

S100P Promotes Pancreatic Cancer Growth, Survival, and Invasion

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Abstract Purpose: In the current study, we examined the functional significance and mechanism of action of S100P in pancreatic cancer cells.

Experimental Design: S100P levels were increased in Panc-1 cells, which do not express S100P, by transfection with an S100P cDNA and S100P levels were reduced in BxPC3 cells, which express high levels of S100P, by small interfering RNA gene silencing. Effects of these manipulations on cell proliferation, resistance to apoptotic insults, cell migration, and invasion were estimated *in vitro* using standard assays. The influences of S100P on tumor growth *in vivo* were studied using xenograft mouse models. To identify the mechanisms involved in these responses, coimmunoprecipitation studies were conducted with S100P with receptor for advanced glycation end products (RAGE) and the effects of inhibiting RAGE using an antagonistic peptide were analyzed.

Results: S100P levels correlated with the rates of cell proliferation, survival, migration, and invasion in both cell models *in vitro*. *In vivo*, increased S100P levels increased the growth of tumors in mice with s.c.-implanted Panc-1 cells and decreased S100P levels decreased tumor growth after orthotopic implantation of BxPC-3 cells. A direct interaction between S100P and RAGE was indicated by coimmunoprecipitation of these molecules from pancreatic cancer cells. A RAGE antagonist peptide inhibited this interaction and also inhibited the biological effects of S100P on these cells *in vitro*.

Conclusions: These data suggest that S100P plays a major role in the aggressiveness of pancreatic cancer that is likely mediated by its ability to activate RAGE. Thus, interference with S100P may provide a novel approach for treatment of pancreatic cancer.

Pancreatic cancer is an almost universally fatal disease, with a 5-year survival rate of <5%, and it has the poorest likelihood of survival among all of the major malignancies (1). Each year in the United States, ~30,000 patients are diagnosed with pancreatic cancer and nearly all will die of this disease. Surgical resection is the only curative treatment. Unfortunately, at the time of diagnosis, only 10% to 20% of tumors are resectable due to the aggressive nature of this disease. Furthermore, pancreatic cancer is markedly resistant to both radiation and chemotherapy (2). The molecular mechanisms underlying the aggressive nature of pancreatic cancer are currently unknown.

The molecule S100P, a 95-amino-acid member of the S100 family of proteins that was first purified from placenta (3), is overexpressed in pancreatic cancer (4–6). Furthermore, it has been reported that the extent of S100P expression increases

during pancreatic cancer progression from PanIN lesions to invasive adenocarcinoma (7). The overexpression of S100P has been suggested to be due to hypomethylation of its gene in pancreatic cancer cells (6). S100P is also expressed in other human cancers, including breast, colon, prostate, and lung. In breast cancer cell lines, S100P levels are associated with cellular immortalization (8). In colon cancer cell lines, its expression level was correlated with resistance to chemotherapy (9). In prostate tumors, S100P levels were found to be androgen sensitive (10). In lung cancer, S100P expression correlated with decreased patient survival (11). However, despite these observations, very little is known about the functional role or mechanism of action of S100P.

We recently reported that transfection of NIH3T3 cells with a cDNA for S100P led to S100P secretion into the culture medium and that extracellular S100P was able to interact with the cell surface receptor for advanced glycation end products (RAGE; ref. 12). RAGE has been shown to participate in a number of important pathologic responses, including Alzheimer's disease, diabetes, inflammation, and cancer (13). Activation of RAGE is known to stimulate Erk and nuclear factor- κ B (NF- κ B) activity and to increase cell proliferation, survival, and motility (12). However, the functional significance of neither S100P nor RAGE has previously been studied in pancreatic cancer cells.

In the current study, we investigated the functions of S100P in pancreatic cancer. We found that S100P was expressed and secreted by nearly all pancreatic cancer cell lines. Gene transfer or extracellular addition of S100P increased, and silencing of S100P expression decreased, cancer cell growth, motility,

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invasion, and survival *in vitro* and tumor growth and metastasis *in vivo*. We also observed that S100P could interact directly with RAGE and that blocking this interaction inhibited all biological effects of S100P *in vitro*. Taken together, these results indicate that S100P contributes to the aggressive nature of pancreatic cancer likely through its ability to activate RAGE.

Materials and Methods

Development of stable cell lines. Panc-1, BxPC3, CTPAC, Mpanc96, Su86.86, HPAC, and Sur99 cells were obtained from the American Type Culture Collection (Manassas, VA) and the nontransformed human pancreatic duct epithelial cell line was kindly provided by Dr. Tsao (University of Toronto, Toronto, Ontario, Canada). Panc-1, CTPAC, Mpanc96, Su86.86, Sur99, and HPAC cells were routinely cultured in DMEM with 10% fetal bovine serum. Human pancreatic duct epithelial cells were cultured in keratinocyte serum-free media. BxPC3 cells were cultured in RPMI 1640 with 10% fetal bovine serum. All cells were maintained at 37°C in a humidified atmosphere of 5% CO₂.

To study the role of S100P in pancreatic cancer, we developed cell lines overexpressing and underexpressing S100P. Panc-1 cells, which did not express endogenous S100P, were transfected using LipofectAMINE reagent (Invitrogen, Carlsbad, CA) with control plasmids and plasmids encoding human S100P (Genbank accession no. X65614) cloned into pcDNA3.1 vector and selected for resistance to G418 (1 mg/mL), and monoclonal lines were selected and used for further study. BxPC-3 cells, which express relatively high levels of S100P, were transfected with a combined small hairpin small interfering RNA (siRNA) synthesized with sense (Genbank accession no. X65614; AATGGAGATGCCAGGTGGAC) and antisense (GTCCACCTGGG-CATCTCCATT) for S100P connected with a loop (AAGCTT) or with a control siRNA that was identical except for the presence of two mismatched nucleotides in the antisense region (A→G/T→C). The siRNAs were designed flanked by *Bbs*1 and *Xba*1 restriction sites and cloned into a mU6 vector that contains the mouse U6 snRNA promoter (RNA polymerase III) as previously described (14). Stable expression of both the S100P siRNA and the control siRNA were established in BxPC-3 cells by puromycin selection (2 µg/mL) and clonal cell lines were developed and used for further study. The effects of siRNA on the expression of S100P were confirmed by Western blotting. For overexpression and silencing studies, the data are shown for a single representative stable clonally selected cell line, but all experiments were reproduced with at least two other monoclonal lines (data not shown).

