

Expression and Activity of Src Regulate Interleukin-8 Expression in Pancreatic Adenocarcinoma Cells: Implications for Angiogenesis

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Abstract

Interleukin-8 (IL-8) is an angiogenic factor that promotes growth of pancreatic tumors. The purpose of this study was to determine if c-Src, a protein tyrosine kinase frequently overexpressed in pancreatic cancer, regulated IL-8 expression and to elucidate the Src-mediated signaling pathways that contribute to angiogenesis in pancreatic adenocarcinoma cells. In a panel of pancreatic cancer cell lines, expression of total and activated Src correlated with IL-8 production. Furthermore, ectopic expression of activated Src in PANC-1 cells with low endogenous Src activity significantly increased IL-8 production ($P < 0.005$). In contrast, pharmacologic inhibition of endogenous c-Src kinase activity or small interfering RNA-mediated “knockdown” of c-Src expression in L3.6pl cells with high Src expression and activity caused significant decreases in IL-8 production ($P < 0.005$). Inhibition of c-Src activity resulted in decreased phosphorylation of Akt, p38, and extracellular signal-regulated kinase (Erk)-1/2. Significant ($P < 0.005$) dose-dependent decreases were observed in IL-8 expression by inhibiting Src-dependent signaling molecules Erk-1/2 and p38 but not phosphatidylinositol 3-kinase. To assess the relevance of Src inhibition to angiogenesis, *in vivo* gelfoam assays were done. Robust infiltration of vessels was observed in gelfoam saturated with conditioned medium from pancreatic carcinoma cells. This angiogenesis was nearly abrogated in gelfoams saturated with conditioned medium from cells treated with the Src family kinase inhibitor, PP2 ($P < 0.001$). Thus, c-Src regulates critical “downstream” signaling pathways that contribute to expression of IL-8 in human pancreatic tumor cells, suggesting c-Src may be a target for therapeutic intervention in pancreatic adenocarcinoma. (Cancer Res 2005; 65(16): 7214-22)

Introduction

Adenocarcinoma of the exocrine pancreas is the fourth most common cause of cancer death in developed countries, with ~30,000 deaths each year in the United States alone (1). Unfortunately, only 5% to 10% of patients present with resectable disease (2) and of these patients only 12% survive for 1 year after

diagnosis and <5% survive 5 years (3, 4). Metastases to the lymphatics, liver, and vessel walls lead to widespread disease resulting in a severe wasting condition that accounts for ~80% of deaths in advanced pancreatic cancer (5). Pancreatic tumors are highly vascularized. Contributing to the vascularization and subsequent progression of pancreatic carcinoma is the expression of several proangiogenic factors including basic fibroblast growth factor, vascular endothelial growth factor (VEGF), and interleukin-8 (IL-8; ref. 6).

Strong evidence supports a role for IL-8 expression in tumor cells promoting progression and metastasis. IL-8 is a prognostic factor for ovarian (7), colorectal (8), prostate (9), breast (10), and hepatocellular cancers (11). IL-8 is often overexpressed in surgical specimens of pancreatic cancer tumor tissue (12, 13) and expression correlates with metastatic potential and tumor growth (14–17). Several studies have suggested that IL-8 contributes directly to tumor progression (18–20), as a primary mediator of angiogenesis (12, 17, 18) via its paracrine effects on endothelial cells (21). Shi et al. recently showed that specific up-regulation of IL-8 expression in pancreatic cancer cells resulted in increased tumor growth and metastasis in nude mice, whereas IL-8 antisense expression caused reduced tumor growth and decreased metastasis (15). In addition, reduction of IL-8 expression by protein tyrosine kinase inhibition results in decreased growth and metastasis of human pancreatic cancer cells in an orthotopic model (22). However, signaling pathways responsible for IL-8 expression in pancreatic tumor cells remain to be elucidated.

Several recent studies have focused on IL-8 expression in various normal cells. For example, in response to nontypeable *Haemophilus influenzae*, which characteristically induces inflammatory responses in infected tissues (23), activation of the mitogen-activated protein kinases (MAPK) extracellular signal-regulated kinase (Erk)-1/2 and p38 were required for IL-8 expression (24). In pulmonary epithelial cells, Src family kinases (SFK) were shown to regulate the MAPKs p38 and Erk-1/2 and subsequent IL-8 protein expression (25). Additionally, SFKs were recently shown to regulate IL-8 promoter activation and protein expression in human aortic endothelial cells independently of nuclear factor- κ B (NF- κ B) through activation of the signal transducer and activator of transcription 3 (26). These results strongly implicate Src activity as important in regulation of IL-8 expression in normal cells. Constitutive activation of several signaling pathways in tumor cells is associated with increased IL-8 expression (14, 16, 17, 27), but as yet, Src activation has not been studied.

c-Src is a 60,000-Da nonreceptor protein tyrosine kinase and the prototype of a nine-member family of structurally related SFKs. Regulation of SFKs has been implicated in multiple processes including cell cycle and cell division, cellular adhesion, cell

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doi:10.1158/0008-5472.CAN-04-3858

migration, and angiogenesis. In pancreatic adenocarcinomas, overexpression of Src has been observed in 70% of tumors (28). Recently, inhibition of Src has been shown to inhibit growth of pancreatic tumor cells in an orthotopic nude mouse model (29). Furthermore, c-Src expression and activation have been shown to correlate with resistance to chemotherapeutic agents in nude mouse models of pancreatic cancer, and inhibition of Src was shown to overcome this resistance (30). Thus, understanding the signaling pathways and biological consequences resulting from Src activation in pancreatic adenocarcinomas is likely to lead to a better understanding of progression of these tumors. In this study, we show that Src regulates IL-8 expression, in part, through the signal transduction molecules Erk-1/2 and p38 MAPKs. We further implicate Src activation as important to pancreatic tumor production of proangiogenic factors using an *in vivo* gelfoam assay.

Materials and Methods

Cell lines. The FG, SG, L3.6pl, and PANC-1 pancreatic cancer cell lines were obtained from Dr. Lee Ellis (University of Texas M.D. Anderson Cancer Center). The SG (slow-growing variant) and FG (fast-growing variant) pancreatic cancer cell lines are derivative tumor cell lines established from the cell line COLO 357 (originating from a human pancreatic adenocarcinoma metastasis) injected intrasplenically in the athymic mice, characterized by progressively increasing metastatic potential (31). The L3.6pl cell line was derived from a repeated cycle of injecting COLO 357 cells into the pancreas of nude mice, selecting for liver metastases, and reinjecting into the pancreas (32). The PANC-1 cells were derived from a ductal adenocarcinoma of exocrine pancreas (33). The cells were plated on 10-cm tissue culture dishes, grown as monolayer cultures, and maintained in culture in MEM supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT), 2 mmol/L L-glutamine, and 0.6% penicillin/streptomycin and incubated in 5% CO₂/95% air at 37°C.

