

Inhibition of TGF β signaling reduces pancreatic adenocarcinoma growth and invasiveness

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Abbreviations: SMAD, mothers against DPP homolog; TGF β , Transforming growth factor- β ; ECM, extracellular matrix; MAPK, mitogen-activated protein kinase; RT-PCR, reverse transcription-polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; CTGF, connective tissue growth factor; PAI-1, plasminogen activator inhibitor, type 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; EMT, epithelial-to-mesenchymal transition.

Abstract

Transforming growth factor β (TGF β) is a pleiotropic factor that regulates cell proliferation, angiogenesis, metastasis, and immune suppression. Dysregulation of the TGF β pathway in tumor cells often leads to resistance to the anti-proliferative effects of TGF β while supporting other cellular processes that promote tumor invasiveness and growth. In the present study SD-208, a small molecule, ATP-competitive inhibitor of the TGF β receptor I kinase (TGF β RI), was used to inhibit cellular activities and tumor progression of PANC-1, a human pancreatic tumor line. SD-208 blocked TGF β -dependent Smad2 phosphorylation and expression of TGF β -inducible proteins in cell culture. cDNA microarray analysis and functional gene clustering identified groups of TGF β -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 TGF β -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 TGF β signaling may promote tumor progression in pancreatic adenocarcinoma. Moreover, they suggest that inhibition of TGF β RI with a small molecule inhibitor may be effective as a therapeutic approach to treat human pancreatic cancer.

Introduction

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 3-8 months following diagnosis. Due to the aggressiveness of the cancer, difficulties in diagnosis, and lack of effective treatment, only 5% of patients diagnosed with 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 85-95% 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 (Friess et al., 1999; Schutte et al., 1996).

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

al., 1999). TGF β signals through the Smad pathway as well as through Smad-independent pathways. Smad signaling is initiated upon binding of TGF β to a type II receptor (TGF β RII), followed by recruitment and transphosphorylation of TGF β RI (reviewed in (Heldin et al., 1997)). Activated TGF β RI 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 TGF β -responsive genes. TGF β signaling through the Smad pathway is tightly controlled by negative feed back loops involving Smad6 and Smad7. TGF β can also signal through MAP kinase (Erk, JNK, p38) cascades and the PI3 kinase pathway (Derynck and Zhang, 2003; Elliott and Blobe, 2005). Cross-talk between these pathways and the TGF β pathway coordinates proliferation and survival signals as well as other signals.

In normal epithelial cells TGF β 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} (Adhikary and Eilers, 2005; Donovan and Slingerland, 2000; Grau et al., 1997). In addition to regulating survival and proliferation, TGF β signaling promotes angiogenesis, fibrosis, metastasis, and immune suppression (Elliott and Blobe, 2005). Alterations that affect expression or activity of components of the TGF β pathway can render cells insensitive to TGF β -mediated growth arrest while enabling other responses that support tumor progression (Dumont et al., 2003; Nicolas and Hill, 2003). During neoplastic conversion, autocrine expression of TGF β is believed to promote tumorigenesis. The cellular response is also influenced by other dysregulated pathways such as the Ras-RAF-MEK-ERK pathway (Ellenrieder et al., 2001), as well as

by stromal cell interactions, growth factors, and cytokines in the tumor cell microenvironment.

The pivotal role of TGF β 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 TGF β RI, 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 (Moore et al., 2001; Villanueva et al., 1998). PANC-1 has also been reported to have altered TGF β RI and Smad7 expression (Nicolas 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 TGF β , which is believed to promote tumor progression and desmoplasia in human pancreatic cancer. Our studies reveal that SD-208 abrogates TGF β -mediated gene responses that may facilitate tumor growth and metastasis. We also demonstrate for the first time that a small molecule inhibitor of TGF β RI attenuates growth and metastasis of established tumors in an orthotopic xenograft model of pancreatic adenocarcinoma.

Materials and Methods

Reagents

Recombinant human TGF β was purchased from R&D Systems. TGF β 1 and VEGF ELISA kits were from Biosource International (Camarillo, CA). The TGF β 2 ELISA was from R&D Systems (Minneapolis, MN). 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, Saint 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 ATCC (Manassas, VA). PANC-1 was cultured in Dulbecco's Modification of Eagle's Medium (DMEM) (Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (FBS). BxPC-3 was cultured in RPMI 1640 (Mediatech, Herndon, VA) supplemented with 10% FBS. TGF β RI kinase inhibitor SD-208 (Scios, Inc, Fremont, CA) was dissolved in DMSO (1000X stock). SD-208 has an IC₅₀ of 49 nM when measured by direct enzymatic assay of TGF β RI kinase activity *in vitro*. It is 100-fold

less specific for TGF β RII 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 ZeocinTM-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). Following selection with ZeocinTM (Invitrogen), a clone with stable luciferase expression and normal growth characteristics was selected for studies.