Reverse transcription-PCR of S100P. Total RNA isolated from pancreatic cancer cell lines was utilized for RT-PCR as previously reported (4). Primers designed for human S100P (Genbank accession no. X65614) were as follows: forward 5'-ATGACGGAAGTAGAGACAGCCATGGGC-3' and reverse, 5'-GGAATCTGTGACATCTCCAGCGCATCA-3'. Primers designed for β-actin (Genbank accession no. BC016045), which was used as a loading control for the RT-PCR reactions, were as follows: forward 5'-ATGATATCGCCGCGCTC-GTCGTC-3' and reverse, 5'-CGCTCGGCCGTGGT GAA-3'. Amplified products were separated on 1.5% agarose gels and visualized by ethidium bromide.

SDS-PAGE and Western blot analysis. Western blot analysis was utilized for the detection of S100P as previously described (12). Briefly, cell lysates and precipitates from coimmunoprecipitation studies were prepared and separated by 15% SDS-PAGE and transferred to nitrocellulose. Membranes were blocked for 1 hour at room temperature in 5% milk solution. S100P was detected by incubating the transferred membrane overnight at 4°C with antihuman monoclonal antibody (Transduction Laboratories, San Diego, CA) at 1:250 dilution in 5% milk solution. RAGE was detected by incubating the transferred membrane for 1 hour at room temperature with antihuman goat polyclonal antibody (Santa Cruz Biotechnologies, Santa Cruz, CA)

at 1:100 dilution in 5% milk solution. Secondary antibody antimouse IgG + horseradish peroxidase antibody and antigoat IgG + horseradish peroxidase was incubated for 1 hour at room temperature and the signal was detected by the ECL detection system (Amersham, Piscataway, NJ) as per the protocol of the manufacturer.

For coimmunoprecipitation experiments with S100P antibody, BxPC3 cell lysates were immunoprecipitated with anti-S100P antibody in the absence or presence of amphoterin-derived peptide (1 µg) at 4°C overnight. Antibody-associated proteins were electrophoresed on 10% polyacrylamide gel and transferred to a nitrocellulose membrane. The transferred membrane was blocked by 5% milk solution overnight at 4°C. RAGE was detected by Western blotting as described above.

Expression and purification of S100P. S100P was prepared as described earlier (12). Briefly, full-length human S100P cDNA was cloned into the pTrcHis2 and transformed into bacteria and isopropyl-β-D-thio-D-galactopyranoside was used to induce the protein expression. His-S100P was purified using a ProBond resin column as described by the manufacturer (Invitrogen). The expression and purity of the S100P protein was confirmed by SDS-PAGE and Western blot.

Cell growth studies. Cell growth was analyzed using the MTS reagent (Promega, Madison, WI) according to the manufacturer's directions. For studies on cell survival, cells were treated with submaximal 5-fluorouracil (5-FU) at the concentrations of 400 µg/mL for Panc-1 cells or 100 µg/mL for BxPC3, based on the LD₅₀ concentration, for the indicated times. As an alternative apoptotic insult, cells were plated on dishes previously coated with polyhydroxymethylmethacrylate (poly-HEMA; Sigma-Aldrich, St. Louis, MO). Poly-HEMA was dissolved at 10 mg/mL in ethanol and 3 mL of solution was added to each well and plates were kept at 37°C for 5 days to evaporate solvent completely. Cell numbers were estimated using MTS, which was added to the wells 1 hour before taking the photometric reading. Fluorescence-activated cell sorting (FACS) analysis and cell counting were done in parallel and gave similar results as the MTS assay (data not shown). Anti-RAGE antibody was kindly provided by Ann Marie Schmidt (Columbia University, New York, NY).

Fluorescence-activated cell sorting analysis. Standard propidium iodide staining by the hypotonic lysis method was used for cell cycle and apoptosis studies. Control and transfected cells were seeded in 100 mm plates. Apoptosis was induced by treating with 5-FU at 400 µg/mL for Panc-1 cells or 100 µg/mL for BxPC3 cells. After 72 hours, cells were collected by trypsinization, washed once with cold PBS, mixed with 500 µL of hypotonic solution (0.1% sodium citrate, 0.1% Triton X-100, 100 µg/mL RNase, 50 µg/mL propidium iodide), and analyzed by flow cytometry after 30-minute incubation. Cell undergoing apoptosis that had lost part of their DNA (due to the DNA fragmentation) were detected as the population of cells with sub-G₁ DNA contents.

Migration and invasion assays. S100P and siRNA stably transfected cells along with control cells were used in migration and invasion experiments. Transwell cell migration plates (Corning, NY) and BIOCOAT matrigel invasion chambers (Becton Dickinson, Franklin Lakes, NJ) were used for this assay. Cell suspensions in serum-free culture media (1 × 10⁴ cells/100 µL top solution) were pipetted into the insert, and the insert was transferred into the well. For control assays, the bottom well was filled with 10% serum-containing media (+), serum-free media (-), or NIH 3T3 conditional media. The plates were incubated in a humidified CO₂ incubator at 37°C for 24 hours. The top solution was then removed, the cells on the top membrane surface were gently scraped with a cotton swab, and the cells on the bottom surface were either fixed and stained with Diff-Quik stain kit (Becton Dickinson) or incubated for one hour with MTS reagent for quantification.

Tumor growth study in nude mice. All animal experiments were reviewed and approved by the University of Michigan Committee for the Use and Care of Animals. The tumorigenic capability of the S100P stably transfected cells along with control cells was assessed in 4-week-old male athymic nude *nu/nu* mice. All animals were maintained in a sterile environment. Cages, bedding, food, and water were all

autoclaved. All animals were maintained on a daily 12-hour light/12-hour dark cycle. Briefly, cells were grown to 80% confluence, harvested by trypsinization, washed twice in PBS (Life Technologies, Inc., Carlsbad, CA), and resuspended to a final concentration of 1×10^6 cells/mL in sterile PBS. Cell suspensions (0.1 mL) were injected s.c. into the left flank of 4-week-old mice. Six mice were inoculated per test group. Tumor volume (in mm^3) was calculated using the formula for the volume of a prolate ellipsoid, $(w^2/2)l$, in which w and l are the width and length of the tumor in millimeters, respectively. Tumors and other organs were harvested from mice after 4 weeks of growth and fixed in formaldehyde for further analysis.

