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Inhibition of Transforming Growth Factor β Signaling Reduces Pancreatic Adenocarcinoma Growth and Invasiveness[S]

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ABSTRACT

Transforming growth factor β (TGFβ) is a pleiotropic factor that regulates cell proliferation, angiogenesis, metastasis, and immune suppression. Dysregulation of the TGFβ pathway in tumor cells often leads to resistance to the antiproliferative effects of TGFβ while supporting other cellular processes that promote tumor invasiveness and growth. In the present study, SD-208, a 2,4-disubstituted pteridine, ATP-competitive inhibitor of the TGFβ receptor I kinase (TGFβRI), was used to inhibit cellular activities and tumor progression of PANC-1, a human pancreatic tumor line. SD-208 blocked TGFβ-dependent Smad2 phosphorylation and expression of TGFβ-inducible proteins in cell culture. cDNA microarray analysis and functional gene clustering identified groups of TGFβ-regulated genes involved in metastasis, angiogenesis, cell proliferation, survival, and apoptosis. These gene responses were inhibited by SD-208. Using a Boyden chamber motility assay, we demonstrated that SD-208 inhibited TGFβ-stimulated invasion in vitro. An orthotopic xenograft mouse model revealed that SD-208 reduced primary tumor growth and decreased the incidence of metastasis in vivo. Our findings suggest mechanisms through which TGFβ signaling may promote tumor progression in pancreatic adenocarcinoma. Moreover, they suggest that inhibition of TGFβRI with a small-molecule inhibitor may be effective as a therapeutic approach to treat human pancreatic cancer.

Pancreatic cancer is the fifth leading cause of cancer-related deaths, resulting in approximately 31,000 deaths annually in the United States alone (Jemal et al., 2006). It is a highly metastatic cancer with an average survival of 3 to 8 months after diagnosis. Because of the aggressiveness of the cancer, difficulties in diagnosis, and lack of effective treatment, only 5% of patients who receive a diagnosis of pancreatic cancer survive longer than 5 years (Jemal et al., 2006). The current treatment, gemcitabine, confers only a modest survival advantage when used as a stand-alone treatment or in combination with other therapies (Eckel et al., 2006).

A number of genetic and epigenetic alterations have been identified in pancreatic cancer. The most common are mutations that affect the activity or expression of K-*ras*, *p15*, *p16*, *p53*, and *DPC4*/Smad4. Activating point mutations in the K-*ras* oncogene are believed to occur early in progression to neoplasia (Hruban et al., 2000) and are found in 85 to 95% of pancreatic cancers (Friess et al., 1999). Mutations in cell cycle inhibitor genes *p15* and *p16* are found at a frequency of approximately 60 and 80%, respectively (Naumann et al., 1996; Villanueva et al., 1998). Aberrations in *p53* and *DPC4*/Smad4 are believed to occur late in tumor progression (Hruban et al., 2000) and are found in approximately half of pancreatic cancers (Schutte et al., 1996; Friess et al., 1999).

Deletion of *DPC4*/Smad4, a key mediator of TGFβ signaling, has been associated with abnormal growth arrest by TGFβ (Yasutome et al., 2005). In addition to altered Smad4 expression, signaling from the Smad pathway may be disrupted by mutations that affect the expression of TGFβ receptors, Smad6, Smad7, and downstream genes (Friess et al., 1999). TGFβ signals through the Smad pathway and through

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ABBREVIATIONS: TGFβ, transforming growth factor β; SMAD, mothers against dipeptidyl peptidase homolog; ECM, extracellular matrix; RT-PCR, reverse transcription-polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; CTGF, connective tissue growth factor; PAI-1, plasminogen activator inhibitor, type 1; DMEM, Dulbecco's modified Eagle's medium; EMT, epithelial-to-mesenchymal transition; SD-208, 2-[(5-chloro-2-fluorophenyl)pteridin-4-yl]pyridin-4-yl-amine; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH_2-terminal kinase; MEK, mitogen-activated protein kinase kinase; DMSO, dimethyl sulfoxide; HRP, horseradish peroxidase; VEGF, vascular endothelial growth factor; IGF2, insulin-like growth factor 2; SB-431542, 4-(5-benzol[1,3]dioxol-5-yl-4-pyrldin-2-yl-1*H*-imidazol-2-yl)-benzamide hydrate.

Smad-independent pathways. Smad signaling is initiated upon binding of TGFβ to a type II receptor (TGFβRII) followed by recruitment and transphosphorylation of TGFβRI (Heldin et al., 1997). Activated TGFβRI then phosphorylates regulatory Smads, Smad2 and Smad3. Once phosphorylated, Smad2 and Smad3 form a complex with Smad4 and translocate to the nucleus, where they activate the transcription of TGFβ-responsive genes. TGFβ signaling through the Smad pathway is tightly controlled by negative feedback loops involving Smad6 and Smad7. TGFβ can also signal through mitogen-activated protein kinase (ERK, JNK, p38) cascades and the phosphatidylinositol-3 kinase pathway (Derynck and Zhang, 2003; Elliott and Blobe, 2005). Cross-talk between these pathways and the TGFβ pathway coordinates proliferation, survival signals, and other signals.

In normal epithelial cells, TGFβ acts as a tumor suppressor, mediating growth arrest through down-regulation of c-Myc, and through transcriptional activation of cell cycle inhibitors p15 and $p21^{Cip1/WAF1}$ (Grau et al., 1997; Donovan and Slingerland, 2000; Adhikary and Eilers, 2005). In addition to regulating survival and proliferation, TGFβ signaling promotes angiogenesis, fibrosis, metastasis, and immune suppression (Elliott and Blobe, 2005). Alterations that affect the expression or activity of components of the TGFβ pathway can render cells insensitive to TGFβ-mediated growth arrest while enabling other responses that support tumor progression (Dumont et al., 2003; Nicolás and Hill, 2003). During neoplastic conversion, autocrine expression of TGFβ is believed to promote tumorigenesis. The cellular response is also influenced by other dysregulated pathways such as the Ras-MEK-ERK-signaling cascade (Ellenrieder et al., 2001) and by stromal cell interactions, growth factors, and cytokines in the tumor cell microenvironment.

The pivotal role of TGFβ in promoting cellular processes that are important for tumor progression suggests that the pathway may be a good target for therapy. In this study, we investigated whether SD-208, a small-molecule inhibitor of TGFβRI, can inhibit tumor progression in pancreatic cancer. We used PANC-1, a human pancreatic ductal carcinoma that harbors genetic alterations (K-*ras*, *p15*, *p16*, and *p53*) commonly found in pancreatic cancer (Villanueva et al., 1998; Moore et al., 2001). PANC-1 has also been reported to have altered TGFβRI and Smad7 expression (Nicolás and Hill, 2003). It is an attractive model for the human disease because PANC-1 tumors are metastatic when grown orthotopically in nu/nu (nude) mice. Furthermore, PANC-1 secretes TGFβ, which is believed to promote tumor progression and desmoplasia in human pancreatic cancer. Our studies reveal that SD-208 abrogates TGFβ-mediated gene responses that may facilitate tumor growth and metastasis. We also demonstrate for the first time that a small-molecule inhibitor of TGFβRI attenuates growth and metastasis of established tumors in an orthotopic xenograft model of pancreatic adenocarcinoma.