Phospho-Smad2 Analysis

Cells were seeded in 6-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 minutes prior to addition of TGF β 1 (2 ng/mL). After 65 minutes, cell lysates were prepared and analyzed by Western blot as previously described (Kapoun et al., 2006).

Measurement of Secreted Proteins

PANC-1 were seeded in 6-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 1X ITS (GibcoTM Insulin-Transferrin-Selenium) and 0.2% BSA. The medium also contained combinations of the following treatments: 0.1% DMSO vehicle control, 400 nM SD-208, and 5 ng/mL TGF β 1. After 24 hours, cell supernatants were collected and assayed for TGF β , VEGF, CTGF and PAI-1 by ELISA. Cells were harvested in MPER

buffer (Pierce), 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 were seeded in DMEM containing 10% serum and grown to ~70% confluency.

The following day cells were treated in complete medium with vehicle (0.1% DMSO), 400 nM SD-208, 2 ng/mL TGF β 1, or a combination of TGF β 1 and SD-208 for 24 hours.

Total RNA was extracted from cells using Qiagen's RNeasy™ kit (Valencia, CA).

Real-time RT-PCR and cDNA microarray analysis were performed as described in (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, 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 TGF β 1 (2 ng/mL) and/or SD-208 (1 μ M) or DMSO (0.1%). 5×10^5 cells were added to each chamber and incubated for 20 hours. Cells that did not pass through the filter were removed with a cotton swab prior to 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 40x magnification and counted manually for each treatment.

Tumor Implantation and Imaging

All of the animal experiments were performed under 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, 6-8 week old male nude mice were anesthetized with ketamine/xylazine cocktail prior to 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 following implantation), animals were randomized and assigned to vehicle and treatment groups (n=12) based on 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 ANOVA with Bonferroni correction using GraphPad Prism, version 4.02 (GraphPad Software Inc., San Diego, CA) unless otherwise stated.

Results

Inhibition of TGF β RI reduces Smad signaling and levels of secreted, TGF β - inducible proteins

We previously observed that SD-208 blocks signaling from TGF β RI in fibroblasts (Kapoun et al., 2006), smooth muscle cells and various tumor cell lines (Uhl et al., 2004; unpublished data). To assess the potency of SD-208 on TGF β RI inhibition in PANC-1, we measured inhibition of Smad2 phosphorylation by SD-208 following induction with TGF β . Maximum phosphorylation of Smad2 occurred at approximately 1 hour following induction with TGF β 1 (data not shown). Pretreatment of cells with SD-208 prior to TGF β 1 induction inhibited phosphorylation in a dose-dependent manner (Figure 1). Fifty-percent inhibition (IC₅₀) was achieved at 62.5-125 nM SD-208. To confirm inhibition of TGF β signaling, downstream events were measured. These included production of CTGF, PAI-1, and VEGF as well as TGF β 1 and TGF β 2, which are regulated in an autocrine manner by TGF β 1. Levels of the secreted proteins increased following TGF β treatment except when cells were cotreated with SD-208 (Figure 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 TGF β signaling (data not shown). In agreement with a previous report (Uhl et al., 2004), these results demonstrate that SD-208 inhibits signaling from the TGF β receptor complex. Furthermore, they demonstrate that SD-208 inhibits expression of proteins that regulate matrix remodeling and angiogenesis.

Inhibition of TGF β RI attenuates gene responses involved in tumor processes

As a first step towards understanding the TGF β -dependent processes important for tumor progression, we assessed regulation of gene expression *in vitro* by TGF β . We employed gene array analysis of PANC-1 cultures grown in serum-containing medium to survey genes that are regulated by TGF β except when cells are cotreated with SD-208. A majority of the TGF β -affected 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, adhesion; cell cycle, proliferation, 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*). TGF β up-regulated the majority of these genes while it down-regulated a few (*CLDN3*, *CLDN4*, *CDH1*). Addition of SD-208 reversed these trends. Collectively, the data show that SD-208 opposed gene responses that promote matrix remodeling and cell motility.

Another large functional group of genes whose expression was regulated by TGF β and reversed by SD-208 are genes involved in proliferation, cell cycle, or apoptosis. Examples of TGF β up-regulated genes in this group are *SNF1LK*, *PDGFA*, *PDGFB*, *CDK6*, *MYC* and *IGF2*. TGF β also down-regulated a number of genes including *TP53\11*, *TNFRSF1B*, *PBEF1*, *NBL1*, and *PTEN*. Because 2 of 4 replicates for *IGF2*

expression (TGF β + 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 TGF β -responsive genes for PANC-1. The gene array analysis, therefore, identified a number of genes in PANC-1 that are regulated by TGF β and may be important for tumor-associated processes. Furthermore, the analysis revealed that SD-208 inhibited regulation of these genes by TGF β .

