structures.^{5,6} The members of the semaphorin family were first identified as axonal growth molecules^{5,8} and were later shown to be involved in a variety of functions including cellular migration, immune regulation, angiogenesis, cellular collapse, apoptosis and cancer.⁹⁻¹⁴

SEMA5A is a transmembrane glycoprotein characterized by unique thrombospondin specific repeats in the extracellular

region of the protein.¹⁵ Originally, mouse Sema5A was identified as a molecule that induces inhibitory responses during optic nerve development.¹⁶ More recent studies indicate that Sema5A controls innate immunity in mice¹⁷ and serves as a candidate gene for the etiology of idiopathic autism in humans.¹⁸ Mice with homozygous null mutations for Sema5A showed embryonic lethality with a specific defect in the cranial vasculature.¹⁹ Recent reports including our analysis demonstrated Plexin B3 as a binding partner for SEMA5A.^{20,21} Furthermore, the role of the *Drosophila* SEMA5A ortholog, *Dsema-5c* in tumorigenicity and metastasis has been reported.²² In a recent study, we have shown the expression of SEMA5A mRNA in aggressive pancreatic cancer cell lines.⁴ However, the expression, localization and functional significance of SEMA5A in pancreatic tumors remain unclear.

In our study, we analyzed the constitutive expression of SEMA5A mRNA and protein and its significance in pancreatic tumor growth and metastasis, using human patient pancreatic tumor and unmatched normal pancreatic tissues and human pancreatic cancer cell lines with different histopathological characteristics. Our data demonstrate constitutive expression of SEMA5A in most of the patient pancreatic tumor tissues and aggressive (highly tumorigenic and metastatic as xenografts in nude mice) pancreatic cancer cell lines. In contrast, there is no expression of SEMA5A in normal pancreatic tissues and less aggressive pancreatic cancer cell lines. Furthermore, our results demonstrate that ectopic expression of mouse full-length Sema5A in the SEMA5A negative pancreatic cancer cell line, Panc1 leads to increased *in vitro* cell proliferation, invasion and homotypic aggregation and *in vivo* tumor growth and metastasis.

Material and Methods

Pancreatic tumor samples and cell culture reagents

Tissue microarray (TMA) of pancreatic adenocarcinoma [Accumax Array, A207(III)] containing 33 cases and 8 unmatched normal pancreatic tissues in duplicate was a generous gift from Petagene (Seoul, South Korea). Sixteen human pancreatic cancer cell lines with different tumorigenic and metastatic potential, Panc89, T3M4, Capan1, Hs766T, HPAF-1, CD11/HPAF (CD11), CD18/HPAF (CD18), AsPC1, Suit2, Suit2-S2013 (S2013), MiaPaca, Capan1, SW1990, QGP1, BxPC3 and Panc1 were maintained in culture as adherent monolayer with RPMI-1640 with 5% fetal calf serum (FCS, Mediatech, Herndon, VA) supplemented with 1× non-essential amino acids, 2 mM L-glutamine, 1× vitamin solution and 40 µg/ml gentamycin (Mediatech). The cultures were free of mycoplasma and pathogenic murine viruses, and were maintained for no longer than 8 weeks after recovery from frozen stock.

Transfection of pancreatic cancer cells

Panc1 cells were transfected with full-length mouse Sema5A cDNA tagged with Flag epitope cloned in pBK-CMV vector (generous gift from Dr. Andreas W. Püschel, Westfälische

Wilhelms-Universität Münster, Münster, Germany) or control pBK-CMV vector (Stratagene, La Jolla, CA) using LipofectAMINE Plus reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. Panc1 cells transfected with Sema5A (Panc1-Sema5A) or its control vector (Panc1-control) were selected and maintained with 400 µg/ml G418 sulfate.

Animals and tumorigenic and metastasis assays

Male athymic BALB/c nude mice (NCI-nu, 6–8 weeks old) were purchased from the National Cancer Institute. The mice were maintained under specific pathogen-free conditions in facilities approved by the American Association of Laboratory Animal Care and in accordance with current regulations and standards of the US Department of Agriculture, Department of Health and Human Services and NIH. All procedures performed in mice were in accordance with institutional guidelines and were approved by the University of Nebraska Medical Center (UNMC) Institution Animal Care and Use Committee (IACUC) guidelines.

For *in vivo* injection, cells were harvested after trypsinization using brief exposure with 0.25% trypsin in 0.02% ethylenediaminetetraacetic acid (EDTA). Trypsinization was stopped with medium containing serum and then cells were washed twice with Hank's Balanced Salt Solution (HBSS). Only single cell suspensions of more than 90–95% viability (tested by trypan blue exclusion assay) were used for injection.

For the tumorigenic assay, mice were injected with 1×10^6 cells/0.05 ml of HBSS/animal into the subcutis of the lateral flank. Tumor growth was monitored and animals were killed when moribund. Tumors were measured with calipers twice a week. Tumor volume was calculated by the following formula: volume = $W^2 \times L/2$, where W = short diameter and L = long diameter. Tumor tissues were harvested and processed for further analysis.

For the tumor growth and metastasis assay, tumor cells were injected orthotopically into the pancreas. Mice were anesthetized with tribromoethanol (Avertin[®]) administration intraperitoneally. A small incision was created and pancreatic tumor cells (5×10^5 /0.05 ml HBSS/animal) were injected into the anterior lobe of the pancreas. A subcapsular intrapancreatic injection was identified as successful by appearance of a fluid bleb without leakage. To prevent leakage, a cotton swab was held over the site of injection for 1 min. The lesion was closed with two separate layers. Abdominal muscular layers were closed using 4-0 or 5-0 vicryl and the outer skin layer was closed with stainless steel wound clips. Wound clips were removed at approximately 10–14 days post-surgical procedure. Mice were monitored for tumor growth and killed when moribund, and primary tumors as well as metastases were resected and processed for further analysis.

mRNA analysis

Total cellular RNA was isolated from pancreatic cancer cell lines using Trizol[®] reagent (Invitrogen)²³ and reverse-

transcription-based polymerase chain reaction (RT-PCR) was performed as described.⁴ First strand cDNA was synthesized using total RNA (5 µg), oligo dT₁₈ primer and superscript II RT (Invitrogen); 2 µl of first strand cDNA (1:10 dilutions) were amplified using PCR primer sets: SEMA5A (755 bp; 30 cycles), 5'-GAA CCG GAA GCG TGT T-3' and 5'-CAG TGA GAT GTG GGT TGA AG-3' and Plexin B3 (232 bp; 30 cycles), 5'-GTG CGG AAC CTT CAA CAT TT-3' and 5'-AAA GAG CAT GGG TGT TGT CC-3'; SEMA3A (266 bp; 30 cycles), 5'-ACC ACC CAA TCA GGA CAG AG-3' and 5'-TGG CAC TGA GCA AAT CAG AC-3; SEMA4D (611 bp; 30 cycles), 5'-TAC CAG TGC CTG TCA GAG GA-3' and 5'-GAC TTT GCT GGT GAT GGT GT-3' and β-actin (246 bp; 25 cycles), 5'-TGA AGT GTG ACG TGG ACA TC-3' and 5'-ACT CGT CAT ACT CCT GCT TG-3'. PCR fragments were separated on a 2% agarose gel containing ethidium bromide (0.25 µg/ml) and visualized and analyzed using the Alpha Imager gel documentation system (Alpha Innotech, San Leandro, CA).