Materials and Methods

Reagents. Recombinant human TGFβ was purchased from R&D Systems (Minneapolis, MN). TGFβ1 and VEGF ELISA kits were from Biosource International (Camarillo, CA). The TGFβ2 ELISA was from R&D Systems. The PAI-1 ELISA was from American Diagnostica, Inc. (Stamford, CT). The rabbit polyclonal antibody for phospho-Smad2 (Ser465/467) was from Cell Signaling Technology (Danvers, MA). The mouse monoclonal antibody against vimentin was from Affinity Bioreagents (Golden, CO). HRP-conjugated donkey anti-rabbit secondary antibody was from Amersham (Pittsburgh, PA), and HRP-conjugated goat anti-mouse secondary antibody was from Santa Cruz Biotechnology (Santa Cruz, CA).

CTGF ELISA. CTGF-specific polyclonal antibodies, which were generated using peptides derived from the C terminus of the protein, were absorbed on a high-binding ELISA plate. After blocking, CTGF standards and cell-culture supernatants were added and incubated overnight at 4°C. Bound CTGF was detected via its heparin-binding site by incubation with biotinylated-heparin (Sigma-Aldrich, St. Louis, MO) and streptavidin-HRP (Chemicon International, Temecula, CA). Quantitation of bound CTGF was extrapolated from a standard curve generated with recombinant human CTGF.

Cell Culture and Inhibitor Treatment. Human pancreatic cancer cell lines PANC-1 (CRL-1469) and BxPC-3 (CRL-1687) were acquired from American Type Culture Collection (Manassas, VA). PANC-1 was cultured in Dulbecco's modified Eagle's medium (DMEM) (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum. BxPC-3 was cultured in RPMI 1640 (Mediatech) supplemented with 10% fetal bovine serum. TGFβRI kinase inhibitor SD-208 (Scios, Inc., Fremont, CA) was dissolved in DMSO (1000× stock). SD-208 has an IC_{50} value of 49 nM when measured by direct enzymatic assay of TGFβRI kinase activity in vitro. It is 100-fold less specific for TGFβRII and is more than 17-fold less specific for related kinases (Kapoun et al., 2006).

Construction of PANC-1 Luciferase Cells. For constitutive expression of luciferase and the Zeocin-resistance gene, PANC-1 were cotransfected with pGL-3 (Promega, Madison, WI) and pSV40-Zeo (Invitrogen, Carlsbad, CA) using FuGENE transfection reagent (Roche Applied Science, Alameda, CA). After selection with Zeocin (Invitrogen), a clone with stable luciferase expression and normal growth characteristics was selected for studies.

Phospho-Smad2 Analysis. Cells were seeded in six-well plates at 2×10^5 cells/well and cultured in serum-containing medium. The next day, they were treated with SD-208 (31.25–1000 nM) for 15 min before the addition of TGFβ1 (2 ng/ml). After 65 min, cell lysates were prepared and analyzed by Western blot as described previously (Kapoun et al., 2006).

Measurement of Secreted Proteins. PANC-1 cells were seeded in six-well plates at 3×10^5 cells/well and cultured in serum-containing medium. The next day, medium was changed to serum-free DMEM containing 1× Insulin-Transferrin-Selenium (Gibco) and 0.2% bovine serum albumin. The medium also contained combinations of the following treatments: 0.1% DMSO vehicle control, 400 nM SD-208, and 5 ng/ml TGFβ1. After 24 h, cell supernatants were collected and assayed for TGFβ, VEGF, CTGF, and PAI-1 by ELISA. Cells were harvested in MPER buffer (Pierce, Rockford, IL), and protein was quantitated by bicinchoninic acid assay (Pierce). Concentrations of secreted proteins were determined by ELISA and normalized to the total cell protein.

Gene Expression Analysis. PANC-1 cells were seeded in DMEM containing 10% serum and grown to ~70% confluence. The next day, cells were treated in complete medium with vehicle (0.1% DMSO), 400 nM SD-208, 2 ng/ml TGFβ1, or a combination of TGFβ1 and SD-208 for 24 h. Total RNA was extracted from cells using an RNeasy kit (QIAGEN, Valencia, CA).

Real-time RT-PCR and cDNA microarray analysis were performed as described by Kapoun et al. (2006). Sequences of primers and probes for real-time RT-PCR can be found in Table 1.

Invasion Assays. Cell invasion was analyzed in 24-well Matrigel-coated invasion chambers (BD Biosciences Discovery Labware, Bedford, MA) according to manufacturer's directions with the following modifications: hydrated chambers were transferred to a new 24-well plate containing DMEM with 10% serum plus TGFβ1 (2 ng/ml) and/or SD-208 (1 μM) or DMSO (0.1%). Cells (5×10^5) were added to each chamber and incubated for 20 h. Cells that did not pass through

the filter were removed with a cotton swab before processing the filter with a Hema-3 staining kit (Fisher, Pasadena, CA) and mounting the filter on a microscope slide. Cells in five fields on the filter were photographed at 40× magnification and counted manually for each treatment.

Tumor Implantation and Imaging. All of the animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee at Scios. Tumors from PANC-1 luciferase were grown subcutaneously in SCID mice to generate trocar fragments. For the orthotopic implantation, 6- to 8-week-old male nude mice were anesthetized with ketamine/xylazine cocktail before implanting one piece of tumor fragment in the tail region of the pancreas using 6-0 vinyl sutures. Tumor growth was monitored weekly with the Xenogen Living Image System (Alameda, CA). When tumors were palpable (at day 10 after implantation), animals were randomized and assigned to vehicle and treatment groups ($n = 12$) based on the intensity of luminescence. SD-208 was administered orally at 20 or 60 mg/kg twice daily. At study termination (day 56), animals were sacrificed, and primary tumors were resected and weighed. Organs harboring metastatic lesions were manually visualized and counted. Metastatic lesions were confirmed with ex vivo Xenogen imaging.

Statistical Analysis. Statistical analysis was performed using one-way analysis of variance with Bonferroni correction using Prism version 4.02 (GraphPad Software Inc., San Diego, CA) unless otherwise stated.

Results

Inhibition of TGFβRI Reduces Smad Signaling and Levels of Secreted TGFβ-Inducible Proteins. We observed previously that SD-208 blocks signaling from TGFβRI in fibroblasts (Kapoun et al., 2006), smooth muscle cells, and various tumor cell lines (Uhl et al., 2004; N. Gaspar and G. Li, unpublished data). To assess the potency of SD-208 on TGFβRI inhibition in PANC-1 cells, we measured inhibition of Smad2 phosphorylation by SD-208 after induction with TGFβ. Maximum phosphorylation of Smad2 occurred at approximately 1 h after induction with TGFβ1 (data not shown). Pretreatment of cells with SD-208 before TGFβ1 induction inhibited phosphorylation in a dose-dependent manner (Fig. 1). IC_{50} was achieved at 62.5 to 125 nM SD-208. To confirm inhibition of TGFβ signaling, downstream events were measured. These included production of CTGF, PAI-1, and VEGF, as well as TGFβ1 and TGFβ2, which are regulated in an autocrine manner by TGFβ1. Levels of the secreted proteins increased after TGFβ treatment except when cells were cotreated with SD-208 (Fig. 2). Similar profiles were observed when we analyzed Smad phosphorylation and levels of secreted proteins from luciferase-expressing cells, indicating that introduction of the gene did not affect TGFβ signaling (data not shown). In agreement with a previous report (Uhl et al., 2004), these results demonstrate that SD-208 inhibits signaling from the TGFβ receptor complex. Furthermore, they demonstrate that SD-208 inhibits expression of proteins that regulate matrix remodeling and angiogenesis.