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 (Figure 3). The validated genes include novel “TGF β -regulated” genes for PANC-1 as well as genes previously reported 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 TGF β RI attenuates TGF β -stimulated invasion *in vitro*

The gene array identified many TGF β -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 TGF β 1 stimulated invasion while cotreatment with SD-208 inhibited TGF β 1-

induced invasion (Figure 4). These results are consistent with gene responses on the array that suggest that TGF β 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 TGF β RI signaling and motility in the Smad4-deficient cell line, BxPC3. Similar to PANC-1, fifty-percent inhibition (IC₅₀) of signaling from the receptor was achieved at 62.5-125 nM SD-208 (Supplemental Figure 1A). Likewise, invasion assays demonstrated that TGF β induced cell motility and that SD-208 inhibited cell motility (Supplemental Figure 1B). Together these results confirm that TGF β signaling and TGF β -stimulated motility are inhibited in another pancreatic tumor cell line harboring different mutations.

Inhibition of TGF β RI reduces primary tumor growth and metastasis in an orthotopic xenograft model

The cell studies revealed that SD-208 inhibited TGF β -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 mg/kg 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 \pm SD) for animals in the vehicle group was $0.7 \pm$

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 (Figure 5A). The difference in tumor weight between vehicle and treated animals was significant ($p < 0.01$ and $p < 0.001$, respectively). Moreover, two tumors completely regressed in animals treated with 60 mg/kg SD-208.

Metastatic lesions were less prevalent in treated groups as compared to 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 fifty percent 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 vs 0.6 ± 0.4 g) and reduction in number of metastatic lesions. The mean tumor weight for the treated group was statistically different ($p = 0.004$) from the mean tumor weight for the vehicle group (Figure 5B). Once again two tumors completely regressed. Nine of the twelve mice had metastatic lesions in the vehicle group, whereas only four 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 TGF β signaling in tumor progression has made the TGF β pathway an attractive target for intervention with soluble receptors (Rowland-Goldsmith et al., 2001; Rowland-Goldsmith et al., 2002), antisense oligonucleotides (Schlingensiepen et al., 2006), and small molecule inhibitors (Halder et al., 2005; Singh et al., 2004; Subramanian et al., 2004; Uhl et al., 2004). 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 appears 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 TGF β signaling inhibitor in an animal model of pancreatic cancer. The observed effect in an immune compromised animal further suggests that inhibition of TGF β 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 TGF β signaling.

The involvement of the TGF β pathway has been established in cancers of many organs including the breast, lung, colon, prostate, and pancreas (Elliott and Blobe, 2005). TGF β signaling is frequently attenuated in pancreatic cancer due to 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 when compared to

epithelial cells that are responsive to the anti-proliferative effects of TGF β (Nicolas and Hill, 2003). Its attenuated signaling may be due to low levels of TGF β RI and to high levels of Smad7 (Nicolas and Hill, 2003). Despite these alterations, the phosphorylation status of the Smad2, a substrate of TGF β RI, can be used to monitor receptor activity. We observed that phosphorylation of Smad2 increased in PANC-1 following treatment with TGF β , 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 TGF β RI 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 TGF β RI in PANC-1. We observed that SD-208 reduced levels of CTGF, PAI-1, and VEGF in the culture medium. Importantly, levels of TGF β 1 and TGF β 2 were also reduced, indicating that SD-208 inhibits autocrine induction of TGF β . The effect of SD-208 on the production of these TGF β -regulated proteins, known modulators of fibrosis, angiogenesis, and metastasis, confirmed that SD-208 is a potent inhibitor of TGF β RI signaling.

Using the gene array data, we identified TGF β -responsive genes in PANC-1 involved in ECM remodeling, cell motility, adhesion, angiogenesis, cell cycle, proliferation, and apoptosis. Regulation of these genes by TGF β is inhibited by SD-208. Some genes, such as *SERPINE* (PAI-1), *CTGF*, and *CDH1*, are known to be regulated by TGF β in pancreatic ductal adenocarcinoma (Geng et al., 1999; Halder et al., 2005). Regulation of other genes such as *JAG1*, *CYR61*, *VEGFC*, *NRP2*, and *IGF2* by TGF β 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 TGF β .