RT-PCR analysis of SEMA5A was confirmed using real-time PCR with SYBR green master mix (Roche, Indianapolis, IN) and 10 µM primer mix utilizing the BioRad iCycler (BioRad, Hercules, CA). The primer sets used for real-time PCR were: SEMA5A (74 bp), 5'-GAT CTA TGG CAT CTT TAC CAC CAA-3' and 5'-TGG CGC TCA GGT TGA AGA C-3' and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (202 bp), 5'-GAG TCA ACG GAT TTG GTC GTA-3' and 5'-ATG GGA TTT CCA TTG ATG ACA-3'. The fluorescence intensity of double-strand specific SYBR Green, reflecting the amount of amplified PCR-product, was monitored at the end of each elongation step. The data collected from these real-time PCRs defined a threshold cycle (Ct) of detection for genes in each cDNA sample. The relative gene expression index was calculated by normalizing the Ct value for each gene normalized with GAPDH (endogenous reference) using the formula $2^{-\Delta Ct}$, where ΔCt represents the difference in Ct between the targeted gene transcript and the housekeeping gene for the same RNA sample.

Generation of SEMA5A antibody

Polyclonal SEMA5A antibody was raised in rabbit using a peptide (KEIGPWLREFRANAVDC)²² from the sema domain of SEMA5A (Synpep Corp, Dublin, CA). Three pre as well as postbleeds were obtained and tested for antibody specificity. An anti-serum direct ELISA was carried out against the immunized peptide using pre and postbleeds from the animal and our data demonstrate a high level of anti-SEMA5A titer (Fig. 1a). For immunohistochemistry, xenograft tumors from mice orthotopically injected with Capan1 (SEMA5A positive) cell lines were immunostained with anti-SEMA5A antibody alone or anti-SEMA5A antibody incubated with SEMA5A peptide used to raise the antibody. We observed that SEMA5A-peptide blocking abrogated anti-SEMA5A immunoreactivity to SEMA5A-expressing pancreatic tumors demonstrating the specificity (Fig. 1b). We confirmed the specificity

of antibody using Western blot analysis using Sema5A over-expressing Panc1 cells (Fig. 1d).

Immunohistochemistry analysis

Cells (1×10^5) were plated on coverslips in 6-well plates, cultured for 48 hr and used for immunostaining. After washing twice with phosphate buffered saline (PBS), the cells were fixed with 4% paraformaldehyde in PBS for 10 min. The samples were washed twice with PBS and incubated with blocking buffer (BD Biosciences) for 30 min. The samples were incubated with primary antibody for 1 hr [anti-SEMA5A antibody (5 µg/ml)] and subsequently incubated with FITC-conjugated secondary antibody. Immunofluorescence was analyzed using a Nikon fluorescent microscope.

Frozen cryosections of xenograft tissue samples were fixed with cold acetone:methanol (1:2) for 10 min. Paraffin fixed tissue samples in slides were deparaffinized, washed with PBS for 5 min three times and quenched for endogenous peroxidase activity by incubating with 3% H₂O₂ in PBS for 5 min. Tissue sections were blocked with blocking buffer (10% horse serum in PBS) for 30 min and incubated with primary antibody (anti-PCNA (1:40, Santa Cruz Biotechnology) or anti-SEMA5A (5 µg/ml) antibodies in PBS overnight at 4°C and then with biotinylated secondary antibody for 30 min to 1 hr after thoroughly washing with PBS. The samples were incubated with ABC reagent (Vector Laboratories) for 15–30 min and immunoreactivity was monitored using DAB substrate (Vector Laboratories). Immunostaining intensity for each tumor and normal specimen was calculated as a score ranging from 0 to 5 based on the intensity of immunostaining; 0 representing no detectable staining and 5+ representing the strongest staining. Two independent observers examined each slide using a Nikon E400 microscope. The mean immunostaining intensity and standard deviation (SD) was calculated for each duplicate specimen and repeated twice.

Western blot analysis

Panc1-control and Panc1-SEMA5A cells were lysed in a buffer containing 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100 and protease inhibitors.²⁴ Fifty micrograms per lane of protein were separated on SDS-PAGE and electrotransferred to 0.45 µm polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA), and blocked with 5% nonfat dry milk in PBS for overnight at 4°C. Blots were incubated with anti-Sema5A or anti-β actin antibody (Sigma Chemicals, St. Louis MO) for 1 hr at room temperature. After washing, the blots were incubated with anti-rabbit antibody conjugated to horseradish peroxidase (GE Healthcare, Piscataway, NJ, 1:3,000) for 1 hr at room temperature. The blots were developed by an enhanced chemiluminescence technique using the ECL plus kit (GE Healthcare) according to the manufacturer's protocol and analyzed using a Typhoon imaging system (GE Healthcare).