Inhibition of TGFβRI Attenuates Gene Responses Involved in Tumor Processes. As a first step toward understanding the TGFβ-dependent processes important for tumor progression, we assessed the regulation of gene expression in vitro by TGFβ. We used gene array analysis of PANC-1 cultures grown in serum-containing medium to survey genes that are regulated by TGFβ except when cells are cotreated with SD-208. A majority of the TGFβ-affected

TABLE 1
Primers and probes used for real-time RT-PCR

Gene	Sequence
SERPINE 1	
Forward	GGCTGACTTCACGAGTCTTTCA
Probe	ACCAAGAGCCTCTCCACGTCGCG
Reverse	GTTCACCTCGATCTTCACTTTCTG
JAG1	
Forward	CTTACACTGGCAATGGTAGTTTCTG
Probe	TCGAGTGCCGCATCTCACAGC
Reverse	GGGTACTGTTGACTAGCTTTTTGCA
ITGB5	
Forward	CCAGGGCCCGCTATGAA
Probe	CCATTATACAGAAAGCCTATCTCCACGCACACT
Reverse	ATTTGTTGAACTTGTTGAAGGTGAAG
ITGAV	
Forward	GCAAAATGTAATGATGAGCTTGGT
Probe	TACCTATGTGCAGCCACTACCCATC
Reverse	TACAAGCTATCCAAGAATGCAAACA
IGF2	
Forward	CCGTGCTTCCGGACAACTT
Probe	CCCAGATACCCCGTGGGCAAGTTCTT
Reverse	GGACTGCTTCCAGGTGTCATATT
F2R	
Forward	AGGCTATTCCTGAGAGCTGCAT
Probe	TCCGCCCCCGATGGAGGAC
Reverse	ATGGCCCTGGCATGTGTCT
CYR61	
Forward	CTTGAGGAGCATTAAGGTATTTCGA
Probe	ACTGCCAAGGGTGCTGGTGCG
Reverse	CGTGGCTGCATTAGTGTCCAT
CTGF	
Forward	TGTGTGACGAGCCCAAGGA
Probe	CTGCCCTCGCGGCTTACCGA
Reverse	TAGTTGGGTCTGGGCCAAAC
COL7A1	Applied Biosystems Assays-on-Demand, assay ABI_Hs00164310_m1

genes (82%) were inhibited by SD-208. These genes were grouped into functional categories based on their involvement in the tumor-promoting processes. The gene responses highlight the major cellular processes such as ECM remodeling, cell motility, and adhesion; cell cycle, proliferation, and apoptosis; and angiogenesis (Table 2).

Included in the ECM remodeling/cell motility/adhesion functional group are genes encoding extracellular matrix proteins (*COL4A2*, *COL7A1*, *COL11A1*, *LAMA4*), regulators of matrix synthesis (*SERPINE 1*, *CTGF*), accessory proteins in the tight junction (*CLDN3*, *CLDN4*), and adhesion proteins (*CDH1*, *CDH5*, *ITGA2*, *ITGAV*, *ITGB2*, *F2R*, *ACTN1*, *CYR61*). TGFβ up-regulated the majority of these genes, whereas it down-regulated a few (*CLDN3*, *CLDN4*, *CDHI*). The addition of SD-208 reversed these trends. Collectively, the data show that SD-208 opposed gene responses that promote matrix remodeling and cell motility.

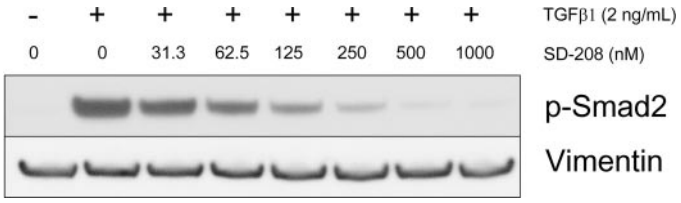

Fig. 1. Dose-response of SD-208 on SMAD2 phosphorylation. Western analysis of lysates from cells treated with 0.1% DMSO (vehicle) in lanes 1 and 2 or with increasing concentrations of SD-208 in lanes 3 to 8 (31.25, 62.5, 125, 250, and 500 nM, and 1 μM) for 15 min before treatment with 2 ng/ml TGFβ (lanes 2–8) for 65 min. Sequential probing with a vimentin antibody verified equal loading. The IC_{50} value for SD-208 is 62.5 to 125 nM.

Another large functional group of genes whose expression was regulated by TGFβ and reversed by SD-208 includes genes involved in proliferation, cell cycle, or apoptosis. Examples of TGFβ up-regulated genes in this group are *SNF1LK*, *PDGFA*, *PDGFB*, *CDK6*, *MYC*, and *IGF2*. TGFβ also down-regulated a number of genes including *TP531*, *TNFRSF1B*, *PBEF1*, *NBL1*, and *PTEN*. Because two of four replicates for *IGF2* expression (TGFβ + inhibitor) on the array did not meet the signal/background criteria, we confirmed the regulation through RT-PCR (see below).

Angiogenesis genes comprise the third functional cluster. Included in this cluster are *NRP2*, *JAG1*, *CYR61*, and *VEGFC*, which are novel TGFβ-responsive genes for PANC-1. The gene array analysis, therefore, identified a