Cell lysis and protein extraction. Cells (1×10^6) were plated in 10-cm dishes and maintained in MEM with 10% FBS. At 70% to 80% confluence, the cells were washed with Dulbecco's PBS (D-PBS) at 37°C and maintained in serum-free medium (5 mL) for 1 hour with the desired concentration of inhibitor or an equal volume of DMSO. After 1 hour, cells were washed and replaced with fresh serum-free medium and inhibitor (or equal volume DMSO) for an additional 23 hours. The cells and supernatants were harvested at 24 hours. The cells were washed with ice-cold $1 \times$ D-PBS, scraped from the plates, lysed, and harvested on ice in radioimmunoprecipitation assay (RIPA) B buffer (20 mmol/L sodium phosphate buffer, 150 mmol/L NaCl, 5 mmol/L EDTA, 1% Triton X-100, 0.5% sodium deoxycholate) supplemented with one tablet complete mini-EDTA protease inhibitor cocktail (Roche Diagnostic, Mannheim, Germany) and sodium orthovanadate (1 mmol/L, pH 7.4). The homogenates were clarified by centrifugation at $15,000 \times g$ for 15 minutes at 4°C and prepared for Western analysis and immunoprecipitation.

Transfection. Subconfluent PANC-1 cells (1.0×10^6) were transfected with Tet-On constitutively activated SrcY527F gene expression plasmids or c-src-targeted small interfering RNA (siRNA) expression plasmids using Transfast transfection reagent (Promega Corp., Madison, WI) and 1 µg of DNA at a 3:1 ratio according to the manufacturer's directions. Twenty-four hours following transfection, the cells were rinsed and selected in medium containing 600 µg/mL of G418 (Mediatech, Inc., Herndon, VA). Single colonies of stable transfectants were isolated and expanded for further analysis.

Creation of Tet-on activated Src gene expression plasmids. A tetracycline-controlled gene expression system was selected to achieve high level and quantitative control of Src gene expression. Tet-On expression plasmids were created using the Clontech TET-On system according to manufacturer's directions (BD Biosciences Clontech, Palo Alto, CA). Subconfluent PANC-1 cells (1.0×10^6) were transfected with

0.5 ng of the pTet-On plasmid and grown in selective medium containing G418 (500 µg/mL). Isolated clones were transiently transfected with the pTRE-LUC plasmid and treated with doxycycline (15 µg/mL) to confirm expression of the tet-responsive protein. Positive clones were transfected with the pTRE-reporter plasmid carrying activated SrcY527F and selected by hygromycin (200 µg/mL). Isolated clones were expanded and assayed by Western blot for total avian c-Src (EC10; 1:1,000; Upstate Biological, Lake Placid, NY) expression following the addition of doxycycline for 48 hours.

Creation of small interfering RNA expression plasmids silencing Src gene expression. siRNA expression plasmids were created using the Ambion pSilencer 1.0-U6 (Austin, TX) according to manufacturer's directions. c-src-specific target sequences were designed using the Ambion siRNA web design tool. The two target sequences used were (52-71 bp) 5'-AACAGAGCAAGCCCAAGGAT-3' and (226-244 bp) 5'-AAGCTGTTCG-GAGGCTTCAAC-3'. Oligonucleotides corresponding to these sequences with flanking *Apa*I (5') and *Eco*RI (3') ends were purchased from Invitrogen/Life Technologies (Carlsbad, CA) and ligated into the expression plasmid at compatible sites. Constructs were confirmed by DNA sequencing. L3.6pl cells were then transfected with 0.5 ng of each siRNA plasmid and 10 ng of pcDNA G418 resistance promoterless plasmid for selection of transfectants. Cells were then grown in selective medium containing G418 as previously described (34). Negative controls were transfected with empty vector target sequences and pcDNA plasmids at identical concentrations. Total c-Src expression levels in siRNA clones were determined by Western blot analysis.

Inhibitors. Selective signal transduction reversible protein kinase inhibitors were used in this study: PP2 for the Src Family (Calbiochem, EMD Biosciences, San Diego, CA), LY294002 for phosphatidylinositol 3-kinase (PI3K, Sigma-Aldrich, St. Louis, MO), PD098059 for MAPK kinase (MEK 1, Cell Signaling Technology, Beverly, MA), and SB203580 for p38 MAPK (Calbiochem, EMD Biosciences). All inhibitors were resuspended in DMSO and were treated at concentrations that were nontoxic, as judged by a lack of effect on cell morphology, proliferation rate, or cell cycle status (data not shown).

Immunoblot analyses. Total protein concentrations were determined via the Bio-Rad D_c protein assay protocol followed by spectrophotometric analysis using the TECAN Genios plate reader and Magellan version 4.0 software. Equal amounts of protein (50 µg) were loaded in each well, separated via 8% SDS-PAGE, and electroblotted onto Immobilon-P membranes. The membranes were blocked with Tris-buffered saline/Tween (TBS-T; 0.1%) + 5% dried milk for 30 minutes and probed with desired primary antibody diluted 1:1,000 in blocking buffer overnight at 4°C. Membranes were probed with polyclonal antibodies to phospho-Akt^{S473} (Cell Signaling Technology), phospho-p44/42 Erk^{T202/Y204} (Cell Signaling Technology), total p44/42 Erk MAPK (Ab-2, Oncogene Sciences, Cambridge, MA), phospho-p38^{T180/Y182} (Cell Signaling Technology), total p38 MAPK (Cell Signaling Technology), and monoclonal antibodies to Akt (5G3, Cell Signaling Technology) and vinculin (Sigma-Aldrich). Primary antibody incubation was followed by incubation with a horseradish peroxidase-conjugated secondary antibody (Bio-Rad goat anti-mouse and sheep anti-rabbit) diluted 1:2,000 in blocking buffer for 1 hour at room temperature with gentle rocking. Western blot analyses of actin and vinculin expression were done as a loading control using anti-actin and anti-vinculin monoclonal antibodies (Sigma-Aldrich). Proteins were visualized by incubation with enhanced chemiluminescence detection reagents (Perkin-Elmer, Boston, MS) and exposed to film (Kodak Biomax MR, Rochester, NY). Membranes were stripped and reprobed. Western blot data were quantitated via densitometric analysis (Scion Image software, Scion Corp., Frederick MD) and presented as the means of triplicate determinations (\pm SD).