Tumor growth and metastasis study in severe combined immunodeficient mice. BxPC3 cells were stably transfected with siRNA against S100P and control siRNA, and silencing of S100P expression was confirmed by Western blotting. Further, these cells were transfected stably with luciferase gene by lentivirus transfection (15). The tumorigenic capability of the siRNA stably transfected cells along with control cells was assessed in 4-week-old male *CB 17 scid* mice. Briefly, cells were grown to 80% confluence, then harvested by trypsinization, washed twice in PBS, and resuspended to a final concentration of 1×10^6 cells/mL. Cell suspensions (0.1 mL) were injected into the pancreas. Six mice were inoculated per test group and tumor growth was accessed by bioluminescence imaging after 6 weeks.

Bioluminescence imaging was conducted using a cryogenically cooled imaging system coupled to a data acquisition computer running LivingImage software (Xenogen Corp., Alameda, CA). Before imaging, animals were anesthetized in an acrylic chamber with 1.5% isoflurane/air mixture and injected i.p. with 40 mg/mL of luciferin potassium salt in PBS at a dose of 150 mg/kg body weight. A digital grayscale animal image was acquired followed by acquisition and overlay of a pseudocolor image representing the spatial distribution of detected photons emerging from active luciferase within the animal. Signal intensity was quantified as the sum of all detected photons within the region of interest per second. Tumor volume, peritoneal dissemination, and metastasis were assessed. After the bioluminescence imaging, animals were dissected and cancer cell dissemination and metastases were visualized and counted. Tissues were also fixed with formaldehyde and histology was conducted to verify the accuracy of the bioluminescence data.

Statistical analysis. Data are presented as mean \pm SE. Statistically significant differences were determined by unpaired *t* test and were defined as $P < 0.05$.

Results

S100P is expressed and secreted by pancreatic cancer cell lines. S100P was previously found to be widely expressed in pancreatic adenocarcinoma but absent in normal and chronic pancreatitis samples (4). To study the function of S100P, we wished to develop cell models with altered expression of this molecule. Thus, we initially determined its expression level in several pancreatic cancer cell lines. S100P mRNA was detected by RT-PCR in six of seven pancreatic cancer cell lines, with Panc-1 cells being the exception that did not express endogenous S100P (Fig. 1A). S100P expression was also not observed in the immortalized normal human pancreatic duct epithelial cell line, as expected. Each of the pancreatic cancer cell lines that expressed significant levels of S100P mRNA also expressed the protein, as indicated by Western blotting, and BxPC3 cells were among the cells with the highest levels of endogenous S100P (Fig. 1B). Therefore, to study its functional effects, we ectopically expressed S100P in Panc-1 cells, which had low endogenous levels (Fig. 1C), by transfection with a

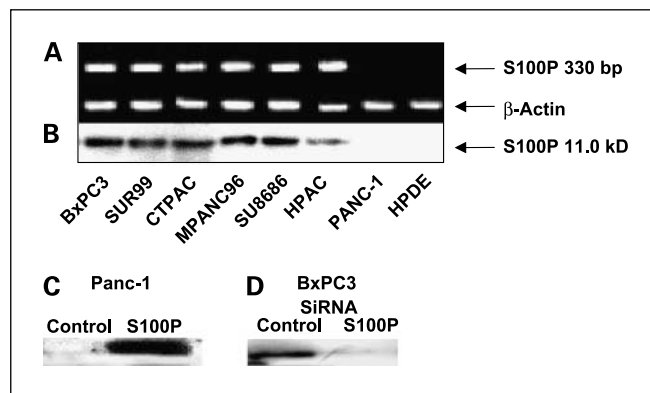


Fig. 1. S100P is expressed in the majority of pancreatic cancer cell lines. *A*, RT-PCR showing the expression of S100P mRNA in pancreatic cancer cell lines along with β -actin as control. *B*, Western blot showing the expression of 11 kDa S100P protein in pancreatic cancer cell lines. *C*, Western blot showing the expression of S100P protein in Panc-1 cells stably transfected with an expression vector bearing S100P (S100P) but not with control vector (control). *D*, Western blot showing the silencing of S100P expression in BxPC-3 cells stably transfected with siRNA against S100P, but not with control siRNA.

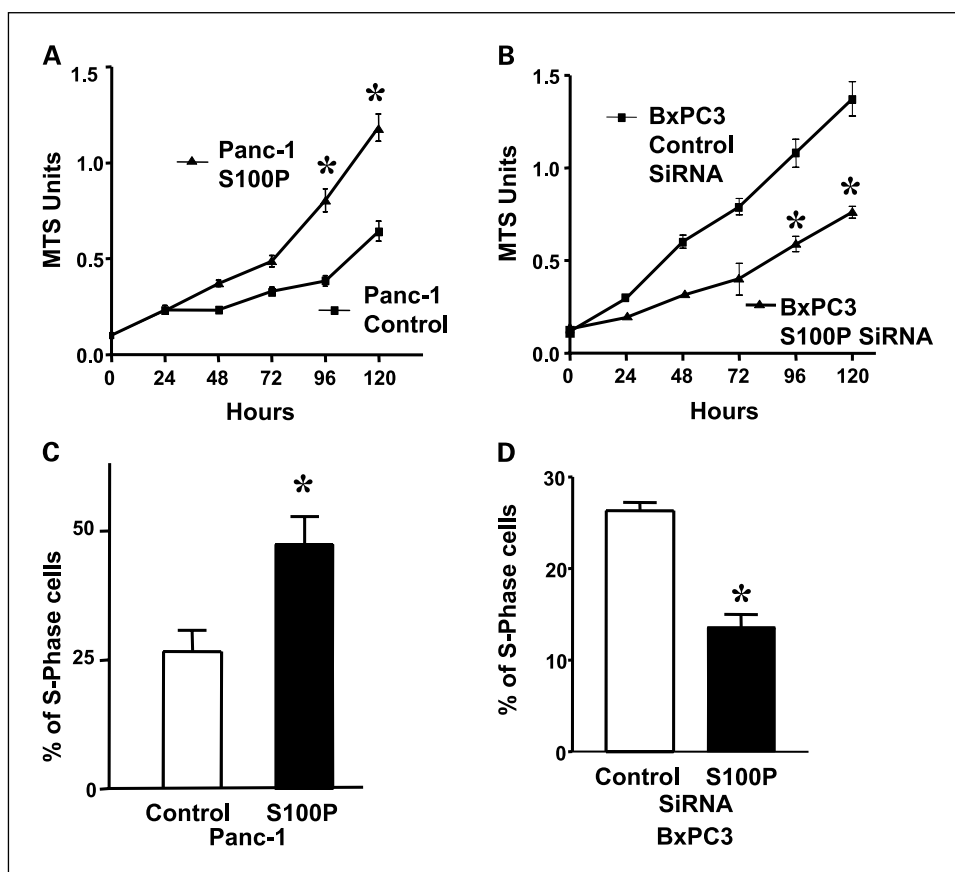
cDNA for S100P. Furthermore, we reduced S100P expression in BxPC3 (Fig. 1D) and Su 86.86 cells (data not shown), which had high endogenous levels, by using a siRNA.