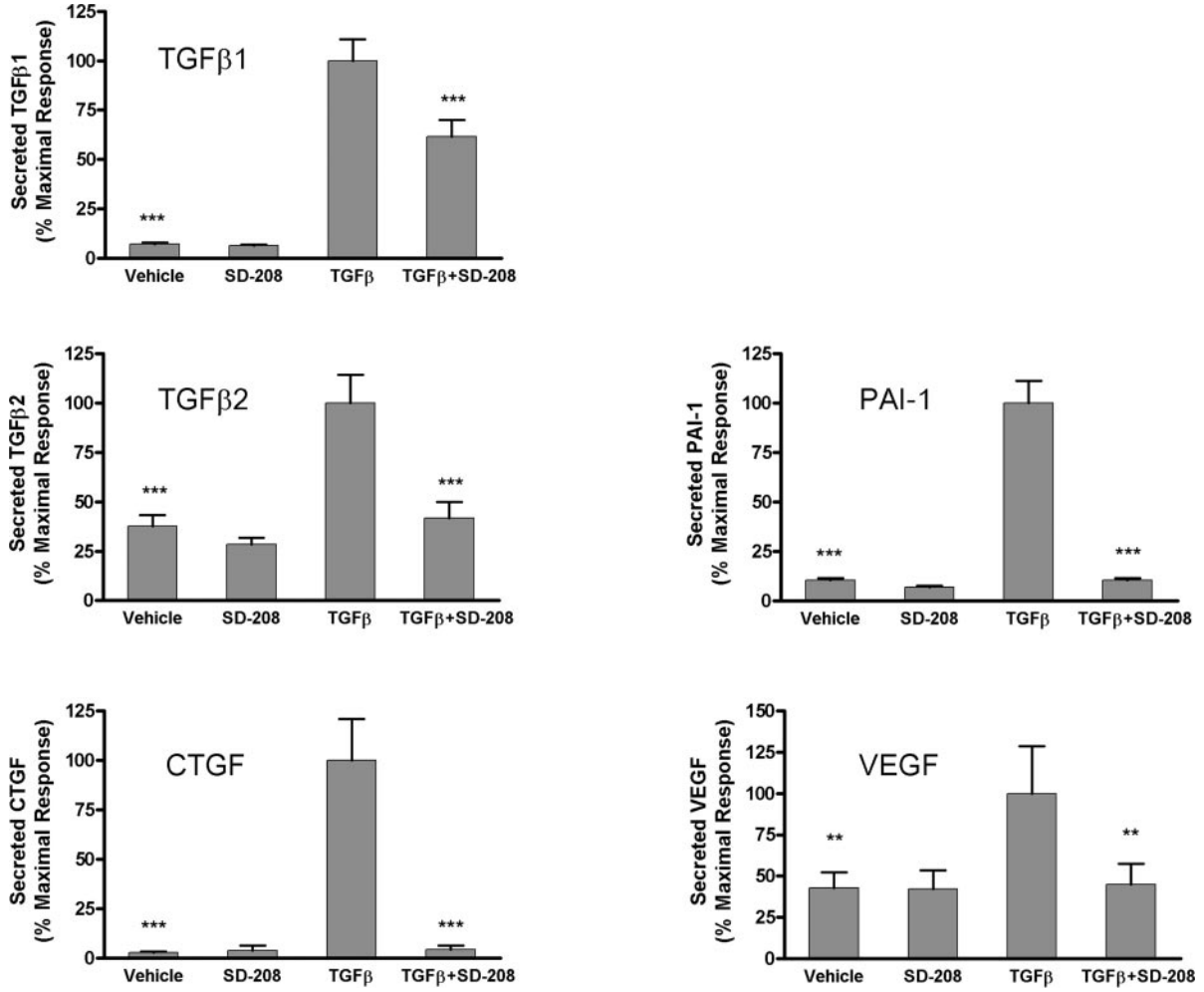

Fig. 2. Effect of TGFβ1 and SD-208 on production of TGFβ1, TGFβ2, CTGF, PAI-1, and VEGF. Quantitation of secreted proteins after culturing PANC-1 for 48 h in the presence of 0.1% DMSO (vehicle), 400 nM SD-208, 5 ng/ml TGFβ1, or TGFβ1 and SD-208. A representative study from three independent studies is shown. Mean ± S.D. values were determined for triplicate biological replicates. Protein levels in vehicle-treated and SD-208-treated samples were compared with levels in TGFβ1-stimulated samples (*, **, and *** represent p values <0.05, <0.01, and <0.001, respectively). SD-208 inhibited TGFβ-stimulated induction of these factors. Analysis of levels of TGFβ1 after stimulation with TGFβ1 is confounded by residual TGFβ1 from treatment; nonetheless, SD-208 significantly decreased TGFβ1 levels.

TABLE 2

Effect of SD-208 on TGFβ-dependent gene responses

The functional gene clusters identify genes that are regulated by TGFβ and are affected by SD-208 in PANC-1 cells: ECM remodeling, cell motility, and adhesion; cell cycle, proliferation, and apoptosis; and angiogenesis. Mean fold expression of TGFβ-regulated genes in PANC-1 from quadruplicate hybridizations: control vs. TGFβ , 24 h (TGFβ); TGFβ vs. TGFβ + SD-208, 24 h (TGFβ + SD-208); control vs. SD-208, 24 h (SD-208). Gene expression profiles were determined from cDNA microarrays as described previously (Kapoun et al., 2006). Functional groups were classified using Gene Ontology terms and published functions.

Gene ID	TGFβ	TGFβ+SD-208	SD-208	Symbol	Name	GenBank Accession No.
Genes involved in ECM remodeling, cell motility, and adhesion						
Up-regulated genes						
P01162_F10	7.9	−6.1	−1.3	NRP2	Neuropilin 2	NM_201266
P01105_C12	6.5	−5	−1.1	COL7A1	Collagen, type VII, α1 (epidermolysis bullosa, dystrophic, dominant and recessive)	NM_000094
P01077_D08	5.9	−6.6	−1.5	CTGF	Connective tissue growth factor	NM_001901
P01140_H03	5.2	−5	−1	JAG1	Jagged 1 (Alagille syndrome)	NM_000214
P01076_A11	4	−3.1	1	SPOCK	Sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican)	NM_004598
P01085_D06	3.1	−3.2	−1	LAMA4	Laminin, α4	NM_002290
P01082_H06	3	−3.2	1.2	COMP	Cartilage oligomeric matrix protein	NM_000095
P01100_A09	3	−2.2	−1.1	HOOK2	Hook homolog 2 (*Drosophila melanogaster*)	NM_013312
P01118_E04	2.9	−3	−1	PLEK2	Pleckstrin 2	NM_016445
P01063_D06	2.9	−2.6	−1	ITGA2	Integrin, α2 (CD49B, α2 subunit of VLA-2 receptor)	NM_002203
P01063_F09	2.8	−2.6	−1.3	TGM2	Transglutaminase 2 (C polypeptide, protein-glutamine-γ-glutamyltransferase)	NM_004613
P00777_A11	2.6	−2.2	−1.1	CYR61	Cysteine-rich, angiogenic inducer, 61	NM_001554
P01162_E08	2.5	−2.5	−1.1	CX3CL1	Chemokine (C-X3-C motif) ligand 1	NM_002996
P01155_A07	2.5	−1.8	−1	BGN	Biglycan	NM_001711
P01088_A02	2.4	−2.2	1	MFAP2	Microfibrillar-associated protein 2	NM_017459
P01109_G02	2.4	−1.7	−1	TRO	Trophinin	NM_016157
P01130_H07	2.3	−2	−1.3	CDH5	Cadherin 5, type 2, VE-cadherin (vascular epithelium)	NM_001795
P01063_D07	2.3	−1.8	−1.2	ITGB5	Integrin, β5	NM_002213
P01069_F04	2	−2.2	−1.2	F2R	Coagulation factor II (thrombin) receptor	NM_001992
P01076_B07	2	−2.2	−1.1	FAT	FAT tumor suppressor homolog 1 (*D. melanogaster*)	NM_005245
P01062_D11	2	−2	−1	SERPINE1	Serine (or cysteine) proteinase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	NM_000602
P01071_A04	2	−1.7	−1.2	TFPI2	Tissue factor pathway inhibitor 2	NM_006528
P01147_F05	1.9	−2.4	−1.1	COL11A1	Collagen, type XI, α1	NM_080630
P01138_G10	1.9	−2.1	−1	TNFRSF12A	Tumor necrosis factor receptor superfamily, member 12A	NM_016639
P01109_B06	1.9	−1.9	−1	ACTN1	Actinin, α1	NM_001102
P01163_B07	1.9	−1.8	−1.1	ITGAV	Integrin, αV (vitronectin receptor, α polypeptide, antigen CD51)	NM_002210
P01093_F03	1.9	−1.5	1.1	COL4A2	Collagen, type IV, α2	NM_001846
P01155_D07	1.8	−2.2	−1	ZYX	Zyxin	NM_003461
P01118_H01	1.8	−1.5	−1.1	GPR56	G protein-coupled receptor 56	NM_005682
Down-regulated genes						
P01091_E06	−4.9	4.3	1.4	CLDN4	Claudin 4	NM_001305
P01136_D03	−4.1	3.5	1.3	PPL	Periplakin	NM_002705
P01110_E06	−2.7	2.2	−1.1	EPB41L4B	Erythrocyte membrane protein band 4.1 like 4B	NM_019114
P01136_C06	−2.4	2.2	1.1	TSPAN1	Tetraspanin 1	NM_005727
P01072_G01	−2.2	1.9	1.1	JUP	Junction plakoglobin	NM_002230
P01091_E05	−2.1	2.4	1.3	CLDN3	Claudin 3	NM_001306
P01103_B11	−2	1.8	−1.1	SORBS1	Sorbin and SH3 domain containing 1	NM_015385
P01072_F10	−1.9	1.7	−1.1	KRT8	Keratin 8	NM_002273
P01068_B06	−1.8	1.8	−1.2	CDH1	Cadherin 1, type 1, E-cadherin (epithelial)	NM_004360
Genes involved in cell cycle, proliferation, and apoptosis						
Up-Regulated genes						
P01140_F07	5.9	−5.6	−1.5	SNF1LK	SNF1-like kinase	NM_173354
P01140_H03	5.2	−5	−1	JAG1	Jagged 1 (Alagille syndrome)	NM_000214
P01087_E02	4	−4.3	−1	PDGFA	Platelet-derived growth factor α polypeptide	NM_033023
P01076_A11	4	−3.1	1	SPOCK	Sparc/osteonectin, cwcv and kazal-like domains proteoglycan (testican)	NM_004598
P01072_F03	3.8	−3.2	−1	LTBP2	Latent transforming growth factor β binding protein 2	NM_000428
P01071_H06	3.3	−2.5	−1.2	PDGFB	Platelet-derived growth factor β polypeptide (simian sarcoma viral oncogene homolog)	NM_002608
P00777_A11	2.6	−2.2	−1.1	CYR61	Cysteine-rich, angiogenic inducer, 61	NM_001554
P01162_A11	2.4	−2.1	−1.4	PGF	Placental growth factor, vascular endothelial growth factor-related protein	NM_002632
P01110_E09	2.4	−1.8	−1.2	PPP1R13L	Protein phosphatase 1, regulatory (inhibitor) subunit 13 like	NM_006663
P01074_B06	2.2	−1.7	−1.1	NOLC1	Nucleolar and coiled-body phosphoprotein 1	NM_004741
P01102_D05	2.1	−2.1	−1.1	TNFRSF11B	Tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	NM_002546