Immunoprecipitation. For detection of Src phosphorylation on Tyr⁴¹⁸ (indicative of an activated form of Src), 500 µg of the samples in 650 µL RIPA buffer were rotated with 6 µL antibody to total c-Src (monoclonal antibody 327, Oncogene Sciences) overnight at 4°C. Fifty microliters of a 1:1 slurry of protein G agarose in RIPA buffer were added and incubated with rotation for an additional hour at 4°C. Bound proteins were

pelleted by centrifugation, washed thrice with RIPA buffer, and eluted by boiling in 1× Laemmli's sample buffer with subsequent immunoblotting with antibodies against phospho-Src^{Y418} (1:1,000; Cell Signaling Technology) then stripped and re probed with antibody to total Src (1:1,000, Oncogene Sciences).

Animals. Specific, pathogen-free female C3H/HeN mice were purchased from the Animal Production Area of the National Cancer Institute-Frederick Cancer Research and Development Center (Frederick, MD). The mice were housed and maintained in specific pathogen-free conditions. The facilities were approved by the American Association for Accreditation of Laboratory Animal Care and meet all current regulations and standards of the U.S. Department of Agriculture, U.S. Department of Health and Human Services, and the NIH. The mice were used between the ages of 8 to 12 weeks, in accordance with Institutional guidelines.

In vivo angiogenesis assay. The development and optimization of a quantitative *in vivo* angiogenesis assay using gelfoam sponges has been previously described (35). Briefly, gelfoam sponges (Pharmacia and Upjohn, Peapack, NJ) were cut into small sections (~0.5 cm × 0.5 cm²) and placed in a 50 mL tube filled with PBS and kept at 4°C overnight. The following day, the hydrated gelfoam sponges were placed on a sterile piece of filter paper to draw out the PBS and transferred to a sterile dish whereas a 0.8% Life Technologies Ultrapure agarose solution (Life Technologies, Grand Island, NY) was prepared in sterile PBS. The agarose solution was sterile filtered with a 0.22-μm filter. To show the use of this assay for evaluation of an antiangiogenic agent, mice were implanted with gelfoam sponges containing different proangiogenic factors as controls and conditioned medium of pancreatic cancer cells treated with and without selective signal transduction inhibitors and appropriate controls. In sterile microcentrifuge tubes, recombinant IL-8 (10 μg/mL, R&D Systems, Minneapolis, MN) and VEGF (10 μg/mL, R&D Systems) were diluted to 2 μg/mL of each cytokine

in 400 μL serum-free medium. Treated and untreated conditioned medium and appropriate controls were sterile filtered and transferred to sterile centrifuge tubes. An equal volume of the agarose solution was added to each group. The solution was vortexed and 100 μL was added to each gelfoam sponge. The sponges were allowed to solidify at room temperature for ~1 hour and implanted *s.c.* into C3H mice. The gelfoam sponges were harvested at 2 weeks and frozen in ornithine carbamyl transferase compound (Sakura Fineter, Torrance, CA), sectioned, probed for CD31 (PharMingen, San Diego, CA) and stained using immunoperoxidase techniques and diaminobenzidine tetrahydrochloride and hematoxylin as chromagens.

Determination of interleukin-8 levels. Culture supernatants were centrifuged for 1 minute at 15,000 rpm to pellet debris and transferred to microcentrifuge tubes. Supernatants not assayed immediately were frozen at -80°C. Quantitative measurements of IL-8 in the cell supernatants were determined using an ELISA kit (Quantikine Human IL-8 Immunoassay, R&D Systems) following the manufacturer's instructions. The IL-8 antibody used in this ELISA was raised against the proteolytically processed 72-amino-acid biologically active form of IL-8 (36). The detection limit of this ELISA was 37 pg/mL. IL-8 concentrations (pg/mL) were determined by spectrophotometric analysis at 450 nm using a TECAN Genios plate reader and Magellan Version 4.0 software and normalized against total protein levels in the corresponding cell lysate. The results are presented as the means of triplicate determinations (±SD).

Statistical analyses. The significance of differences in IL-8 expression between control cells and treatment groups (Tet-On and siRNA clones or inhibitor treated) were determined using a Student's *t* test (two tailed). The significance of differences in inhibitor-treated versus control samples in the angiogenesis assay was determined using a Mann-Whitney *U* test. *P* < 0.05 was deemed significant.