We previously observed that S100P could be secreted from NIH3T3 cells stably expressing this molecule (11). Therefore, we determined whether S100P was also secreted from pancreatic cancer cells. S100P was detected in the media bathing Panc-1 cells after, but not before, stable transfection with an S100P expression vector (Panc-1 wt: not detectable; Panc-1 S100P transfected: 34.4 ± 6.5 ng/mL). Furthermore, S100P was detected in culture media bathing several wild-type pancreatic cancer cell lines including HPAC (29 ± 6 ng/mL) and BxPC3 cells (332 ± 54 ng/mL).

S100P stimulates pancreatic cancer cell growth in vitro. To evaluate the potential role of S100P in pancreatic cancer cell proliferation, we initially evaluated the effect of S100P expression on Panc-1 cells *in vitro*. Panc-1 cells stably expressing S100P proliferated at a significantly more rapid rate than control cells, such that cell numbers were twice those control cells within 96 hours (Fig. 2A). The stimulation of proliferation was further confirmed by FACS analysis that indicated a $>80\%$ increase in the number of cells in S-phase cells in Panc-1 cells expressing S100P (Fig. 2C). We further observed that these effects could be duplicated by treatment of wild-type Panc-1 cells with exogenous S100P (data not shown), as was previously observed in NIH3T3 cells (12). These data indicated that S100P had the potential to be a growth stimulant for pancreatic cancer cells.

To determine whether endogenous S100P promoted growth in pancreatic cancer cells, we examined the effect of silencing S100P expression in BxPC3 cells. BxPC3 cells with siRNA-silenced S100P production proliferated at a significantly reduced rate compared with control siRNA-expressing cells, such that cell numbers were $<50\%$ of controls within 96 hours (Fig. 2B). A similar result was observed with another pancreatic cancer cell line, SU 86.86 cell (data not shown), indicating that this result was not confined to a single cell model. The reduction in growth rate was further confirmed by FACS analysis that indicated a 50% decrease in the proportion of

Fig. 2. Expression of S100P stimulates pancreatic cancer cell proliferation. *A*, proliferation of Panc-1 cells stably transfected with control or S100P-expressing vector. *B*, proliferation of BxPC3 cells stably transfected with control siRNA or S100P siRNA. Cells were plated at an equal density and cultured for the indicated time periods before cell numbers were estimated using the MTS assay. Similar results were obtained using cell counting (data not shown). *C*, comparison of the percentage of cells in S-phase in Panc-1 cells stably transfected with control or S100P-expressing vectors. *D*, comparison of the percentage of cells in S phase in BxPC3 cells stably transfected with control siRNA or S100P siRNA. Cells were plated at an equal density and the percentage of cells in the S phase was analyzed by FACS after 96 hours. Columns, mean for three experiments (* $P < 0.05$); bars, SE.



cells in the S-phase in cells expressing the siRNA against S100P (Fig. 2D).

S100P promotes pancreatic cancer cell survival in vitro. We next examined the effects of S100P on pancreatic cancer cell survival following two different cellular insults. In one set of experiments, cells were treated with the chemotherapeutic agent 5-FU to induce apoptosis. The 5-FU dose was selected to be submaximal and was based on the LD₅₀ concentration for each cell type. 5-FU treatment efficiently reduced the numbers of control Panc-1 cells (Fig. 3A). In contrast, there was a significant reduction in the efficiency of 5-FU cell killing in Panc-1 cells stably expressing S100P (Fig. 3A). In complementary experiments, 5-FU-induced cell killing was much more pronounced in BxPC3 cells expressing a siRNA for S100P compared with their counterparts expressing a control siRNA (Fig. 3B). These survival benefits of S100P were further confirmed using FACS analysis of 5-FU-treated cells to estimate the number of apoptotic cells that were found to be inversely correlated with S100P expression (Fig. 3C and D).

In another set of experiments, the effects of S100P were evaluated on anoikis, the form of apoptosis resulting from the lack of cell anchorage. Panc-1 cells plated on dishes coated with poly-HEMA, which prevents cell attachment, underwent anoikis as indicated by a reduction in cell numbers. The level of anoikis in Panc-1 cells was significantly reduced by expression of S100P (Fig. 3E). In complementary experiments, S100P silencing by siRNA expression increased the extent of anoikis in BxPC3 cells plated on poly-HEMA-coated dishes (Fig. 3F).

S100P stimulates pancreatic cancer cell migration and invasion in vitro. Next, we analyzed the effects of S100P on cell migration and invasion. S100P expression increased the rate of migration (Fig. 4A-B) and invasion (Fig. 4E-F) of Panc-1 cells at 24 hours. In complementary experiments, specific silencing of S100P reduced the rate of BxPC3 cell migration (Fig. 4C-D) and invasion (Fig. 4G-H). Similar results of S100P silencing were observed in an independent pancreatic cancer cell line, SU.86.86 cells (data not shown). Furthermore, exogenous addition of S100P increased the rates of Panc-1 cell migration and invasion in a dose-dependent manner (data not shown).

S100P increases pancreatic tumor growth and metastasis in vivo. Based on the effects of S100P on pancreatic cancer cell growth, survival, and motility observed *in vitro*, we wished to further analyze its impact on tumors developed *in vivo* in immune deficient mice. In one set of experiments, tumor formation was compared between Panc-1 cells with and without stable expression of S100P. For these studies, cells were inoculated s.c. and tumor growth was measured after 4 weeks. Panc-1 cells overexpressing S100P showed a dramatic increase (~5-fold) in the size of tumors when compared with its control counterpart (Fig. 5A and B).

In a complementary set of experiments, BxPC3 cells expressing siRNA for S100P, or a control mismatch siRNA, which for monitoring purposes were also modified to express luciferase, were orthotopically implanted into the pancreas and cancer progression was monitored using bioluminescence imaging. Silencing of S100P significantly reduced tumor

volume by $\geq 80\%$ after 6 weeks (Fig. 5C and D). Silencing of S100P also reduced peritoneal dissemination compared with the control siRNA-bearing cells (two of six versus four of six) and distant metastasis to liver (one of six versus two of six) and lung (none of six versus one of six).