TABLE 2 *Continued*

Gene ID	TGFβ	TGFβ+SD-208	SD-208	Symbol	Name	GenBank Accession No.
P01104_C09	2.1	−1.9	−1.1	CKLF	Chemokine-like factor	NM_016951
P01099_G10	2.1	−1.5	−1.1	DAB2	Disabled homolog 2, mitogen-responsive phosphoprotein (*D. melanogaster*)	NM_001343
P01069_F04	2	−2.2	−1.2	F2R	Coagulation factor II (thrombin) receptor	NM_001992
P01068_C05	2	−1.6	−1.2	IER3	Immediate early response 3	NM_003897
P01106_G06	2	−1.5	−1	PTHLH	Parathyroid hormone-like hormone	NM_198965
P01069_E03	1.9	−2.2	−1	CDK6	Cyclin-dependent kinase 6	NM_001259
P01138_G10	1.9	−2.1	−1	TNFRSF12A	Tumor necrosis factor receptor superfamily, member 12A	NM_016639
P01081_E11	1.9	−1.8	−1	MYC	v-myc myelocytomatosis viral oncogene homolog (avian)	NM_002467
P01063_F06	1.9	−1.6	−1.1	VEGFC	Vascular endothelial growth factor C	NM_005429
P01065_B07	1.8	−1.6	−1.1	ARHGEF6	Rac/Cdc42 guanine nucleotide exchange factor (GEF) 6	NM_004840
Down-regulated genes						
P01078_F08	−3.9	3	1.1	TP53I11	Tumor protein p53 inducible protein 11	NM_006034
P01086_E12	−3.2	3.7	1.4	TNFRSF1B	Tumor necrosis factor receptor superfamily, member 1B	NM_001066
P01165_C12	−2.6	1.8	−1	PBEF1	Pre-B-cell colony enhancing factor 1	NM_005746
P01136_C06	−2.4	2.2	1.1	TSPAN1	Tetraspanin 1	NM_005727
P01064_F03	−2.2	2.1	1.1	TOB1	Transducer of ERBB2, 1	NM_005749
P01096_D02	−2.2	1.9	1.1	ADAMTS1	A disintegrin-like and metalloprotease (reprolysin type) with thrombospondin type 1 motif, 1	NM_006988
P01072_D04	−2.2	1.8	1.2	MX1	Myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	NM_002462
P01091_G06	−2.2	1.5	1.1	PARD6A	Par-6 partitioning defective 6 homolog α (*Caenorhabditis elegans*)	NM_016948
P01100_F10	−2.1	2.3	1.1	FLT1	Fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor)	NM_002019
P01065_F11	−2.1	2.1	−1	PPP1CA	Protein phosphatase 1, catalytic subunit, α isoform	NM_206873
P01073_F03	−2.1	2.1	1.2	NBL1	Neuroblastoma, suppression of tumorigenicity 1	NM_182744
P01140_F10	-2.1	1.7	1	IFITM1	Interferon-induced transmembrane protein 1 (9–27)	NM_003641
P01090_E08	−2.1	2	1.3	MAP2K6	Mitogen-activated protein kinase kinase 6	NM_002758
P01129_B02	−2	1.6	1	EPHB4	EPH receptor B4	NM_004444
P01115_C03	−1.9	2	1.1	IL2RA	Interleukin 2 receptor, α	NM_000417
P01067_D03	−1.8	2.2	−1	BNIP3	BCL2/adenovirus E1B 19-kDa interacting protein 3	NM_004052
P01095_E03	−1.8	1.8	−1	CREG1	Cellular repressor of E1A-stimulated genes 1	NM_003851
P01087_E12	−1.8	1.7	−1	PTEN	Phosphatase and tensin homolog (mutated in multiple advanced cancers 1)	NM_000314
P01163_B03	−1.8	1.7	1.2	IL6R	Interleukin 6 receptor	NM_000565
Genes involved in angiogenesis						
Up-regulated genes						
P01162_F10	7.9	−6.1	−1.3	NRP2	Neuropilin 2	NM_201266
P01140_H03	5.2	−5	−1	JAG1	Jagged 1 (Alagille syndrome)	NM_000214
P01087_E02	4	−4.3	−1	PDGFA	Platelet-derived growth factor α polypeptide	NM_033023
P01071_H06	3.3	−2.5	−1.2	PDGFB	Platelet-derived growth factor beta polypeptide (simian sarcoma viral oncogene homolog)	NM_002608
P01096_H11	2.9	−2.2	1.4	EPAS1	Endothelial PAS domain protein 1	NM_001430
P00777_A11	2.6	−2.2	−1.1	CYR61	Cysteine-rich, angiogenic inducer, 61	NM_001554
P01162_A11	2.4	−2.1	−1.4	PGF	Placental growth factor, vascular endothelial growth factor-related protein	NM_002632
P01138_G10	1.9	−2.1	−1	TNFRSF12A	Tumor necrosis factor receptor superfamily, member 12A	NM_016639
P01063_F06	1.9	−1.6	−1.1	VEGFC	Vascular endothelial growth factor C	NM_005429
Down-regulated genes						
P01061_C08	−3.4	3.1	1.3	TGFBR3	Transforming growth factor, β receptor III (betaglycan, 300 kDa)	NM_003243
P01100_F10	−2.1	2.3	1.1	FLT1	Fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor)	NM_002019
P01162_G04	−2	1.9	−1	TNFAIP2	Tumor necrosis factor, α-induced protein 2	NM_006291