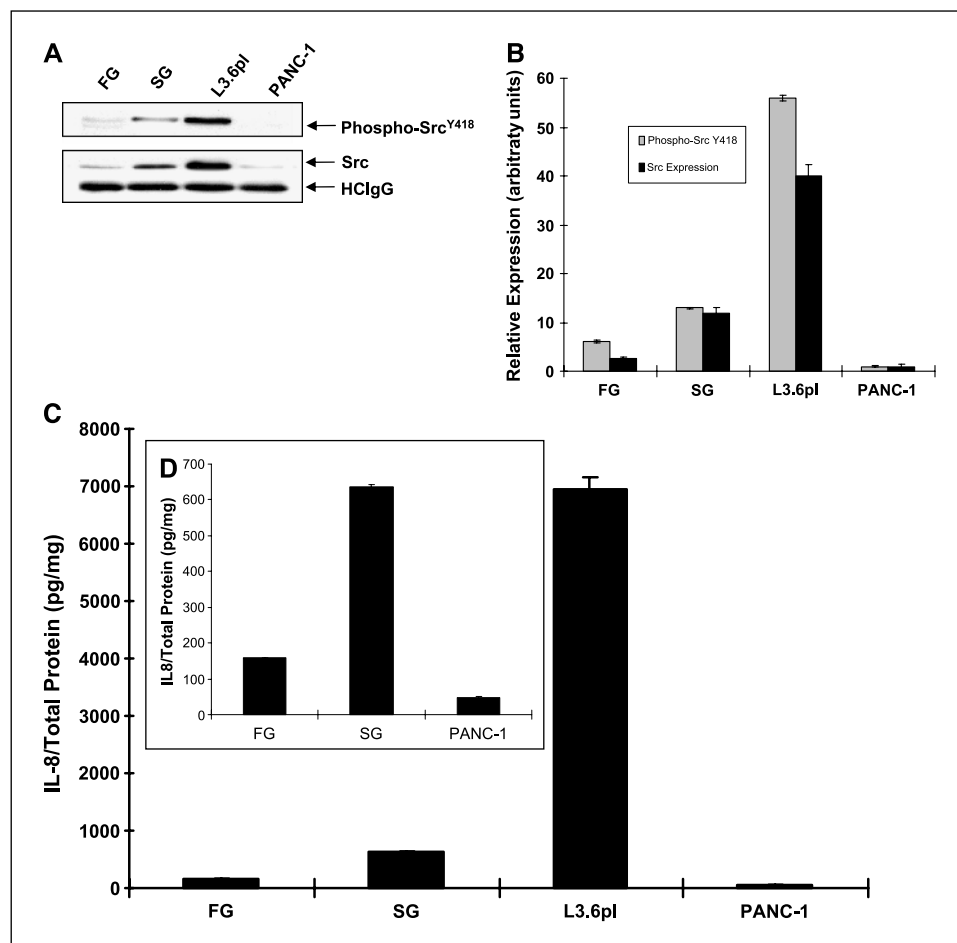


Figure 1. Expression of total Src, Src^{Y418}, and IL-8 in pancreatic adenocarcinoma cell lines. Cells were grown to 70% to 80% confluence then serum starved. After 24 hours, cell lysates and supernatants were collected as described in Materials and Methods. *A*, c-Src activation was determined by immunoprecipitation of total c-Src and subsequent Western blot analysis with anti-phospho-Src^{Y418}. The blots were subsequently stripped and re probed for total c-Src. *B*, quantitation of differences of relative expression of total Src and phospho-Src^{Y418}. Differences are expressed relative to PANC-1 (*n* = 3). *C*, culture supernatants were assayed for IL-8 as described in Materials and Methods. IL-8 levels were expressed as the amount of IL-8 per total cellular protein (pg/mg; *n* = 3). *D*, inset of IL-8 levels from FG, SG, and PANC-1 cells.

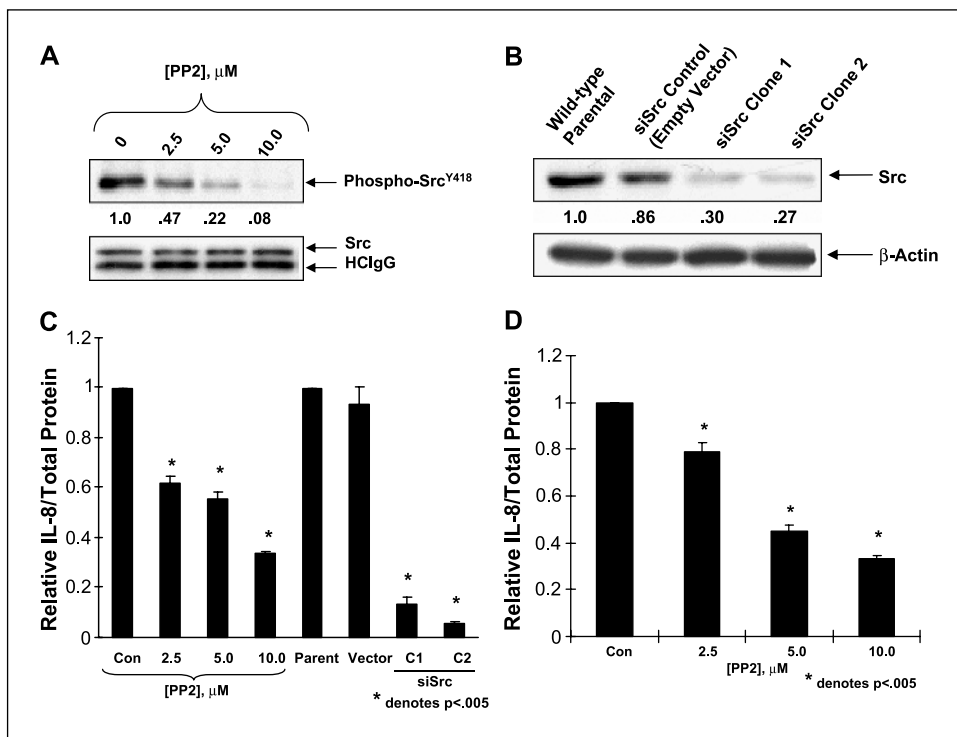


Figure 2. Effects of c-Src inhibition on IL-8 levels in pancreatic cancer cells. *A*, L3.6pl cells were plated and maintained as described in Materials and Methods. Twenty-four hours after plating, the cell culture medium was replaced with serum-free medium containing indicated concentrations of PP2 or DMSO as a control. Cell lysates and cell culture supernatants were harvested after an additional 24 hours. Expression of total and activated Src were determined as described in Fig. 1. Levels of phosphorylated Src were quantitated via scanning densitometry and normalized to total Src expression. Values expressed are relative to DMSO controls ($n = 3$; SDs < 0.01). *B*, stable G418 resistant clones expressing either *c-src*-targeted siRNA (siSrcC1 and siSrcC2) or vector controls were generated from parental L3.6pl cells as described in Materials and Methods. Cells were plated and serum starved and cell lysates and cell culture supernatants were harvested as described above. Membranes were probed for total Src and reprobed for β -actin as a loading control. Total Src levels were quantitated via scanning densitometry and expressed relative to wild-type parental cells ($n = 3$; SDs < 0.05). *C*, IL-8 levels were assayed as described in Materials and Methods. *D*, PANC-1 cells were cultured for 24 hours in serum-free medium containing either DMSO or the indicated concentrations of PP2. Cell lysates and culture supernatants were harvested at the end of this time period, and cell culture supernatants were assayed for IL-8 expression as described in Materials and Methods. The significance of differences in IL-8 expression was determined using Student's *t* test (two tailed). *, $P < 0.005$.

Results

c-Src expression and activation correlates with interleukin-8 protein expression. To determine whether Src activation correlates with IL-8 protein expression, the pancreatic adenocarcinoma cell lines FG, SG, L3.6pl, and PANC-1 were examined for levels of total and activated Src as well as corresponding IL-8 protein levels in cell culture supernatants as described in Materials and Methods. Src activation was determined via Western blot analysis against Y418, the autophosphorylation site in c-Src. PANC-1 cells were the lowest in Src expression and activity (Fig. 1A). Relative to PANC-1, 6.1-, 13-, and 56-fold increases in Src expression and 2.7-, 12-, and 40-fold increases in Src activity were observed in FG, SG, and L3.6pl cells, respectively (Fig. 1B). Expression of IL-8 (Fig. 1C-D) correlated with expression of c-Src. PANC-1 cells expressed the lowest levels of IL-8, as determined by ELISA (pg/mL), with levels increased 3-, 13-, and 140-fold for FG, SG, and L3.6pl respectively. These results suggested that Src activity might be an important contributor to IL-8 expression in pancreatic cancer cell lines.