S100P interacts with and acts through receptor for advanced glycation end products. Our previous studies revealed the interaction of S100P molecules with RAGE (12). Both Panc-1 and BxPC3 cells were found to express RAGE (data not shown). However, it is not known whether S100P can act through this receptor in pancreatic cancer cell lines; to investigate this possibility, we initially did coimmunoprecipitation assays using lysates from BxPC3 cells. Lysates were immunoprecipitated with anti-S100P monoclonal antibody, and the isolated proteins were separated on an SDS-PAGE gel, transferred to nitrocellulose, and blotted with an antibody specific for RAGE (Fig. 6A). RAGE was identified in the precipitate, indicating an interaction between S100P and RAGE. We then tested whether this

interaction could be inhibited by coincubating the samples with a peptide derived from amphoterin that has previously been found to block the interaction between RAGE and amphoterin (16). Inclusion of the amphoterin peptide (1 μg) greatly inhibited the coimmunoprecipitation of S100P and RAGE (Fig. 6A). To further confirm this interaction, we did the reverse immunoprecipitation. BxPc3 cell lysates were immunoprecipitated with anti-RAGE antibody and S100P was identified in the precipitate by Western blotting. Again, inclusion of the amphoterin peptide almost completely blocked the coimmunoprecipitation of RAGE and S100P (Fig. 6B).

To determine whether S100P activation of RAGE was required for the effects of S100P on pancreatic cancer cell growth, we utilized two different approaches to inhibit the activation of RAGE by S100P and investigated the effects on cell growth. Incubation of wild-type BxPC3 cells with the inhibitory amphoterin-derived peptide or with anti-RAGE antibodies (17) inhibited the cell growth (Fig. 6C), migration, and invasion

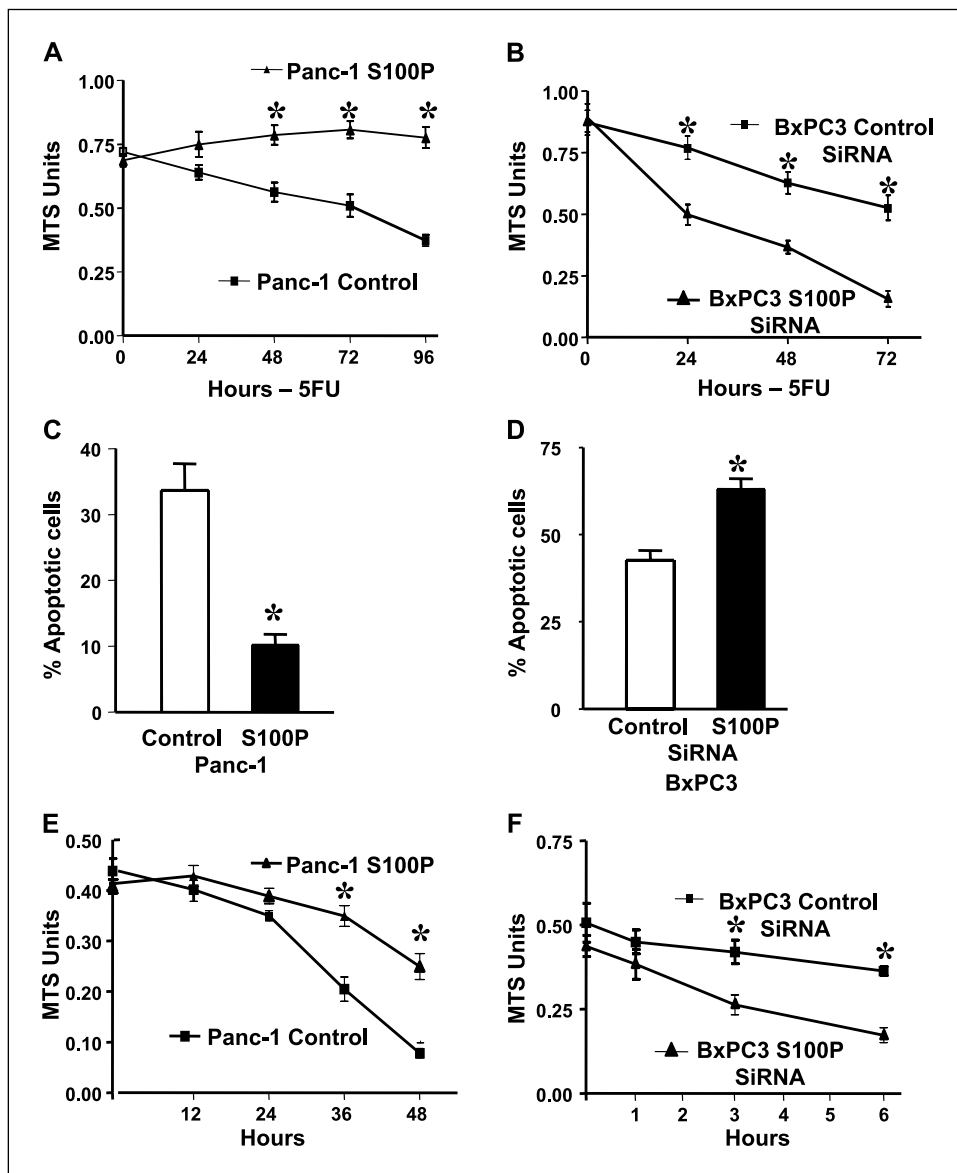
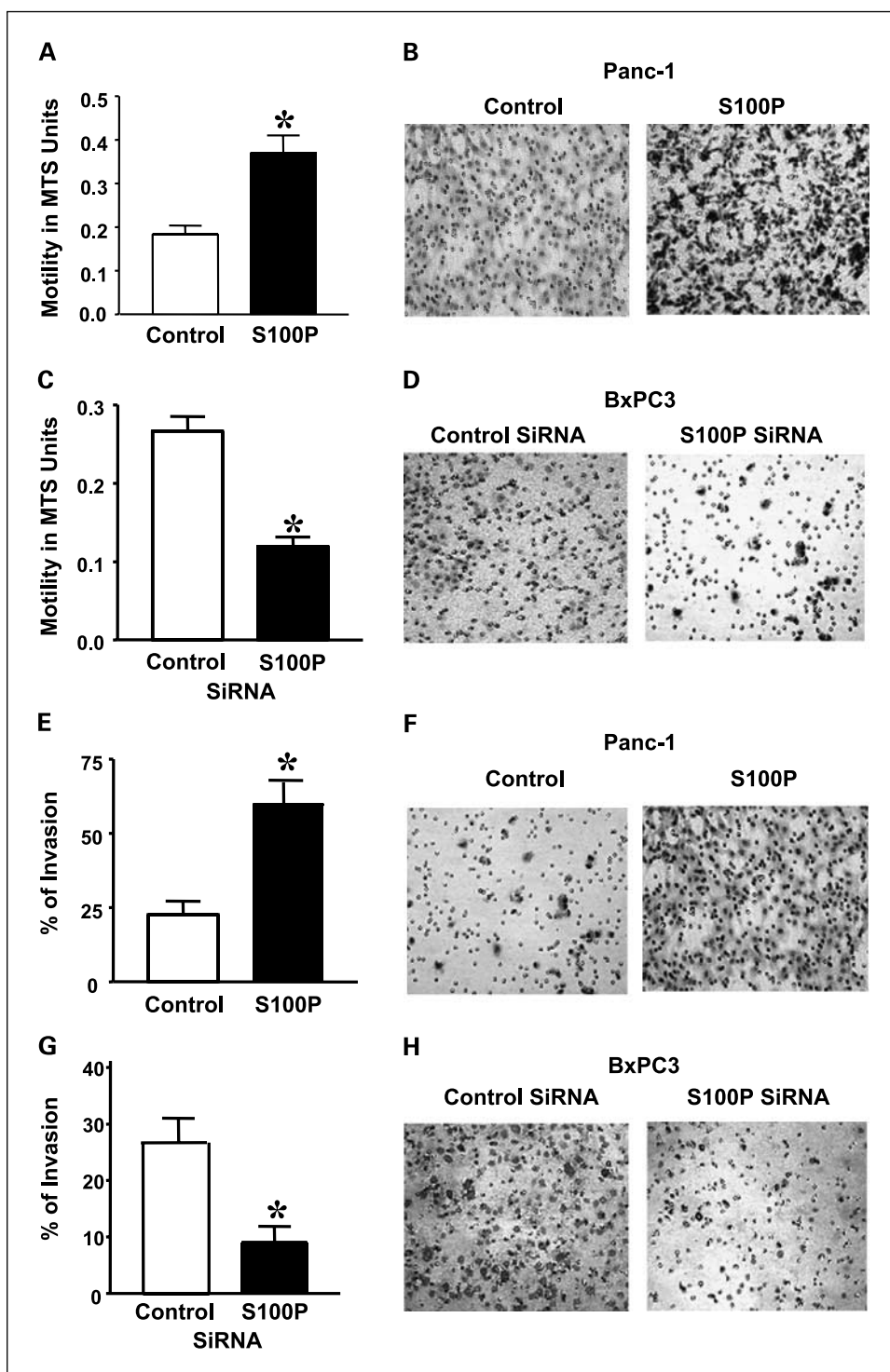