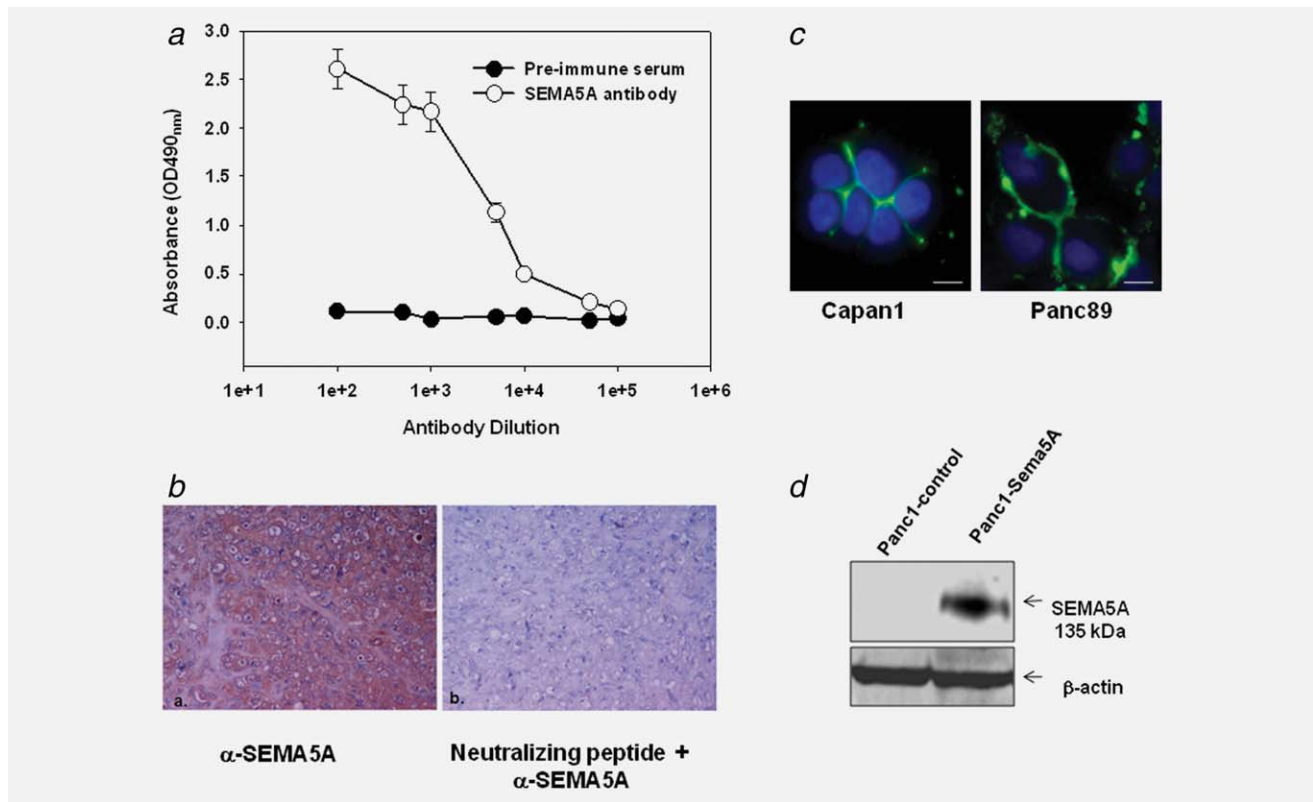


Figure 1. SEMA5A protein expression in pancreatic cancer cells. (a and b) Determination of SEMA5A antibody specificity. (a) Anti-SEMA5A antibody and preimmune serum was titrated against SEMA5A peptide using ELISA. (b) Orthotopic xenograft tumors from mice injected with Capan1 cell line were immunostained with (i) anti-SEMA5A antibody or (ii) anti-SEMA5A antibody incubated with SEMA5A peptide used to raise the antibody (at $\times 200$ magnification). (c) Cellular localization of SEMA5A using immunostaining with anti-SEMA5A antibody showed the membrane localization of SEMA5A in Capan1 (i) and Panc89 (ii) cell lines (at $\times 400$ magnification). (d) Western blot analysis to show antibody specificity. Panc1-control and Panc1-Sema5A cell lysates were used. We observed 135 kDa band of Sema5A in Panc1-Sema5A cells but not in Panc1-control cells. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

In vitro cell proliferation assay

Cells (5×10^3) were seeded in 96-well flat-bottomed plates in triplicate and allowed to adhere overnight. The cultures were then washed and re-fed with media containing different concentrations of serum. After 72 hr of incubation, proliferative activity was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, EMD Biosciences, La Jolla, CA) assay using a microtiter plate reader (Bio-Tek Instruments, Inc.) at 570 nm. The absorbance of the treated and untreated cells was plotted as a bar graph. Growth stimulation or inhibition was calculated as (%) = $[(A/B) - 1 \times 100]$, where A is the absorbance of Sema5A-treated cells and B is the absorbance of untreated control cells.²⁵

Invasion assay

Cells (1×10^5) were plated onto transwell chambers coated with Matrigel (6.5 mm; Corning Costar Corp., Cambridge, MA) with media (serum-free) alone or media containing different concentrations of serum in duplicate and incubated at 37°C in 5% CO₂ for 24 hr. MTT was added and cells were incubated for an additional 2 hr. Cells from the top of the

transwell chambers were removed using a cotton swab (residual cells). The transwell chambers (migrated cells) and cotton swab containing residual cells were plated in separate wells of a 24-well plate containing 400 μ l of DMSO. After 1 hr of gentle shaking, 100 μ l samples were removed and absorbancy was determined at 570 nm using a microtiter plate reader. The percent invasive activity was calculated²⁶ as: percent migration = $[(A/B) - 1 \times 100]$, where A is the number of migrated cells and B is the number of residual cells.

Aggregation assay

The ability of pancreatic cancer cells to aggregate was tested using hanging drop suspension cultures as described.²⁷ Single-cell suspensions of pancreatic cells were resuspended at 2.5×10^5 cells/ml in the appropriate media containing serum and 20 μ l was pipetted as single drops (5,000 cells/drop) onto the inner surface of the lid of a Petri dish (100 \times 20 mm; Nunc). The lid was then inverted and placed on top of a Petri dish containing 8 ml of serum free media (to avoid evaporation) and the drops were hanging from the lid with the cells suspended within them. The cell suspensions