c-Src is essential for maximum constitutive interleukin-8 expression in L3.6pl cells. To determine directly if c-Src regulated IL-8 expression, two approaches were taken: expression and activity of endogenous c-Src were decreased in L3.6pl cells and expression of activated Src was increased in PANC-1 cells. To decrease Src activity, L3.6pl cells were incubated with increasing

doses of the SFK inhibitor, PP2, for 24 hours, and cell lysates and supernatants were harvested. As seen in Fig. 2, in response to increased PP2 concentrations, autophosphorylation of Src was decreased in a dose-dependent manner, with maximal inhibition detected at 10 μ mol/L (Fig. 2A). The IC₅₀ for inhibition of Src autophosphorylation by PP2 was determined to be 3.75 μ mol/L in these experiments. IL-8 expression in culture supernatants was also significantly decreased in a dose-dependent manner in the presence of PP2. IL-8 levels from cells treated with 10 μ mol/L PP2 were 3.4-fold lower than controls ($P < 0.005$). The IC₅₀ of PP2 for IL-8 production was determined to be 4.4 μ mol/L in these experiments, suggesting that inhibition of Src is proportional to inhibition of IL-8. Interestingly, the importance of c-Src kinase activity for IL-8 production was not dependent on high levels of c-Src expression or activation, as similar results were observed in PANC-1 cells (Fig. 2D), which express much lower levels of c-Src. These results suggest that constitutive IL-8 production in pancreatic cancer cells occurs in a Src-dependent fashion.

To specifically examine the role of c-Src in regulating IL-8 expression, L3.6pl cells were stably transfected with siRNA constructs targeting the *c-src* gene as described in Materials and Methods. As seen in Fig. 2B, clones expressing the siRNA expressed significantly lower levels (4- to 5-fold) of total c-Src than vector controls or parental L3.6pl cells. The reduced expression of c-Src in the siRNA clones did not affect cell viability or growth rates, as

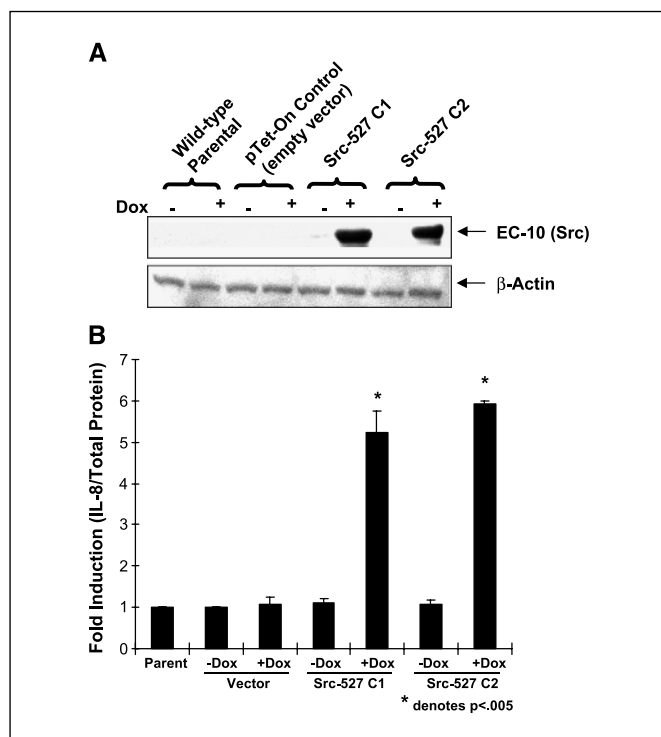


Figure 3. Expression of activated c-Src and effects on IL-8 expression in pancreatic cancer cells. Stable clones expressing either empty vector or constitutively active c-Src (SrcY527F) in the Tet-On system were generated as described in Materials and Methods. Cells were plated and maintained for 48 hours in complete medium with doxycycline (15 μ g/mL). After 48 hours, cell lysates and cell culture supernatants were harvested as described in Materials and Methods. **A**, expression of SrcY527F in the presence and absence of doxycycline was assessed by Western blot analysis using mAb EC10. **B**, IL-8 levels from parental PANC-1 cells or clonal variants expressing empty vector or SrcY527F in the Tet-On system in the presence or absence of doxycycline were determined by ELISA as described in Materials and Methods. Results are shown for one representative vector control and two representative clones expressing SrcY527F (clones 1 and 2; $n = 3$). The significance of differences in IL-8 expression was determined using Student's *t* test (two tailed). *, $P < 0.005$.

determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay.⁴ In these clones, IL-8 levels were significantly reduced (7-fold) in comparison with parental cells and cells expressing vector alone ($P < 0.005$; Fig. 2C). These results are consistent with those obtained by pharmacologic inhibition of SFKs, and taken together, indicate that c-Src is required for maximal constitutive IL-8 production in L3.6pl pancreatic cancer cells.

Induction of Src activity in PANC-1 Tet-on cell line with doxycycline increases interleukin-8 expression. We next determined if overexpression of Src in PANC-1 cells with low levels of endogenous c-Src expression and activation led to increased IL-8 expression. For these experiments, PANC-1 cells were transfected with a plasmid encoding constitutively active avian c-Src (SrcY527F) in a tetracycline-controlled gene expression system (Tet-On). SrcY527F expression was induced in Tet-On cell lines through addition of doxycycline. After 48 hours of incubation with doxycycline and 1% serum, the supernatants and cell lysates were harvested. Western blot analysis using EC10, specific for avian c-Src, indicated successful induction of SrcY527F expression in the presence of doxycycline (Fig. 3A). IL-8 levels were determined by

ELISA as described in Materials and Methods. SrcY527F induction resulted in a 5- to 6-fold increase in IL-8 protein levels in PANC-1 cells compared with vector controls and parental PANC-1 cells ($P < 0.005$; Fig. 3B). These results further show that Src activity regulates IL-8 expression.

Effects of Src small interfering RNA on extracellular signal-regulated kinase-1/2, p38 mitogen-activated protein kinase, and Akt kinase activity. To examine the signaling pathways required for Src-mediated IL-8 expression, the effect of Src inhibition on signaling intermediates important to IL-8 production in other systems was determined. Several previous studies have shown that Src activity regulates members of the MAPK family as well as PI3K/Akt. Therefore, the phosphorylation of p44/42 Erk, p38 MAPK, and Akt was determined from cell lines expressing c-src-targeted siRNA. As seen in Fig. 4, decreases in Akt, Erk-1/2, and p38 phosphorylation were observed in the Src siRNA clones, relative to parental L3.6pl cells and vector controls, with no change in expression of these signaling enzymes (data not shown). Similar results were seen upon SFK inhibition with PP2 (data not shown). Concomitant increases in Erk-1/2, p38 MAPK, and Akt phosphorylation were observed in PANC-1 Tet-On cell lines upon induction of SrcY527F expression with doxycycline (data not shown). These data implicate c-Src as an upstream regulator of the Erk-1/2, p38 MAPK, and PI3K/Akt signaling pathways in pancreatic adenocarcinoma cell lines.