Fig. 3. Expression of S100P protects pancreatic cancer cells against cell death induced by detachment or 5-FU. *A* and *B*, effect of 5-FU treatment on cell viability in the presence and absence of S100P expression. Panc-1 cells stably transfected with control or S100P expressing vector (*A*) and BxPc3 cells stably transfected with control siRNA or S100P siRNA (*B*) were cultured in the presence of 5-FU (400 $\mu\text{g}/\text{mL}$ for Panc-1 and 100 $\mu\text{g}/\text{mL}$ for BxPC3 cells) for indicated time points and cell viability was analyzed using the MTS method. *C* and *D*, effect of 5-FU treatment on cell apoptosis in the presence and absence of S100P expression. Panc-1 cells stably transfected with control or S100P expressing vector (*C*) and BxPc3 cells stably transfected with control siRNA or S100P siRNA (*D*) were cultured in the presence of 5-FU for indicated time points and the number of apoptotic cells was estimated as the percentage of cells with sub-G₁ DNA contents as quantitated using FACS analysis. *E* and *F*, effect of detachment on cell viability in the presence and absence of S100P. Panc-1 cells stably transfected with control or S100P expressing vector (*E*) and BxPc3 cells stably transfected with control siRNA or S100P siRNA (*F*) were plated on poly-HEMA-coated dishes to prevent cell attachment and were incubated for indicated time points. Cell viability was analyzed using the MTS method. Points, mean for three experiments (* $P < 0.05$); bars, SE.

Fig. 4. S100P stimulates pancreatic cancer cell migration and invasion. Panc-1 cells stably transfected with control or S100P-expressing vector (*A, B* and *E, F*) and BxPc3 cells stably transfected with control siRNA or S100P siRNA (*C, D* and *G, H*) were placed in serum-free culture media and added into the upper compartment of a migration or invasion chamber. After 24 hours, cells in the upper chamber were removed and cells that had migrated or invaded onto the lower surface of the membrane were quantified photometrically using MTS reagent for migration studies and cells in three different areas were counted for invasion studies. Columns, mean for three experiments ($*P < 0.05$); bars, SE. Photographs of representative membranes after Diff-Quick staining are also shown.



(data not shown). Furthermore, the inhibitory amphotericin-derived peptide dramatically inhibited the ability of exogenous S100P to stimulate cell growth (Fig. 6D), migration, and invasion (data not shown) in Panc-1 cells.

Discussion

There is a great need to understand the mechanisms behind the aggressiveness and resistance to therapy of

pancreatic cancer. The current study investigated the role played by S100P in this disease. We previously observed that S100P is highly expressed in the neoplastic epithelial cells of pancreatic adenocarcinoma (4). In the current study, we observed that pancreatic cancer cell lines secrete S100P and that S100P expression stimulates proliferation, survival, motility, and invasiveness of these cells. These results extend the biological effects previously shown for S100P by over-expression studies in NIH3T3 cells (12) to the pancreatic

cancer cell. The current study also extends our previous investigations on the role of S100P by examining its effects on tumor growth *in vivo*. We observed that the S100P expression was associated with high levels of tumor growth and metastasis *in vivo*. These data suggest that S100P acts as an autocrine growth factor for pancreatic cancer, which is important in its growth, resistance to therapy, and its metastatic spread.