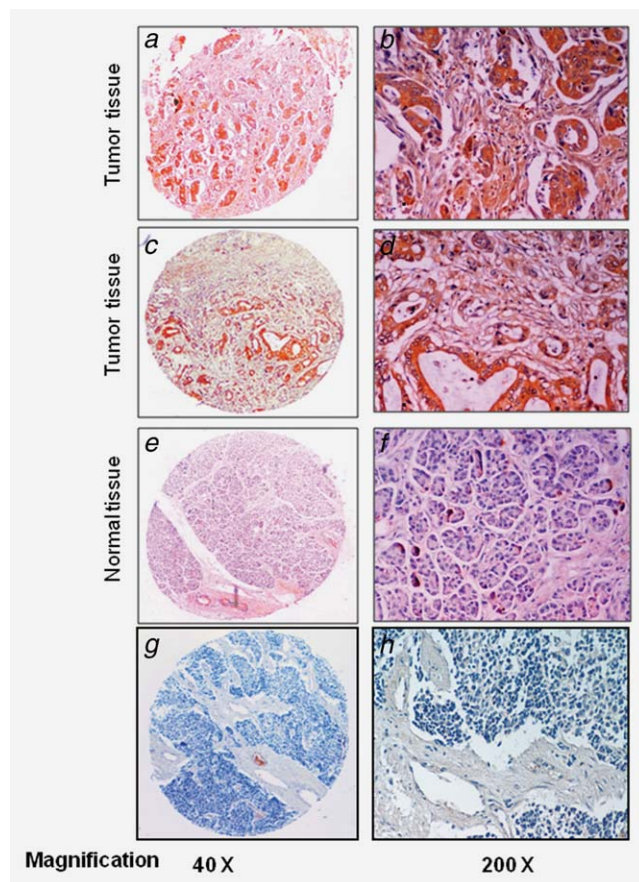


Figure 2. Expression of SEMA5A protein in human pancreatic tumors. Immunohistochemical analysis of pancreatic tumors from TMA using anti-SEMA5A antibody was performed. (a and c) Pancreatic tumors stained with anti-SEMA5A antibody (shown at $\times 40$ magnification), (b and d) at higher magnification ($\times 200$), (e) noninvolved (normal) tissue from pancreatic tumors (shown at $\times 40$ magnification) and (f) at higher magnification ($\times 200$); (g and h) low and higher magnification tissue showing negative control staining. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

hanging on the lid were incubated overnight at 37°C and were photographed using a Nikon microscope at $20\times$ magnification after inverting the lid.

Statistical analysis

The significance of the data was determined by the Student's *t*-test (two-tailed) for all *in vitro* studies. *In vivo* analysis was performed using Mann-Whitney *U*-test of significance. A value of $p < 0.05$ was deemed significant. All statistical analyses were done using SPSS software (SPSS, Chicago IL).

Results

Constitutive expression of SEMA5A protein in pancreatic tumors but not in normal pancreas

We examined the expression of SEMA5A protein in human pancreatic tumor tissues from patients using immunohisto-

Table 1. SEMA5A expression in pancreatic tumor and normal specimens

(A) Comparison between tumors with different stages and normal tissues			
Stage of disease	TNM classification	Number	SEMA5A expression
Normal	T0 N0 M0	8	0.5 ± 0.27
Stage IB	T2 N0 M0	2	$2.0 \pm 0.0^*$
Stage IIA	T3 N0 M0	6	$2.33 \pm 0.42^*$
Stage IIB	T1-3 N1 M0	10	$1.6 \pm 0.27^*$
Stage IV	T1-4 N0/1 M1	12	$1.67 \pm 0.41^*$
(B) Samples between tumors with different cellular differentiation and normal tissues			
Differentiation status	Number	SEMA5A expression	
Well-differentiated	8	$2.25 \pm 0.37^{***}$	
Moderately differentiated	30	$1.81 \pm 0.21^{***}$	
Poorly/undifferentiated differentiated	16	1.34 ± 0.37	
Others	12	0.45 ± 0.16	
Normal	8	0.5 ± 0.27	

SEMA5A expression in tumor tissue ($n = 33$ in duplicates) and normal pancreas ($n = 8$) in a tissue microarray was examined using immunohistochemistry. Immunostaining was scored from 0 to 5 based on the intensity of immunostaining; 0 representing no detectable staining and 5+ representing the strongest staining. The average intensity of immunostaining and standard deviation (SD) was calculated for each duplicate specimen. The values are mean intensity of immunostaining \pm SD. The samples are compared (A) between tumors with different stages and normal tissues and (B) between tumors with different cellular differentiation and normal tissues. *p* values ($p < 0.05$) significantly different than normal controls are shown with one asterisk (*) and those different than poorly/undifferentiated tumors are shown with 2 asterisks (**).

chemical staining of TMA sections. The expression and distribution of SEMA5A protein in the pancreatic tumor and normal pancreatic tissues showed different patterns. The expression of SEMA5A protein was observed mainly in pancreatic tumor tissues (Figs. 2a–2d). In contrast, most of the normal pancreatic tissue (Figs. 2e and 2f) samples did not show any expression of SEMA5A protein. There was a significant ($p < 0.05$) difference in the PEI of SEMA5A between pancreatic tumors with different stages of disease and normal tissue samples (Table 1A). Similarly, the PEIs (Table 1B) of SEMA5A were associated with the differentiation status of the disease. The moderately and well-differentiated tumors were observed to have significantly ($p < 0.05$) higher PEI compared to poorly/undifferentiated tumors or normal tissues (Table 1B). On the other hand, there was no significant difference in expression of SEMA5A protein between tumors from different stages of the disease (Table 1A) or between pancreatic tumors with poorly/undifferentiated status and normal tissues (Table 1B). Overall, the TMA data shows that SEMA5A protein is expressed in moderately or well-differentiated pancreatic tumor tissues as compared to poorly/undifferentiated tumors or normal tissues.

SEMA5A mRNA is constitutively expressed in aggressive pancreatic cancer cell lines

To show the tumorigenic and metastatic potentials (aggressiveness) of a panel of pancreatic cancer cell lines as xenograft tumors, we performed subcutaneous and orthotopic metastatic (xenograft) assays in athymic nude mice for 8 weeks (Supporting Information Table 1). Of 13 pancreatic cancer cell lines tested, 10 of them demonstrated higher tumorigenic potentials when xenografted (subcutaneously and/or orthotopically) into nude mice; whereas, 3 cell lines were poorly tumorigenic and nonmetastatic at the cell inoculums used in our study. In addition, all (7 of the tested cell lines) of the pancreatic cancer cell lines established from metastases and only 3 of 6 cell lines established from primary tumors were observed to have higher tumorigenic (subcutaneously and orthotopically) and metastatic (orthotopically) potentials (Supporting Information Table 1).

To expand our study, we examined SEMA5A mRNA expression using standard and real-time RT-PCR in a total of 16 pancreatic cancer cell lines including those tested for xenograft assays. SEMA5A mRNA expression was predominantly observed in 9 of 10 (90%) cell lines with higher incidence of tumorigenic and metastatic potential in nude mice (Figs. 3a and 3b). In contrast, none of the 3 cell lines with low tumorigenic and metastatic potential in nude mice expressed SEMA5A mRNA (Figs. 3a and 3b). Furthermore, 100% (10 of 10) of cell lines established from metastases and only 33% (2 of 6) of cell lines from primary tumors expressed SEMA5A mRNA (Figs. 3a and 3b). Moreover, 91% (10 of 11) moderately or well-differentiated pancreatic cancer cell lines constitutively expressed SEMA5A mRNA; whereas only 33% (1 of 3) of the poorly differentiated cell lines expressed SEMA5A mRNA. This observation of differentiation status and SEMA5A mRNA expression in pancreatic cancer cell lines is similar to that of patient pancreatic tumors (Table 1B). Together, these data suggest the association of SEMA5A expression with tumorigenesis and metastasis of pancreatic cancer cell lines in nude mice and cellular differentiation.