Inhibition of mitogen-activated protein kinases inhibits expression of interleukin-8 in pancreatic adenocarcinoma cell lines. Having established that Src regulates the Erk-1/2, p38 MAPK, and PI3K/Akt pathways, we next determined the importance of these signaling intermediates for IL-8 expression. For these studies, the effect of selective inhibitors on IL-8 expression was determined. The Erk-1/2 pathway was inhibited with PD098059, an inhibitor of the upstream kinase MEK 1. The p38 MAPK pathway was inhibited with SB203580, a direct inhibitor of p38 MAPK. The PI3K/Akt pathway was inhibited with LY294002, an inhibitor of PI3K. Inhibition of the Erk-1/2, p38 MAPK, and PI3K/Akt pathways was

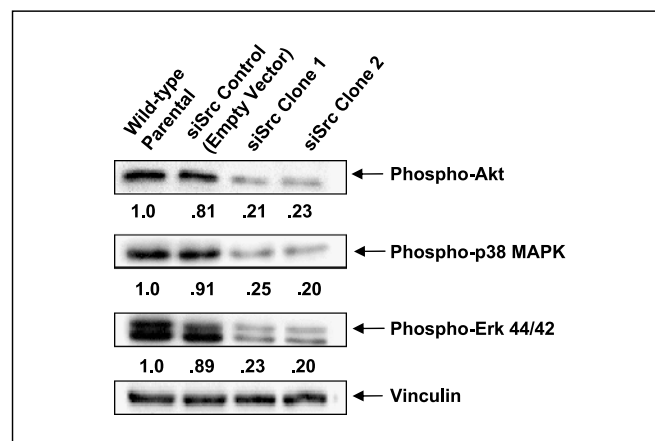


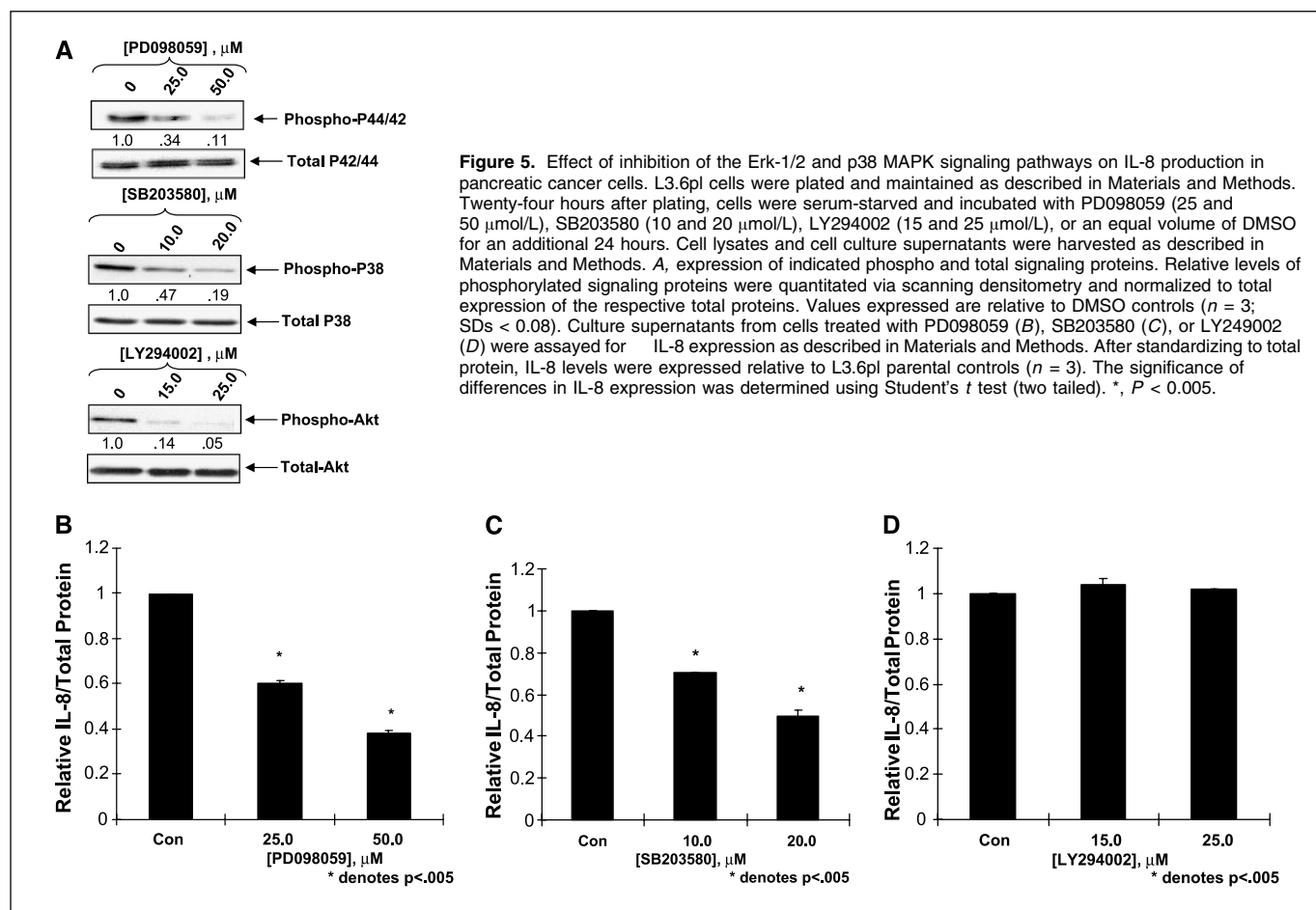
Figure 4. Effects of c-Src knockdown on downstream signaling pathways. Parental L3.6pl cells, or clonal variants expressing empty vector or c-src-targeted siRNA (siSrc clones 1 and 2) were plated and maintained as described in Fig. 1. Twenty-four hours after plating, cells were serum-starved for 24 hours, and cell lysates were harvested as described in Materials and Methods. Expression of phosphorylated Akt, p38, and Erk-1/2 was determined by Western blot analysis. Levels of phosphorylated signaling proteins were quantitated via scanning densitometry and normalized to total expression of the respective proteins. Values expressed are relative to wild-type parental cells ($n = 3$; SDs < 0.05). Anti-vinculin Western blot analysis was done as a loading control.