number of genes in PANC-1 that are regulated by TGFβ and may be important for tumor-associated processes. Furthermore, the analysis revealed that SD-208 inhibited regulation of these genes by TGFβ.

To confirm the expression patterns observed in our microarray studies, we used real-time RT-PCR to validate gene responses of representative genes in each of the functional groups (Fig. 3). The validated genes include novel "TGFβ-regulated" genes for PANC-1 and genes reported previously to be involved in tumor progression. The same genes were

analyzed in PANC-1 luciferase (data not shown). Real-time RT-PCR results confirm the gene responses seen on the array. Furthermore, they demonstrate that PANC-1 and PANC-1 luciferase respond similarly.

Inhibition of TGFβRI Attenuates TGFβ-Stimulated Invasion in Vitro. The gene array identified many TGFβ-responsive genes that may promote metastasis. To test whether SD-208 inhibits metastasis in vitro, we measured invasion using Boyden chambers. Membranes in the chambers are coated with ECM to mimic basement membrane. Movement of cells through the membrane requires not only migration but also degradation of matrix, a critical step in the metastatic process. Treatment of cells with TGFβ1 stimulated invasion, whereas cotreatment with SD-208 inhibited TGFβ1-induced invasion (Fig. 4). These results are consistent with gene responses on the array that suggest that TGFβ signaling promotes cell motility and matrix remodeling.

To confirm that other pancreatic cancer cells are similarly affected by SD-208, we tested the effect of SD-208 on TGFβRI signaling and motility in the Smad4-deficient cell line, BxPC3. Similar to PANC-1, the IC_{50} value of signaling from the receptor was achieved at 62.5 to 125 nM SD-208 (Supplemental Fig. S1A). Likewise, invasion assays demonstrated that TGFβ induced cell motility and that SD-208 inhibited cell motility (Supplemental Fig. S1B). Together, these results confirm that TGFβ signaling and TGFβ-stimulated motility are inhibited in another pancreatic tumor cell line harboring different mutations.

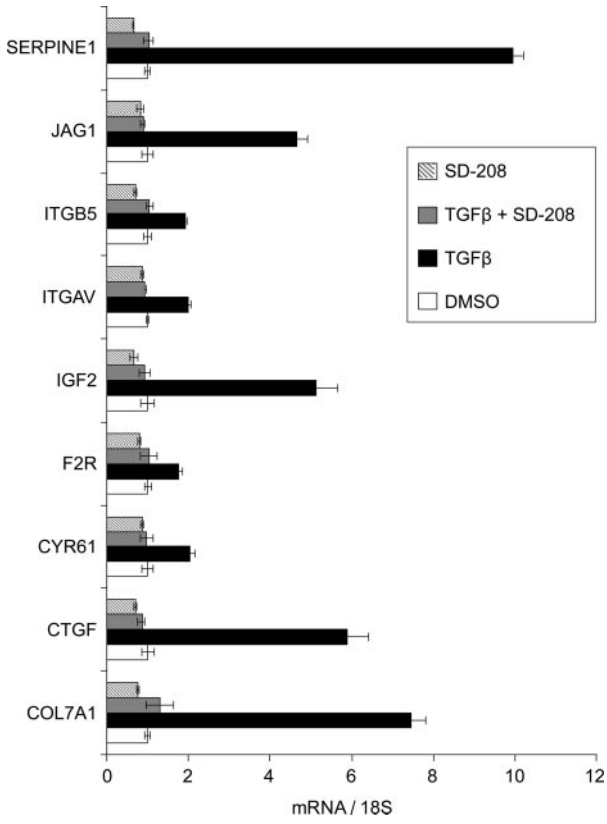

Fig. 3. Validation of TGFβ-regulated gene responses for PANC-1 by real-time RT-PCR. Expression results for *COL7A1*, *CTGF*, *CYR61*, *F2R*, *ITGAV*, *IGF2*, *ITGB5*, *JAG1*, and *SERPINE1*. Expression levels were normalized to 18S rRNA. For each gene, the control (DMSO) was set to 1. All real-time RT-PCR reactions were performed in triplicate on each of the three biological replicates. TGFβ positively regulated all genes, whereas SD-208 inhibited induction.

Inhibition of TGFβRI Reduces Primary Tumor Growth and Metastasis in an Orthotopic Xenograft Model. The cell studies revealed that SD-208 inhibited TGFβ-dependent processes in PANC-1. To determine whether SD-208 affected these processes in vivo, we tested this inhibitor in a mouse model of pancreatic cancer. In this model, tumor cells were labeled with luciferase so tumor progression could be followed by luminescent imaging. Oral treatment with SD-208 (20 or 60 mg/kg twice daily) began at day 10 when tumors were established. Weekly monitoring revealed that tumors in the vehicle group continued to increase in luminescence throughout the 56-day period. At day 56, tumors in the SD-208 group had lower luminescence intensity compared with the vehicle group (data not shown). Furthermore, tumors in the SD-208 group were smaller than tumors in the vehicle group: the tumor weight (mean ± S.D.) for animals in the vehicle group was 0.7 ± 0.4 g, whereas the tumor weight for animals treated with 20 or 60 mg/kg SD-208 was only 0.3 ± 0.2 and 0.2 ± 0.2 g, respectively (Fig. 5A). The difference in tumor weight between vehicle and treated animals was significant ($p < 0.01$ and < 0.001, respectively). Moreover, two tumors completely regressed in animals treated with 60 mg/kg SD-208.

Metastatic lesions were less prevalent in treated groups compared with the vehicle group (Table 3, study 1). Nine mice in the vehicle group ($n = 12$) had metastatic lesions in the lymph nodes, spleen, liver, and peritoneum, whereas only five mice (42%) in the 60 mg/kg treatment group ($n = 12$) had metastatic lesions. These were found primarily in the lymph node. The 20 mg/kg dose had metastatic lesions in 50% of the mice with several in the spleen and peritoneum, suggesting a dose effect.