Expression of SEMA3A, SEMA4D and Plexin B3 mRNA in pancreatic cancer cells

Next, we examined the expression of other members of the semaphorin family, which have been implicated in the regulation of tumor progression, angiogenesis and metastasis and Plexin B3, a functional SEMA5A receptor. SEMA3A mRNA was observed in 15 of 16 (93.75%) pancreatic cancer cell lines examined (Fig. 3c). Similarly, SEMA4D mRNA expression was observed in 14 of 16 (87.5%) pancreatic cancer cell lines (Fig. 3c). The expression of Plexin B3 was observed in all of the 16 (100%) pancreatic cancer cell lines irrespective of their origin and aggressiveness (Fig. 3c). This shows that semaphorin family members, SEMA3A and SEMA4D, and Plexin B3 mRNA are ubiquitously expressed and do not show any differential expression with respect to aggressiveness and/or differentiation status of the pancreatic cancer cell lines.

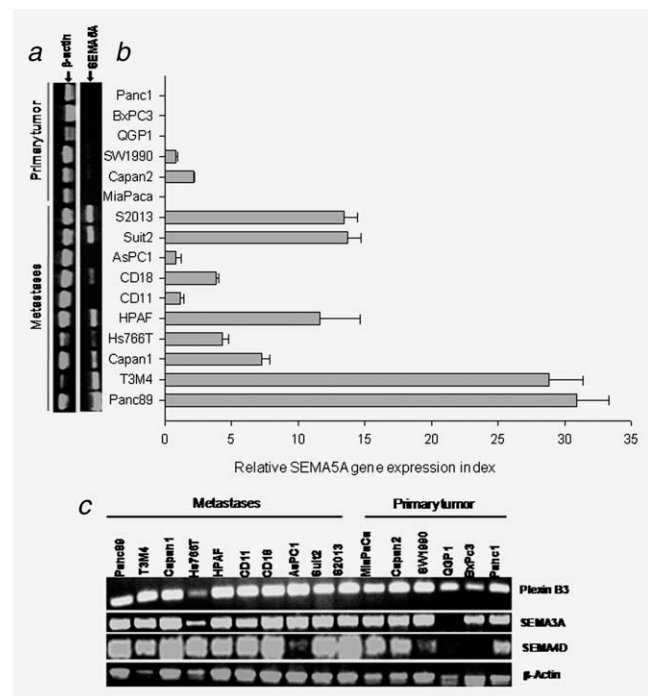


Figure 3. Expression of SEMA5A mRNA and its related molecules. (a) A representative standard RT-PCR analysis of SEMA5A expression in pancreatic cancer cell lines with different tumorigenic and metastatic potential as xenografts in nude mice. (b) A quantitative real-time RT-PCR analysis of SEMA5A expression in pancreatic cancer cell lines showing the similar pattern of SEMA5A expression as that of standard RT-PCR. See text for relative SEMA5A gene expression index. (c) A representative RT-PCR analysis showing the expression of the putative SEMA5A receptor, Plexin B3 and other semaphorins, SEMA3A and SEMA4D in pancreatic cancer cell lines.

SEMA5A is localized on the membrane of pancreatic cancer cells

To show the cellular localization of SEMA5A protein in pancreatic cancer cell lines, indirect immunofluorescence was performed in Capan1 and Panc89 (SEMA5A positive) pancreatic cancer cell lines. The transmembrane staining of SEMA5A was observed in a significant proportion of Capan1 cells, and the intensity of the fluorescent staining was high at the cell-cell junctions of the adjacent interacting cells. The cell surface that is not in contact with the adjacent cells had no/low staining (Fig. 1c). This demonstrates that membrane and cell-cell junction expression of SEMA5A and suggests its role as a cell-adhesion molecule.

Ectopic expression of Sema5A *in vitro* in pancreatic cancer cells

To investigate the functional role of SEMA5A, Panc1 was stably transfected with a mammalian expression vector containing full-length mouse Sema5A tagged to the Flag epitope

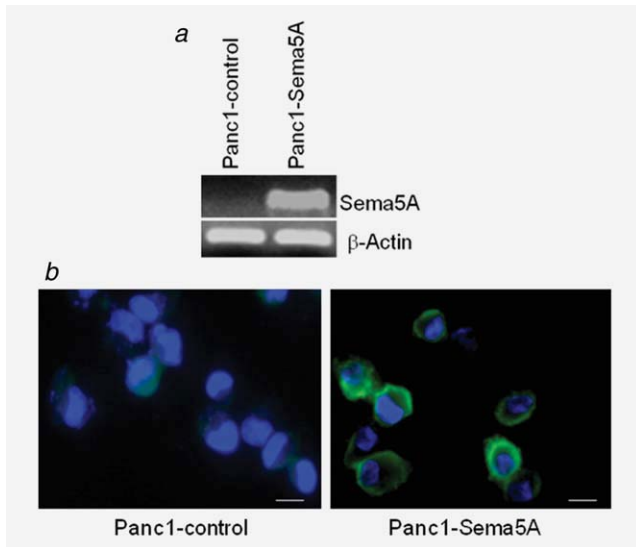


Figure 4. Ectopic expression of Sema5A in Panc1 cells. (a) Standard RT-PCR analysis showing Sema5A expression in Panc1 transfected with mammalian expression vector containing full-length Sema5A or its control vector. β -actin has been used as internal control. (b) Immunocytochemistry in (i) Panc1-control and (ii) Panc1-Sema5A-Flag using anti-Sema5A and secondary fluorescence antibodies (at $\times 400$ magnification). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

(Panc1-Sema5A) or with the empty vector alone (Panc1-control). Expression of Sema5A in the transfected cells was evaluated at the mRNA level by RT-PCR using specific primers. We observed Sema5A mRNA in Panc1-Sema5A cells whereas Panc1-control cells did not express Sema5A mRNA (Fig. 4a).

Similarly, we confirmed the cellular localization of Sema5A by immunofluorescent staining with an anti-SEMA5A antibody. Results showed no immunoreactivity in Panc1-control cells whereas membrane localization of SEMA5A protein was clearly observed in Panc1-Sema5A (Fig. 4b).

Sema5A expression enhanced density-dependent cell proliferation

We determined whether expression of Sema5A regulates cell proliferation in pancreatic cancer cells. Panc1-Sema5A and Panc1-control cells were seeded and cellular proliferation was measured using the MTT assay. Our data showed significantly ($p < 0.05$) higher proliferation in Panc1-Sema5A compared to Panc1-control cells with media alone or media containing different concentrations of serum (Fig. 5a). These data suggest that Sema5A expression promotes the cell density-dependent pancreatic cancer cell proliferation.