S100 molecules are small (9-12 kDa) calcium-binding proteins that display 30% to 50% homology within the family. There are at least 19 members of the S100 family and most map closely together on chromosome 1q21, with the notable exception of S100P, which is located on 4p16 (18). S100 proteins have been reported to serve a number of important functions, many of which are isoform specific (19). S100 proteins have recently become of major interest owing to their differential expression in a variety of tumors (7-10, 19) and their putative involvement in the metastatic process (20). In our previous study (4), we found that several S100 proteins were present in neoplastic cells within pancreatic tumors. Similarly, other studies have indicated the presence of several S100 molecules in pancreatic tumors

(5, 6). However, we found that the S100P isoform was the most highly differentially expressed member of the S100 protein family in pancreatic cancer compared with normal pancreas and chronic pancreatitis. In contrast, several other S100 isoforms were also elevated in chronic pancreatitis. S100P is a small 95-amino-acid protein first isolated from human placenta (3). S100P is also expressed in some noncancerous diseases, including inflammatory bowel disease, ulcerative colitis, and Crohn's disease (21).

In the current study, we observed that S100P stimulated pancreatic cancer cell proliferation and provided survival benefits similar to what we previously described in transfected NIH3T3 cells (12). More importantly, in the current study, we observed that the silencing of endogenous S100P inhibited the growth and survival of pancreatic cancer cells. Silencing is more specific than overexpression for determining the role of a factor in cell biology because it avoids the problems associated with overexpression. The current studies also indicate that the growth stimulatory effects of S100P on pancreatic cancer are not limited to the *in vitro* environment, as the same effect was also observed *in vivo*. The current study also extended the effects of S100P to increases in

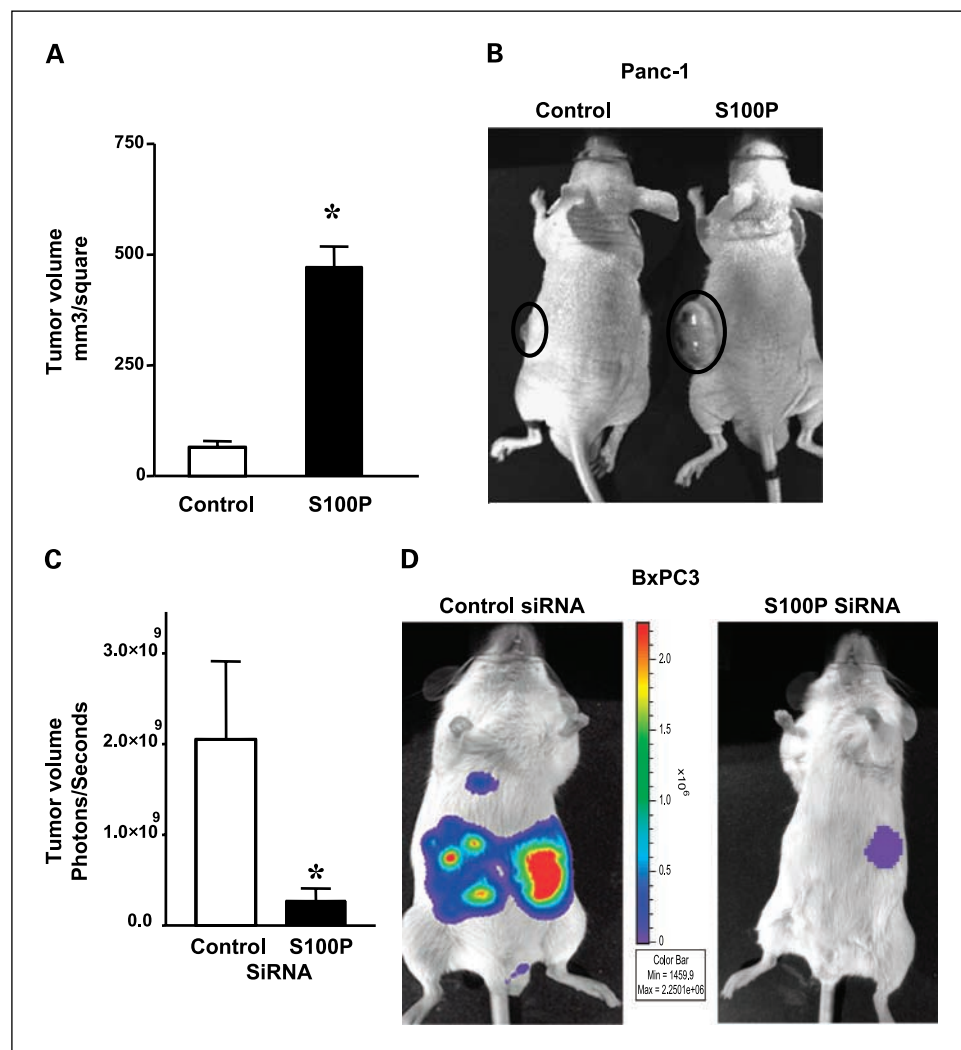


Fig. 5. S100P expression increases and silencing reduces tumor growth and cancer metastasis *in vivo*. The growth of tumors formed from Panc-1 cells stably transfected with control or S100P-expressing vector was analyzed in nude mice. Four-week-old male athymic nude mice *nu/nu* were inoculated s.c. with 1×10^6 cells and maintained in a sterile environment for 4 weeks. At the end of the experiment, tumor was removed and tumor volume was calculated (A). A representative image to indicate tumor size is shown (B). BxPC3 cells stably transfected with control siRNA and S100P siRNA and also expressing the luciferase gene were transplanted orthotopically into 4-week-old male *CB 17 scid* mice. Bioluminescent imaging was utilized to estimate tumor volume and metastasis from six animals in each group (C). A representative image using pseudocolor to indicate tumor size is shown (D). Columns, mean for six animals (* $P < 0.05$); bars, SE.