⁴ J.G. Trevino et al., submitted for publication.

confirmed by Western blot analysis of inhibited cell lysates using antibodies directed against phospho-Erk-1/2, phospho-p38, and phospho-Akt, respectively. The minimum concentrations of each inhibitor necessary to achieve maximum inhibition of the target enzymes were used in these experiments. The inhibitors did not induce cytotoxic effects over the time course of the experiments, as determined by cell cycle analysis (data not shown). As seen in Fig. 5A, the pharmacologic inhibitors induced dose-dependent decreases in activation of their respective target pathways. Both PD098059 and SB203580 induced significant ($P < 0.005$) dose-dependent reductions in IL-8 production (2.6- and 2.1-fold, respectively, at maximal concentrations; Fig. 5B and C). Inhibition of the PI3K/Akt pathway by LY294002, however, did not affect IL-8 production at either concentration tested (Fig. 5D), despite nearly complete inhibition of Akt phosphorylation (Fig. 5A). Similar results were observed in the PANC-1 cell line (data not shown). These results indicate that both the Erk-1/2 and p38 MAPK pathways regulate IL-8 production in L3.6pl cells, whereas the PI3K/Akt pathway does not. Thus, c-Src may regulate IL-8 production in pancreatic cancer cells, in part through activation of the Erk-1/2 and p38 MAPK pathways.

Src family kinases regulate angiogenesis promoted by pancreatic adenocarcinoma cell lines. The results presented above indicate that Src plays an important role in the production of the proangiogenic molecule IL-8 in pancreatic cancer cells. We next sought to determine if Src inhibition could block the ability of cell culture supernatants from these cells to induce angiogen-

esis *in vivo*. Gelfoam sponges were saturated with a solution of 0.4% agarose and either serum-free medium; serum-free medium with 1 $\mu\text{g}/\text{mL}$ recombinant IL-8 (rIL-8); serum-free medium with 1 $\mu\text{g}/\text{mL}$ each of recombinant IL-8 (rIL-8) and recombinant VEGF (rVEGF); serum-free medium with VEGF, IL-8, and 10 $\mu\text{mol}/\text{L}$ PP2; conditioned medium from control L3.6pl cells; or conditioned medium from L3.6pl cells treated with 10 $\mu\text{mol}/\text{L}$ PP2. The sponges were implanted *s.c.* and harvested 14 days later for analysis. The sponges were frozen, and sections were stained for CD31. Representative fields of CD31⁺ cells at 200 \times magnification are shown in Fig. 6. Vessel counts, as determined by CD31⁺ staining, are summarized in Table 1. The number of CD31⁺ cells (staining brown) in 10 fields of 0.159 $\text{mm}^2 \times 100$ was determined. L3.6pl conditioned medium induced maximal angiogenesis with a median of 53 and range of 31 to 73. Angiogenesis into the gelfoam was nearly abrogated in the Src-inhibited L3.6pl conditioned medium, which was no better at supporting angiogenesis than serum-free medium alone. Previously determined optimal concentrations of rIL-8 (35), alone or in the presence of rVEGF, with or without PP2, induced robust angiogenesis (median vessel density, 43). No significant differences were discernable among treatments that supported angiogenesis (L3.6pl conditioned medium or serum-free medium supplemented with proangiogenic factors; $P < 0.01$). These results show that residual PP2 in the medium does not act directly on endothelial cells to disrupt angiogenesis but rather blocks the production of angiogenic factors from the pancreatic tumor cells.



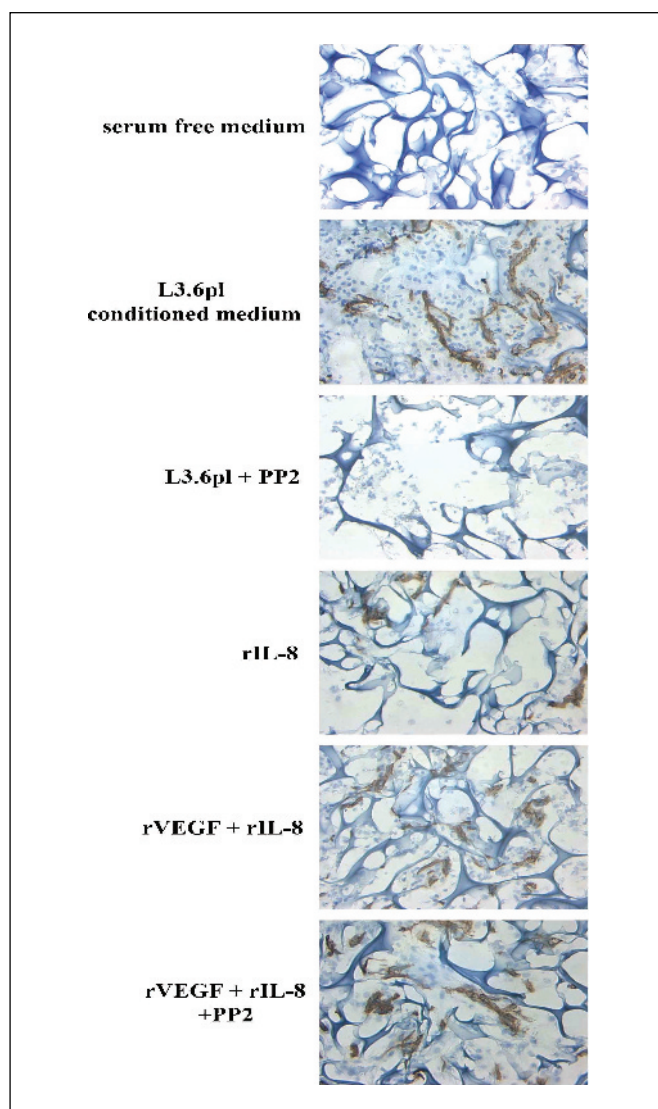


Figure 6. Endothelial cell staining in gelfoam. L3.6pl cells were plated and maintained as described in Materials and Methods. Twenty-four hours after plating, the cell culture medium was replaced with serum-free medium containing either 10.0 $\mu\text{mol/L}$ PP2 or an equal volume of DMSO. Cell culture supernatants were harvested after an additional 24 hours. Gelfoam sponges were saturated with serum-free medium, L3.6pl conditioned medium, L3.6pl conditioned medium from PP2-treated cells, serum-free medium with recombinant IL-8 (2 $\mu\text{g/mL}$), recombinant VEGF and IL-8 (2 $\mu\text{g/mL}$), or recombinant VEGF and IL-8 plus 10 $\mu\text{mol/L}$ PP2. Gelfoams were implanted s.c. into C3H/HeN mice as described in Materials and Methods. Gelfoams were harvested 2 weeks after implantation, sectioned, and stained for CD31 as described in Materials and Methods.