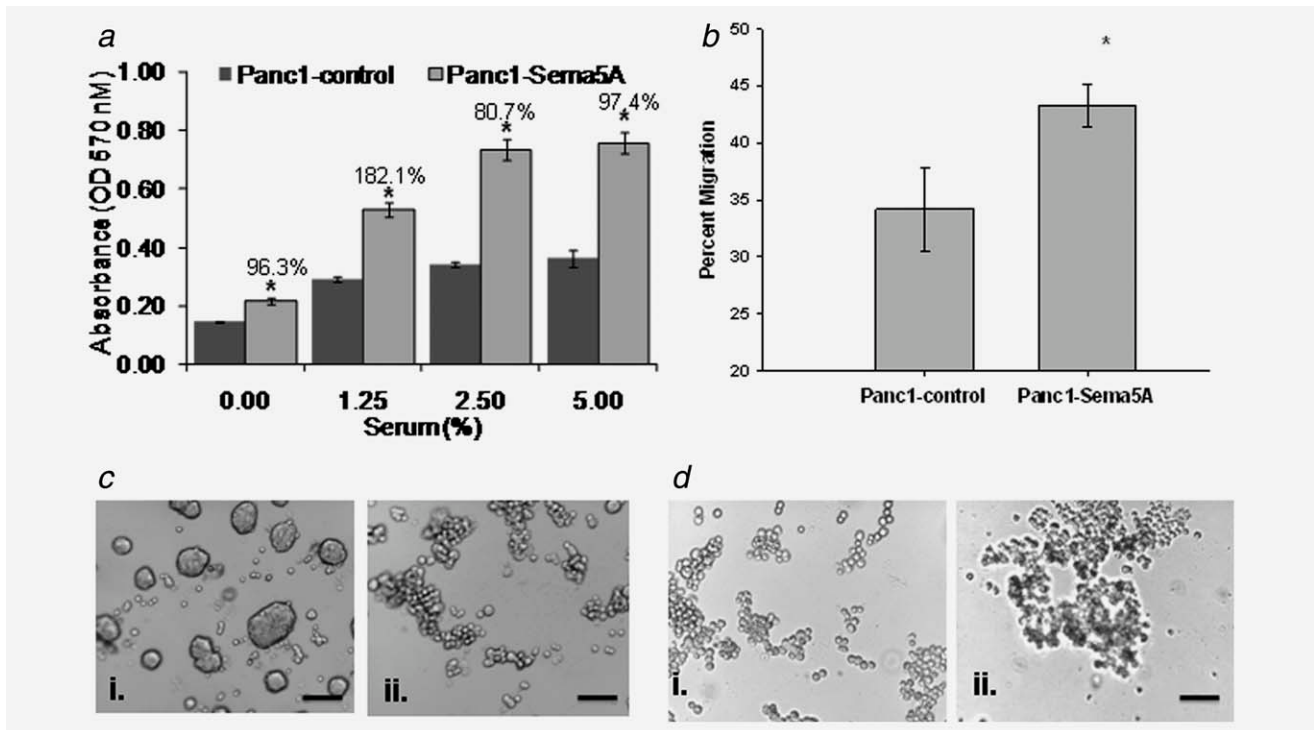


Figure 5. Sema5A stimulates pancreatic cell proliferation, invasion and homotypic cellular aggregation. (a) Proliferation assay by adding MTT after 72 hr in the presence of different concentrations of serum using 5,000 cells/well of Panc1-control or Panc1-Sema5A. (b) Matrigel invasion assay to show differential migration between Panc1-Sema5A and Panc1-control cells. The percentage difference in proliferation or migration between the control and experimental samples are shown. *Significant difference compared to controls with $p < 0.05$. Aggregation assay using hanging-drop method showing [c (i)] well-aggregated Capan1 cells and [c (ii)] loosely aggregated Panc1 cells. [d (i)] Panc1-control and [d (ii)] Panc1-Sema5A cells. Bar is 0.01 mm.

Sema5A expression enhanced tumor cell invasion and aggregation

Next, we examined the invasive potential of modulated Panc1 cells using Matrigel invasion assay. There was a significant ($p < 0.05$) increase in the invasive potential of Panc1-Sema5A cells as compared to Panc1-control cells (Fig. 5b). Thus, the data suggest that Sema5A provides migratory cues for pancreatic cancer cells through autocrine or paracrine signaling.

Aggregation and metastatic potential have been reported to be associated in malignant tumors.^{28,29} SEMA4D, a close relative of SEMA5A, was shown to induce homotypic cellular

aggregation in B- and T-cells.³⁰ The ability of SEMA5A-expressing cells to form homotypic aggregates as compared to SEMA5A nonexpressing cells was evaluated using a hanging-drop assay. The results demonstrated that Capan1 (SEMA5A positive) cells formed more aggregated colonies of cells as compared to Panc1 (SEMA5A negative) cells (Fig. 5c). Similarly, there was higher aggregation potential of Panc1-Sema5A cells compared to Panc1-control cells (Fig. 5d). These data suggest that expression of SEMA5A provides enhanced aggregation potential to pancreatic cancer cells.

Sema5A expression enhanced tumorigenicity and tumor growth

To test the hypothesis that SEMA5A expression enhances tumor formation and growth, we injected Panc1-Sema5A or control cells (1×10^6 cells/mice) subcutaneously into nude mice. Tumor growth was monitored twice a week for 8 weeks. Panc1 cells were nontumorigenic when injected subcutaneously in nude mice (Supporting Information Table 1). Similar to parental Panc1 cells, Panc1-control cells did not form tumors. In contrast, 100% (10/10) of the mice injected with Panc1-Sema5A cells developed tumors (Fig. 6a), and we observed significantly enhanced tumor growth in mice injected with Sema5A-expressing pancreatic cancer cells (Panc1-Sema5A) as compared to their controls (Panc1-controls, Fig. 6a).

We confirmed the expression of Sema5A in tumors produced by Panc1-Sema5A or their control cells by immunocytochemistry. We observed significantly higher expression of Sema5A in Panc1-Sema5A compared to its control cells (Fig. 6b). Together, these results demonstrate that Sema5A enhances tumorigenicity and tumor growth in pancreatic cancer using xenograft assays.