A second study using a 60 mg/kg dose confirmed the results of the first study. Examination of tumors revealed that the 60 mg/kg group responded similarly to treated animals in the first study, with a dramatic decrease in mean tumor weight (0.2 ± 0.2 versus 0.6 ± 0.4 g) and reduction in number of metastatic lesions. The mean tumor weight for the treated

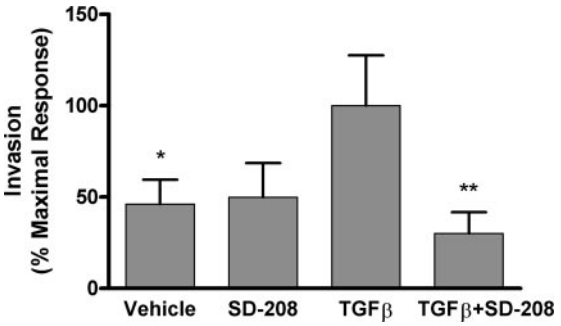

Fig. 4. Effect of SD-208 on TGFβ-dependent invasion. PANC-1 cells were cultured for 20 h in the presence of 0.1% DMSO (vehicle), SD-208 (1 μM), and TGFβ1 (2 ng/ml) alone or in combination with SD-208. Cells that crossed chamber membranes were quantified as described under *Materials and Methods*. Mean ± S.D. values were determined for triplicate wells per condition from a representative study. Responses for vehicle-treated and SD-208-treated samples were compared with the response for the TGFβ-stimulated sample (* and ** represent p values <0.05 and <0.01, respectively). The analysis reveals that TGFβ induced invasion and that SD-208 inhibited invasion. The response for the SD-208-treated sample was also compared with the response for the vehicle-treated sample and shows no statistical significance between the two groups.

group was statistically different ($p = 0.004$) from the mean tumor weight for the vehicle group (Fig. 5B). Once again, two tumors completely regressed. Nine of the 12 mice had metastatic lesions in the vehicle group, whereas only 4 mice had lesions in the treatment group (Table 3, study 2). Lesions in the treated animals resided in the lymph node and spleen but not in distal organs. Together, these studies demonstrate that treatment with SD-208 reduced both tumor growth and invasiveness in an orthotopic model of human pancreatic cancer.

Discussion

The lack of effective treatment for pancreatic cancer underscores the need to develop new therapies. In the last decade, our understanding of the role of TGFβ signaling in tumor progression has made the TGFβ pathway an attractive target for intervention with soluble receptors (Rowland-Goldsmith et al., 2001, 2002), antisense oligonucleotides (Schlingensiepen et al., 2006), and small-molecule inhibitors (Singh et al., 2004; Subramanian et al., 2004; Uhl et al., 2004; Halder et al., 2005). Previously, SD-208 has been shown to reduce tumor progression in an orthotopic syngeneic model of glioblastoma (Uhl et al., 2004). The increased survival of animals treated with SD-208 seems to have been driven by the host mounting an immune response against the tumor. Here we extend these findings and demonstrate that SD-208 can inhibit tumor progression in pancreatic cancer, which, like glioblastoma, has no effective therapy. To our knowledge, this is the first example to demonstrate the efficacy of a small-molecule TGFβ-signaling inhibitor in an animal model of pancreatic cancer. The observed effect in an immune-compromised animal further suggests that inhibition of TGFβ signaling can reduce tumor progression and, in some cases, cure animals of tumor in the absence of a normal immune response. This is significant in that it demonstrates an additional mechanistic rationale for inhibiting TGFβ signaling.

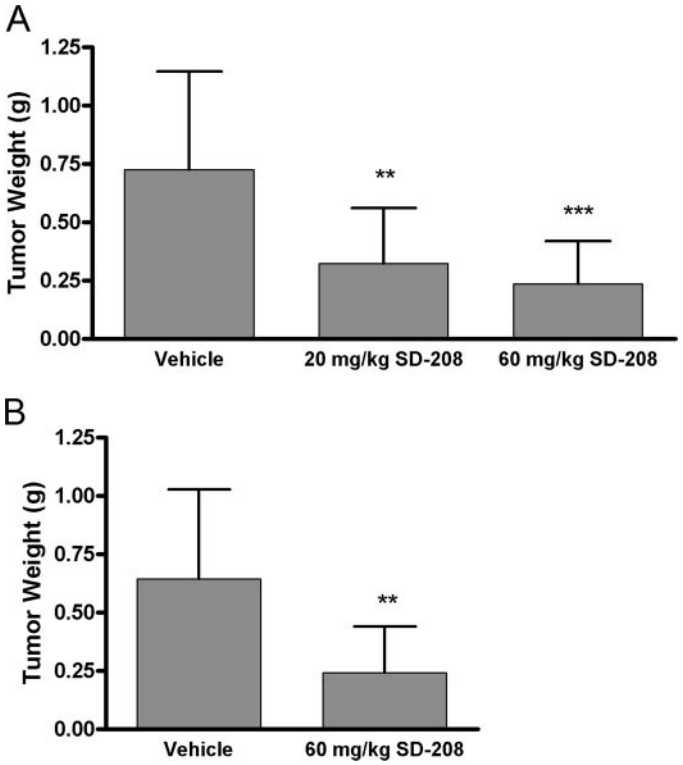

Fig. 5. Effect of SD-208 on tumor growth in an orthotopic xenograft model. Mean tumor weights of vehicle-treated and SD-208-treated tumors from two independent studies (A and B). Tumor weights were measured at study termination on day 56. Mean ± S.D. was determined for 12 mice in each treatment group. Statistical significance was determined using one-way analysis of variance with Bonferroni correction (A) or Student's *t* test (B). Comparison of tumor weights between vehicle and SD-208 treatment groups indicates that SD-208 reduced tumor growth (** and *** represent *p* values <0.01 and <0.001, respectively).

The involvement of the TGFβ pathway has been established in cancers of many organs including the breast, lung, colon, prostate, and pancreas (Elliott and Blobe, 2005). TGFβ signaling is frequently attenuated in pancreatic cancer because of alterations in components of the pathway (Jonson et al., 2001). Although PANC-1 has functional Smad4, it has been reported to have attenuated Smad signaling compared with epithelial cells that are responsive to the antiproliferative effects of TGFβ (Nicolás and Hill, 2003). Its attenuated signaling may be caused by low levels of TGFβRI and by high levels of Smad7 (Nicolás and Hill, 2003). Despite these alterations, the phosphorylation status of the Smad2, a substrate of TGFβRI, can be used to monitor receptor activity. We observed that phosphorylation of Smad2 increased in PANC-1 after treatment with TGFβ, whereas nanomolar levels of SD-208 inhibited induction. Similar receptor activation and inhibition profiles were observed when we tested the Smad4-deficient cell line BxPC-3. These results demonstrate that SD-208 is a potent inhibitor of TGFβRI signaling in two pancreatic cancer cell lines that differ with respect to tumorigenic mutations and Smad4 activity. We also tested the efficacy of SD-208 inhibition on the expression of proteins regulated by TGFβRI in PANC-1 cells. We observed that SD-208 reduced levels of CTGF, PAI-1, and VEGF in the culture medium. It is noteworthy that levels of TGFβ1 and TGFβ2 were also reduced, indicating that SD-208 inhibits autocrine induction of TGFβ. The effect of SD-208 on the production of these TGFβ-regulated proteins, known modulators of fibrosis, angiogenesis, and metastasis, confirmed that SD-208 is a potent inhibitor of TGFβRI signaling.