Discussion

Despite aggressive treatment of pancreatic adenocarcinoma, prognosis remains poor, in part due to most patients presenting with untreatable metastatic disease. Thus, new therapeutic strategies are needed to inhibit continued growth and spread of pancreatic carcinomas. Recent success in chronic myelogenous leukemia with inhibitors that target Src (37), coupled with increased evidence that Src activation is important in pancreatic tumor progression and resistance to therapy, suggest that Src may be an important therapeutic target (30). Src regulates diverse biological properties, including secretion of proangiogenic factors (38). As tumors often secrete multiple proangiogenic molecules, an

understanding of the signaling pathways that regulate them may lead to new targets for therapeutic development.

Most pancreatic cancer cells constitutively express IL-8, and its expression correlates with angiogenic potential and prognosis (14, 16, 17, 27). Whereas the transcriptional activators of IL-8 are well understood, the signaling pathways leading to IL-8 expression in pancreatic cancer remain unclear. The work presented in this study shows for the first time that c-Src activation is a critical mediator of IL-8 expression in pancreatic tumor cells and that c-Src inhibition results in a biologically significant reduction in IL-8 secretion. Both endogenous and ectopic c-Src expression and activity correlate with IL-8 protein expression, and inhibition of c-Src expression via siRNA or activity via the pharmacologic inhibitor PP2 significantly reduce IL-8 levels in cell culture supernatants. These data are consistent with studies in normal cell systems that show that Src mediates IL-8 expression downstream of diverse stimuli through many of the same signaling molecules used to regulate IL-8 expression in pancreatic cancer cells (24, 26, 39). Our data suggest that constitutive activation of Src in pancreatic tumor cells deregulates these pathways and leads to constitutive IL-8 expression and further that the extent of Src activation correlates with the amount of IL-8 produced.

In this study, we show an apparent requirement of Erk-1/2 and p38 MAPKs and a corresponding dispensability of PI3K/Akt activity for IL-8 expression in pancreatic cancer cells. The relative importance of these kinase pathways for IL-8 expression was determined via the use of selective kinase inhibitors targeting the activities of these enzymes, or in the case of Erk-1/2, the upstream kinases that directly regulate their activity. It should be noted that whereas no small molecule inhibitor is absolutely specific exclusively for its intended target, in these studies, the inhibitors were used at the minimum concentrations that provided maximum inhibition of the intended targets in our system. These concentrations used here are similar to other studies in which these three inhibitors were used (40) and did not induce any apparent cytotoxic

Table 1. Vessel density in gelfoam induced by L3.6pl-conditioned media in the presence and absence of Src inhibition

Gelfoam/0.4% agarose with:	Vessel density (CD31 ⁺), median (range)
Serum-free medium	5 (0-30)
L3.6pl conditioned medium	53* (31-73)
L3.6pl + PP2	6 [†] (0-33)
rIL-8	48* (16-99)
rVEGF + rIL-8	41* (31-104)
rVEGF + IL-8 + PP2	39* (20-110)

NOTE: Gelfoam sponges containing different proangiogenic conditions were implanted s.c. in C3H/HeN mice. The sponges were harvested on day 14 and the number of CD31⁺ cells in 10 fields of 0.159 $\text{mm}^2 \times 100$ was determined.

*No significant differences were discernable among sponges containing proangiogenic conditions ($P < 0.01$).

[†]Treatment with PP2 significantly inhibited angiogenesis induced by L3.6pl conditioned medium ($P < 0.001$, Mann-Whitney U test).

effects. Additionally, these results are supported by several studies implicating these kinases in the regulation of IL-8 expression in other systems. Erk-1/2 are well-established activators of transcriptional factors regulating proliferation, differentiation, and migration of both normal and tumor cells and have been shown to regulate IL-8 expression through activation of transcription factors such as activator protein and NF- κ B (41–43). p38 MAPK, primarily activated during cellular “stress,” has been shown, through several recent studies, to play a multifaceted role in mediating IL-8 expression, both through activation of transcription factors and posttranscriptional stabilization of the IL-8 mRNA (17, 44–46).

In these studies, inhibition of PI3K/Akt did not affect IL-8 expression, suggesting that this pathway may not contribute to IL-8 expression in pancreatic tumor cells. Similar results have been reported recently in other systems (24, 45, 47). Inhibition of PI3K in human glioblastoma cells reduces IL-8 expression through decreased NF- κ B activity (46). Le et al. showed that IL-8 expression is driven by constitutive NF- κ B activity in pancreatic cancer cells (14). The overexpression and constitutive activity of NF- κ B in pancreatic tumor cells (48) may thus limit the effectiveness of inhibiting upstream components of this pathway, such as PI3K/Akt.

Finally, in these studies, we have also shown that Src inhibition resulted in a significant decrease in the ability of cell culture supernatants from L3.6pl cells to support angiogenesis *in vivo*, through use of the gelfoam assay. It should be noted that the inability of Src-inhibited cell culture supernatants to support *in vivo* angiogenesis is not due exclusively to reduced IL-8 levels. Work from this laboratory and others have shown that Src activation also leads to increased expression of the proangiogenic factor, VEGF (38, 49, 50). We have shown further that c-Src plays

an important role in both constitutive and epidermal growth factor–induced VEGF expression in pancreatic cells.⁵ C-Src may represent a central mediator of the expression of multiple proangiogenic molecules in pancreatic adenocarcinoma cells thus emphasizing the importance of Src for pancreatic cancer angiogenesis.

Recently, several small molecule inhibitors of c-Src have been developed and are in various stages of preclinical and clinical trials. Duxbury et al. recently showed that Src activity mediates gemcitabine chemoresistance in pancreatic cancer cells (30). These data, coupled with the data presented here, suggest that c-Src may represent an important candidate for targeted therapy in pancreatic cancer. C-Src inhibition may serve the dual function of increasing the sensitivity of pancreatic tumors to established chemotherapeutic agents and inhibiting the ability of these tumors to induce angiogenesis, a crucial step for the progression and subsequent metastasis of pancreatic tumors.

Acknowledgments

Received 10/26/2004; revised 4/12/2005; accepted 5/24/2005.

Grant support: NIH grants 2R01 CA65527 (G.E. Gallick), U54 CA 090810 (G.E. Gallick), and T32 CA 09599 (J.G. Trevino); The Lockton Fund (G.E. Gallick); Eleanor B. Pillsbury Fellowship, University of Illinois Hospital (J.G. Trevino); and Lockton Fellowship for Pancreatic Cancer Research (M.J. Gray).

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We thank Nila U. Parikh and Marjorie Johnson for excellent technical support.

⁵ J.M. Summy et al. *Pancreas*. In press, 2005.

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Expression and Activity of Src Regulate Interleukin-8 Expression in Pancreatic Adenocarcinoma Cells: Implications for Angiogenesis

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Cancer Res 2005;65:7214-7222.

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