Sema5A expression enhances distant metastasis in orthotopic model

To examine the impact of Sema5A expression in pancreatic tumor growth and metastasis, we performed orthotopic tumor growth and metastatic assays using Panc1-Sema5A or its control cells. Mice were injected orthotopically with Panc1-Sema5A or Panc1-control (5×10^5 cells/mice) and were sacrificed until the mice become moribund. We did not observe a statistically significant difference in tumor growth (measured as weight of tumors) as well as incidence of primary tumor between mice injected with Panc1-Sema5A and Panc1-control cells (Table 2). Interestingly, the majority of the mice bearing Panc1-Sema5A tumors developed metastasis to

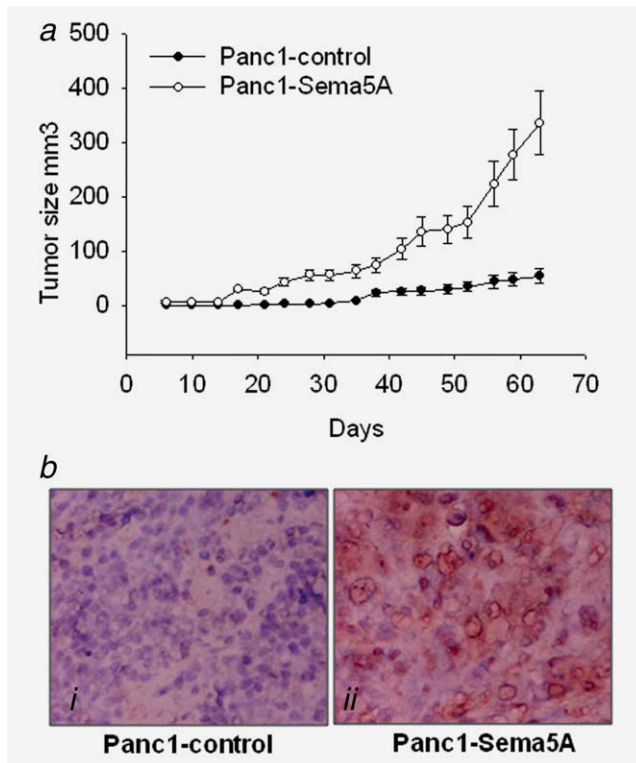


Figure 6. Tumorigenesis and growth kinetics of Sema5A-expressing Panc1. Nude mice injected subcutaneously with Panc1-control or Panc1-Sema5A. Tumor growth was monitored twice a week and tumor volume determined for 8 weeks. (a) A significantly enhanced tumor growth in Panc1-Sema5A injected mice as compared to control cells injected mice at different days are shown. (b) Sema5A expression in modulated Panc1 cells (i) Panc1-control and (ii) Panc1-Sema5A tumors were analyzed using immunocytochemistry (at $\times 200$ magnifications). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Table 2. Tumor growth and metastatic potential of Sema5A-expressing pancreatic cancer cells

Cell line	Incidence of primary tumor	Average weight (mg)	Incidence of metastases			
			Lymph node	Liver	Spleen	Peritoneum
Panc1-control	100	24.91 \pm 1.29	40	0	0	0
Panc1-Sema5A	100	25.67 \pm 3.71	75	50	50	50

Orthotopic injection of Panc1-Sema5A or its Panc1-control in nude mice ($n = 10$ per group) was performed as described in "Material and Methods." Average weight of tumor, incidence of primary tumor and metastasis to distant organs was examined in mice bearing orthotopic tumors.

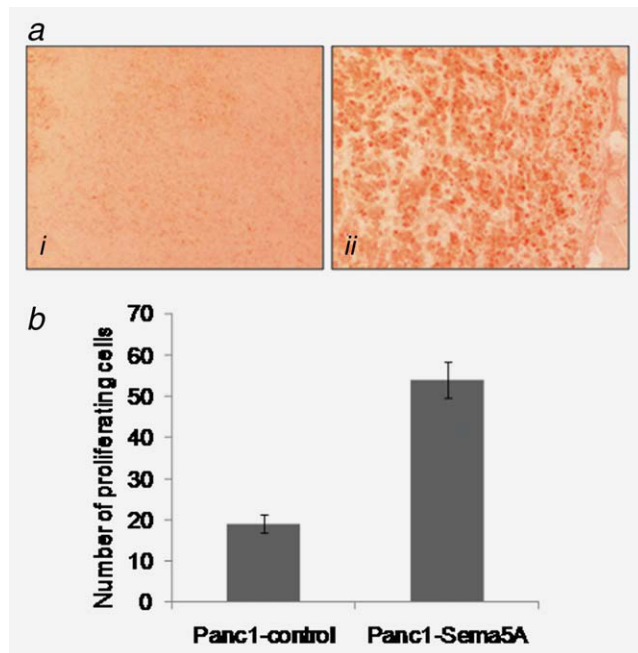


Figure 7. Proliferation of orthotopic tumors from Panc1-Sema5A and its control. (a) Immunohistochemistry showing PCNA expression in (i) Panc1-control and (ii) Panc1-Sema5A. (b) The number of cells positive for PCNA. The values are mean number of cells \pm SEM (bars) of 3 slides. *Significant difference with $p < 0.05$. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

multiple sites compared to mice bearing Panc1-control tumors (Table 2). Panc1-Sema5A tumor bearing mice developed significantly higher metastases to lymph node, liver, spleen and peritoneal cavity as compared to Panc1-control tumor bearing mice (Table 2). These data suggest that Sema5A expression enhanced metastasis of pancreatic tumor cells. We confirmed the expression of Sema5A in primary tumors from Panc1-Sema5A mice and not in the Panc1-control mice by immunocytochemistry using rabbit polyclonal anti-SEMA5A antibody (data not shown).

Sema5A expression increased *in situ* tumor cell proliferation

Panc1-Sema5A and Panc1-control tumors were examined for *in situ* cell proliferation by immunostaining using anti-PCNA antibody. Our results demonstrated an intense staining for PCNA in Panc1-Sema5A tumor samples as compared to control (Figs. 7a and 7b). The number of PCNA positive cells was significantly higher in Panc1-Sema5A tumors as compared to Panc1-control (Figs. 7a and 7b) demonstrating that Sema5A expression provides a growth advantage to the pancreatic cancer cells *in vivo*.

Discussion

In spite of advances in therapeutic strategies, pancreatic cancer remains a leading cause of cancer death in the United

States. Most of the patients still die within 6 months to 2 years after diagnosis due to metastasis of the malignant cells to the same organ or distant organs. The main reason for the failure of current therapy and the major cause for cancer-related mortality in general is the lack of appropriate molecular marker(s) for primary tumor or metastases. Cell adhesion molecules play a major role in tumor progression and metastasis.³¹ In our study, we demonstrate that the cell adhesion molecule, SEMA5A, protein and mRNA is constitutively expressed in majority of human pancreatic tumor tissues and aggressive pancreatic cancer cell lines with higher tumorigenicity, growth and metastatic potential as xenografts in nude mice. We did not observe expression of SEMA5A protein and mRNA in normal pancreatic tissues and less aggressive pancreatic cancer cell lines. Moreover, ectopic expression of mouse Sema5A in pancreatic cancer cells enhanced proliferation, aggregation and invasion *in vitro* and tumor growth and metastasis *in vivo*. Together, these studies demonstrate the importance of SEMA5A expression in pancreatic cancer growth and metastasis.