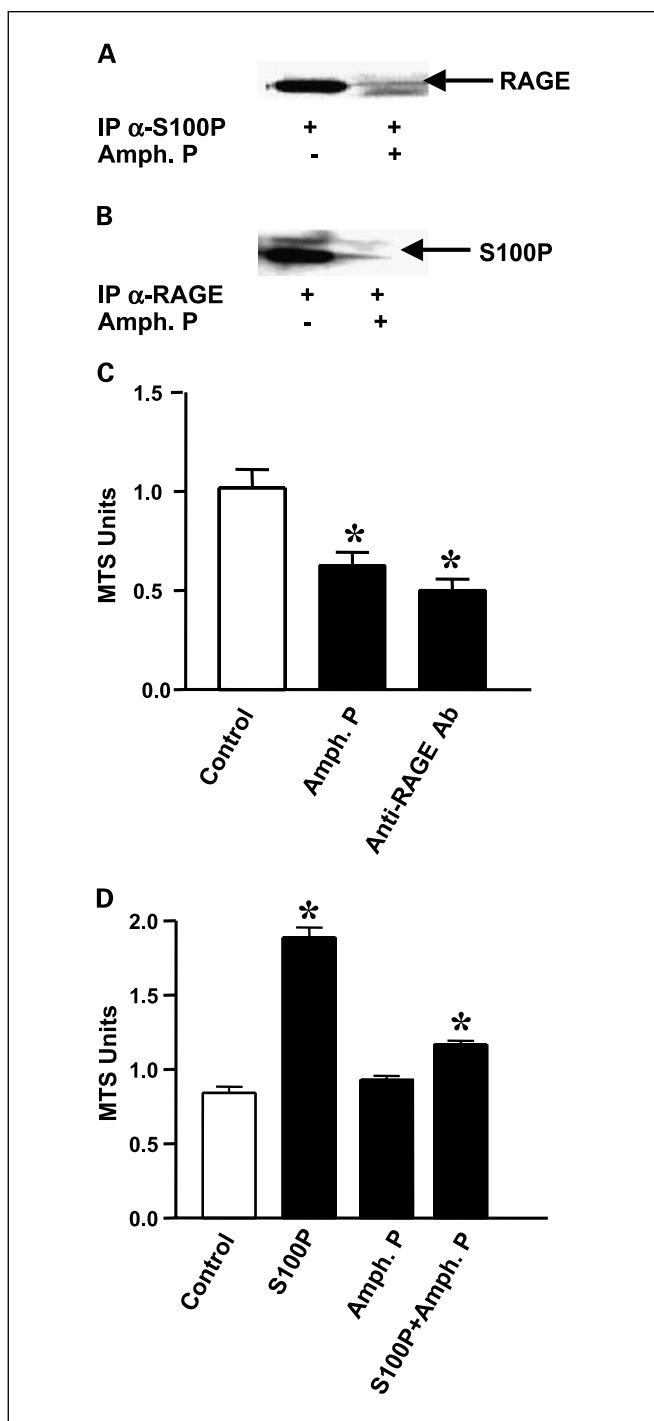


Fig. 6. S100P interacts with and functions through RAGE. *A*, BxPC3 cell lysates were immunoprecipitated (IP) with anti-S100P monoclonal antibodies (IP-S100P) and RAGE was identified in the immunoprecipitates by Western blotting with an anti-RAGE antibody. Addition of an amphoterin-based antagonist peptide previously shown to block amphoterin activation of RAGE (*Amph. P*, 1 μ g) interfered with the interaction between S100P and RAGE. *B*, BxPC3 cell lysates were immunoprecipitated with anti-RAGE antibody and S100P was identified in the immunoprecipitates by Western blotting with an anti-S100P antibody. Addition of an amphoterin-based antagonist peptide (1 μ g) interfered with the interaction between RAGE and S100P. *C*, wild-type BxPC3 cells were treated with an amphoterin-based antagonist peptide (150 μ mol/L) and anti-RAGE antibody (1 μ g) and effects on cell proliferation were analyzed after 48 hours. *D*, wild-type Panc-1 cells were treated with 100 nmol/L S100P with or without an amphoterin-based antagonist peptide (150 μ mol/L) and the effects on cell proliferation were analyzed after 48 hours. Columns, mean for three experiments; bars, SE.

motility and invasiveness. Thus, these data strongly suggest that S100P acts as an autocrine factor that increases the aggressiveness of pancreatic cancer. Of course, S100P is not the only regulator of pancreatic cancer aggressiveness and we found one cancer cell line, Panc-1 cells, which do not express S100P. However, data from our laboratory and others indicated that S100P is expressed in ~90% of pancreatic tumors and the current data support the hypothesis that S100P is an important factor contributing to aggressiveness in the vast majority of cases of pancreatic cancer.

S100P protected pancreatic cancer cells from apoptosis initiated by either a chemotherapeutic agent, 5-FU, or from detachment from the substrate (anoikis). The ability of S100P to inhibit cell death induced by 5-FU supports the hypothesis that S100P may provide pancreatic cancer cells with resistance to therapeutic agents and that interference with S100P function may be a useful approach to improving the effectiveness of therapies. Further studies will be required to test this hypothesis. S100P also protected pancreatic cancer cells from anoikis. This is potentially of great importance, because anoikis is a major limiting factor in cancer metastasis (22). This may help explain the observation that metastasis was reduced by silencing of S100P in an orthotopic pancreatic cancer model.

Metastasis is the leading cause of treatment failure and the most significant predictor of poor clinical outcome in patients with pancreatic cancer. We observed a reduction in metastasis *in vivo* when S100P was silenced. There are several possible explanations for this observation as S100P was found to protect pancreatic cancer cells against anoikis and also to stimulate motility and invasiveness. Taken together, these data strongly suggest that this molecule is important in pancreatic cancer metastasis. Interestingly, S100P was previously found as one of a small number of genes whose expression level correlated with poor outcome in patients with lung cancer (11). Therefore, S100P levels may have prognostic value in pancreatic cancer, but this will need to be further evaluated.

S100 proteins are known to affect cell function by both intracellular and extracellular mechanisms (19). Several lines of evidence suggest that it is likely that the actions of S100P observed in the current study on pancreatic cancer cells were mediated by the ability of S100P to activate RAGE. First, RAGE is ubiquitously expressed, and the presence of RAGE receptors has previously been reported in pancreatic cancer cell lines (23). Second, S100P was secreted from pancreatic cancer cells and, therefore, is available to bind RAGE extracellularly. Third, S100P and RAGE were observed to coimmunoprecipitate in the cell lysates from wild-type pancreatic cancer cells. Fourth, we also observed that inhibitors of RAGE were able to abrogate the effects of S100P on BxPC3 cell proliferation and invasion. RAGE has previously been suggested to stimulate growth, survival, and metastatic spread of other cancers (22–26). However, little is known concerning the function of RAGE in the context of pancreatic cancer. The current data suggest that RAGE activation may be an important mechanism responsible at least in part for the aggressiveness of pancreatic cancer. However, it remains possible that S100P also has additional effects and further studies will be necessary to fully understand S100P actions on pancreatic cancer cells.

In summary, our results show that S100P is an important factor that contributes to the aggressive nature of the majority of pancreatic cancers. S100P is a potent pleiotropic factor that promotes tumor growth and metastasis through the following three mechanisms: (a) direct stimulation of tumor cell growth, (b) stimulation of survival in the face of apoptotic insults, and (c) stimulation of motility and

invasiveness. Thus, development of treatments directed toward S100P may be an important approach for control of tumor growth and metastasis.

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