Using the gene array data, we identified TGFβ-responsive genes in PANC-1 involved in ECM remodeling, cell motility, adhesion, angiogenesis, cell cycle, proliferation, and apoptosis. Regulation of these genes by TGFβ is inhibited by SD-208. Some genes, such as *SERPINE* (PAI-1), *CTGF*, and *CDH1*, are known to be regulated by TGFβ in pancreatic

TABLE 3

Reduced incidence of metastasis with SD-208 treatment

Incidence and distribution of metastatic lesions from two independent studies. Treatment with SD-208 reduced the incidence and distribution of metastatic lesions in a dose-dependent manner.

	Incidence of Metastasis ($n = 12$)	Distribution of Metastasis			
		Lymph Node	Spleen	Liver	Peritoneum
		mice			
Study 1					
Vehicle	9 (75%)	8	5	2	4
20 mg/kg	6 (50%)	5	2	1	3
60 mg/kg	5 (42%)	5	1	1	0
Study 2					
Vehicle	9 (75%)	8	4	3	3
60 mg/kg	4 (33%)	3	2	0	0

ductal adenocarcinoma (Geng et al., 1999; Halder et al., 2005). Regulation of other genes such as *JAG1*, *CYR61*, *VEGFC*, *NRP2*, and *IGF2* by TGFβ is novel for PANC-1 and, to our knowledge, pancreatic cancer. An interesting finding in the expression analysis is that *MYC* was induced instead of repressed by TGFβ.

The TGFβ-dependent induction of *SERPINE* and *CTGF*, known regulators of matrix remodeling, is consistent with the responses we observed in invasion assays and in cells studies when we measured secreted PAI-1 and CTGF. Negative regulation of E-cadherin (*CDH1*) by TGFβ and reversal with TGFβRI inhibitor SB-431542 has been observed previously in PANC-1 cultures (Halder et al., 2005). Down-regulation of E-cadherin is required for epithelial-to-mesenchymal transition (EMT) and metastasis (Hay, 1995) and has been associated with lymph node metastasis in high-grade and advanced-stage pancreatic cancer (Pignatelli et al., 1994). The TGFβ-dependent regulation of *JAG1* on the array is interesting because its gene product, the Notch-ligand Jagged1, has been reported to be induced by TGFβ in cultured epithelial cells, in which it is important for EMT (Zavadil et al., 2004). Thus, two gene responses suggest that SD-208 may inhibit EMT in pancreatic cancer. It is interesting that *JAG1* has also been found to play an important role in vascular angiogenic remodeling (Xue et al., 1999).

Several of the other genes affected by TGFβ (*CYR61*, *VEGFC*, and *NRP2*) regulate angiogenesis or lymphangiogenesis. *CYR61* encodes a secreted, matricellular protein that binds to integrin αvβ3 in human umbilical vein endothelial cells and promotes angiogenesis and cell adhesion, migration, and tumor growth (Kireeva et al., 1996; Babic et al., 1998). The gene product of *VEGFC* (VEGF-C) plays a role in the early development of the vascular system and lymphangiogenesis (Kukk et al., 1996). It is implicated in tumor progression because its expression in pancreatic cancer correlates with increased lymphatic vessel invasion and lymph node metastasis (Tang et al., 2001). The last gene, neuropilin (*NRP2*), encodes a coreceptor for VEGF-C and VEGF-A and is highly expressed in pancreatic cancer, in which it is believed to promote tumor angiogenesis and progression (Fukahi et al., 2004). Although *VEGFA* was not on the array, the regulation of *VEGFC* and *NRP2* in PANC-1 is consistent with the TGFβ induction of total VEGF protein observed in this study and reported previously (Teraoka et al., 2001). The gene analysis, therefore, indicates that SD-208 is a potent inhibitor of TGFβ-dependent gene responses that support angiogenesis or lymphangiogenesis.

The observation that TGFβ signaling induced expression of *IGF2* and *MYC* in PANC-1 is notable because both genes are involved in cell proliferation and are frequently dysregulated in tumors. IGF2 has been shown to promote DNA synthesis and cell survival and to reduce apoptosis in various pancreatic cell types (Hogg et al., 1993; Petrik et al., 1998). It is a ligand for the IGF1 receptor (IGFR1) and is overexpressed in most primary tumors (Fürstenberger and Senn, 2002). Regulation of c-Myc by TGFβ is essential for cell-cycle arrest: G_1 arrest requires TGFβ-mediated down-regulation of c-Myc to relieve transcriptional suppression of p15 and $p21^{Cip1/WAF1}$ (Donovan and Slingerland, 2000; Adhikary and Eilers, 2005). Tumor cells escape TGFβ-mediated growth arrest by overexpressing or activating c-Myc (Adhikary and Eilers, 2005). Thus, the induction of these genes by TGFβ could explain why we have observed (M. Henson and N. Gaspar, unpublished data) and others have reported (Nicolás and Hill, 2003; Subramanian et al., 2004) that PANC-1 is unresponsive or weakly responsive to TGFβ growth arrest.

When we evaluated TGFβ-dependent motility of PANC-1, we found that TGFβ induced migration (G. Li, unpublished data) and invasion (this report), whereas SD-208 inhibited TGFβ-dependent motility. Similar results were seen when we evaluated motility of the pancreatic adenocarcinoma cell line, BxPC3. Contrary to our study, PANC-1 has been reported to be unresponsive in motility assays to induction with TGFβ or inhibition with SD-093, another TGFβRI inhibitor (Subramanian et al., 2004). BxPC3, on the other hand, was reported to be responsive to induction and inhibition in invasions assays but only inhibition in migration assays (Subramanian et al., 2004). The differences observed in our study and the previous study may reflect differences in assay conditions or assay sensitivity. The responses we observed for PANC-1 in motility assays are consistent with our gene array results, which reveal that motility-promoting gene responses are induced by TGFβ and inhibited by SD-208. Moreover, they are consistent with the observation that SD-208 inhibits metastasis in the animal model and provide an explanation for the effect.

TGFβ signaling has been targeted previously in animal models of human pancreatic ductal adenocarcinoma (Rowland-Goldsmith et al., 2001, 2002). The studies demonstrated that when COLO-357 or PANC-1 tumor cells expressing soluble TGFβRII receptor (sTβRII) were injected into mice, they formed smaller tumors than sham-transfected cells. From these studies, it is not possible to determine whether inhibiting TGFβ signaling attenuated tumor progression or prevented the establishment of tumors. Because we treated animals with established tumors, our studies demonstrate that inhibiting TGFβ signaling can in fact attenuate tumor growth and metastasis and, in some cases, cure animals of tumor.

In summary, we have demonstrated that SD-208 inhibits tumor-associated processes in a human pancreatic adenocarcinoma at the cellular level and in an animal model. The study identifies potential mechanisms through which inhibition of TGFβRI signaling with a small-molecule inhibitor can reduce tumor progression. Furthermore, it provides hypotheses that can be tested in animals or in the clinical setting. The results of this study are encouraging because they demonstrate that SD-208 not only reduced TGFβ-mediated gene expression, protein expression, and invasion, but that it also reduced both primary tumor growth and metastasis in vivo. Thus, these findings suggest that molecules similar to SD-208 and, moreover, small-molecule inhibitors of TGFβRI, may be effective as new therapies to treat human pancreatic cancer.

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