In our previous study, we observed that SEMA5A mRNA is expressed in aggressive pancreatic cancer cell lines and thus, predicted as marker for pancreatic cancer.⁴ In our current study, we screened the expression of SEMA5A protein in human pancreatic tumor tissues with different histopathology and normal pancreatic tissues. The results showed that SEMA5A protein is expressed in majority of pancreatic tumor tissues irrespective of the stage of the disease and is not expressed in normal pancreatic tissues. In spite of the physiological complexities being posed by species difference, the xenograft experiments (subcutaneous and orthotopic injections) using immune deficient animals ultimately help to evaluate tumor growth and metastatic ability (aggressiveness) of any cancer cell in relation to human tumor progression and metastasis.³² In our current study, we performed both subcutaneous and orthotopic injections to assess the aggressiveness of 13 human pancreatic cancer cell lines. This analysis identified 10 pancreatic cancer cell lines to be aggressive based on their tumor growth and/or metastasis as xenograft tumors. On the basis of RT-PCR analysis, we observed that SEMA5A mRNA is constitutively expressed in most of the aggressive pancreatic cancer cell lines whereas it is less or not expressed in nonaggressive pancreatic cancer cell lines. Moreover, most of the pancreatic cancer cell lines established from metastases expressed SEMA5A mRNA whereas the majority of cell lines established from primary tumors did not express SEMA5A. This along with our previous observation⁴ suggests that SEMA5A could be a marker for pancreatic cancer.

The vast majority of pancreatic cancers are ductal adenocarcinoma (>90%), which are generally characterized according to morphological criteria such as the grade of differentiation from undifferentiated to well-differentiated tumors.³³ A previous report from our laboratory demonstrated a correlation between the expression of MUC4, a pancreatic cancer

marker, and the degree of cellular differentiation.³⁴ In our study, elevated expression of SEMA5A protein and mRNA was observed in moderately and well-differentiated pancreatic tumors and cell lines as compared to poorly/undifferentiated tumors and cell lines. Interestingly, most of the pancreatic cancer cell lines established from metastases and that expressed SEMA5A mRNA were moderately to well-differentiated as shown in Supporting Information Table 1. Although the differentiation status of tumors and cell lines are not comparable, there is an association between SEMA5A expression and the differentiation status of pancreatic cancer. This suggests that SEMA5A is mainly expressed in moderately to well-differentiated pancreatic cancer cells and cell lines established from secondary tumors. Furthermore, we have used only normal and cancer tissue for the current analysis, which excludes a progressive model of pancreatic cancer development going through pancreatic intraepithelial neoplasia (PanIn) lesions. Further studies need to be performed to address the role of SEMA5A mRNA and protein expression using archival tissue with different PanIn lesions.

Next we examined cellular localization and observed that SEMA5A is predominantly localized at cell–cell junctions. Furthermore, SEMA5A expressing cells underwent higher homotypic aggregation as compared to cells not expressing SEMA5A. Earlier reports have demonstrated that homotypic aggregation is one of the phenotypes of highly aggressive and metastatic cells.^{28,29} Also, it is known that growing as aggregates is a major phenotype of differentiated cells. Hence, our data suggest that pancreatic cancer cells expressing SEMA5A show higher homotypic aggregation.

Previously, we predicted the expression of SEMA5A in human pancreatic cancer using phage display peptide library assay and bioinformatic approaches but the functional role of SEMA5A was not known. In this report, we demonstrate that mouse Sema5A expression in Panc1 (not expressing SEMA5A) induced tumorigenesis and metastasis. All the mice with Panc1-Sema5A cells developed tumors after subcutaneous implantation in nude mice and the control cells did not form any tumors. On the other hand, we did not observe a significant difference in tumor size and incidence in Panc1-Sema5A and Panc-1 control cells injected orthotopically in mice. Nevertheless, upregulation of Sema5A enhanced proliferation of tumor cells and incidence of distant metastasis to lymph node, liver, spleen, and peritoneal cavity after orthotopic injection in mice. These results demonstrate the associ-

ation of Sema5A with tumor growth, metastatic potential and aggressiveness.

The role of SEMA5A in modulating the proliferation and migration of cancer cells has not been examined earlier. An earlier report suggests that Sema5A is expressed in areas of proliferating neuronal precursors and not in the postmitotic neuronal cells in the developing mouse brain,¹⁴ suggesting that Sema5A may be involved in the proliferation of cells. Our *in vitro* proliferation assay using ectopic expression of Sema5A suggest that the increase in proliferation of pancreatic cancer cells occurs through direct cell–cell interactions. In addition, the proliferation of tumor cells ectopically expressing full-length Sema5A was significantly higher compared to control when orthotopically injected into nude mice. In addition, Matrigel invasion assay showed increased invasive potential of Sema5A-expressing Panc1 cells as compared to control Panc1 cells. Together these data demonstrate that upregulation of Sema5A regulates cell proliferation and invasion, and suggests the role of SEMA5A as a mitogenic and motogenic factor for pancreatic cancer cells.

The functions of semaphorins are exerted by binding to a family of transmembrane proteins called plexins, which share the sema domain with semaphorins.^{35,36} A recently published report²⁰ and our analysis²¹ identified Plexin B3 as a high affinity functional receptor for Sema5A. In our study, we demonstrate that all the pancreatic cancer cell lines examined express Plexin B3 with no association between expression and aggressiveness of tumors. This data suggest an autocrine role for SEMA5A in aggressive pancreatic cancer cells. The precise downstream signaling events in SEMA5A-mediated pancreatic cancer cell proliferation and migration is currently under investigation in our laboratory.

In summary, our data demonstrate SEMA5A mRNA expression and to a lesser degree protein expression as markers for moderately to well-differentiated pancreatic cancer. Sema5A expression in pancreatic cancer cells promotes growth, proliferation, aggregation, invasion and metastatic potential and suggests that targeting SEMA5A expression and/or activity might be a novel diagnostic/therapeutic approach for pancreatic cancer.

Acknowledgements

This work was supported in part by Cancer Glycobiology Program from Nebraska Research Initiative and by grants CA72781 (R.K.S.) and CA78590 (S.K.B.) from the National Institutes of Health.

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