The TGF β -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 TGF β and reversal with TGF β RI inhibitor SB-431542 has previously been observed 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 TGF β -dependent regulation of *JAG1* on the array is interesting because its gene product, the Notch-ligand Jagged1, has been reported to be induced by TGF β in cultured epithelial cells where it is important for EMT (Zavadil et al., 2004). Thus, two gene responses suggest that SD-208 may inhibit EMT in pancreatic cancer. Interestingly, *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 TGF β (*CYR61*, *VEGFC* and *NRP2*) regulate angiogenesis or lymphangiogenesis. *CYR61* encodes a secreted, matricellular protein that binds to integrin $\alpha v \beta 3$ in human umbilical vein endothelial cells and promotes angiogenesis as well as cell adhesion, migration, and tumor growth (Babic et al., 1998; Kireeva et al., 1996). 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 where 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 TGF β induction of total VEGF protein observed in this study and previously reported (Teraoka et al., 2001). The gene analysis, therefore, indicates that SD-208 is a potent inhibitor of TGF β -dependent gene responses that support angiogenesis or lymphangiogenesis.

The observation that TGF β 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 as well as 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 (Furstenberger and Senn, 2002). Regulation of c-Myc by TGF β is essential for cell-cycle arrest: G(1) arrest requires TGF β -mediated down-regulation of c-Myc to relieve transcriptional suppression of *p15* and *p21^{Cip1/WAF1}* (Adhikary and Eilers, 2005; Donovan and Slingerland, 2000). Tumor cells escape TGF β -mediated growth arrest by overexpressing or activating c-Myc (Adhikary and Eilers, 2005). Thus, the induction of these genes by TGF β could explain why we have observed (unpublished data) and others have reported (Nicolas and Hill, 2003; Subramanian et al., 2004) that PANC-1 is unresponsive or weakly responsive to TGF β growth arrest.

When we evaluated TGF β -dependent motility of PANC-1, we found that TGF β induced migration (unpublished data) and invasion (this report) while SD-208 inhibited TGF β -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 TGF β or inhibition with SD-093, another TGF β RI 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 TGF β 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.

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

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 TGF β -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 TGF β RI, may be effective as new therapies to treat human pancreatic cancer.

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Figure Legends:

Figure 1. Dose response of SD-208 on SMAD2 phosphorylation: Western analysis of lysates from cells treated with 0.1% DMSO (Vehicle) in lanes 1-2 or with increasing concentrations of SD-208 in lanes 3-8 (31.25, 62.5, 125, 250, 500 nM, 1 μ M) for 15 minutes prior to treatment with 2 ng/mL TGF β (lanes 2-8) for 65 minutes. Sequential probing with a vimentin antibody verified equal loading. The IC₅₀ for SD-208 is 62.5-125 nM.

Figure 2. Effect of TGF β 1 and SD-208 on production of TGF β 1, TGF β 2, CTGF, PAI-1 and VEGF: Quantitation of secreted proteins after culturing PANC-1 for 48 hours in the presence of 0.1% DMSO (Vehicle), 400 nM SD-208, 5 ng/mL TGF β 1, or TGF β 1 and SD-208. A representative study from 3 independent studies is shown. Mean \pm SD values were determined for triplicate biological replicates. Protein levels in Vehicle-treated and SD-208-treated samples were compared to levels in TGF β 1-stimulated samples (*, **, and *** represent $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively). SD-208 inhibited TGF β -stimulated induction of these factors. Analysis of levels of TGF β 1 following stimulation with TGF β 1 is confounded by residual TGF β 1 from treatment; nonetheless SD-208 significantly decreased TGF β 1 levels.

Figure 3. Validation of TGF β -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 3 biological replicates. TGF β positively regulated all genes, whereas SD-208 inhibited induction.

Figure 4. Effect of SD-208 on TGF β -dependent invasion: PANC-1 were cultured for 20 hours in the presence of 0.1% DMSO (Vehicle), SD-208 (1 μ M), and TGF β 1 (2ng/mL) alone or in combination with SD-208. Cells that crossed chamber membranes were quantified as described in “Material and Methods”. Mean \pm SD values were determined for triplicate wells per condition from a representative study. Responses for Vehicle-treated and SD-208-treated samples were compared to the response for the TGF β -stimulated sample (* and ** represent $p < 0.05$ and $p < 0.01$, respectively). The analysis reveals that TGF β induced invasion and that SD-208 inhibited invasion. The response for the SD-208-treated sample was also compared to the response for the Vehicle-treated sample and shows no statistical significance between the two groups.

Figure 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 & B). Tumor weights were measured at study termination on Day 56. Mean \pm SD was determined for 12 mice in each treatment group. Statistical significance was determined using One-way ANOVA variance with Bonferroni correction (A) or Students t-Test (B). Comparison of tumor weights between Vehicle and SD-208 treatment groups indicates that SD-208 reduced tumor growth (** and *** represent $p < 0.01$ and $p < 0.001$, respectively).

MOL #29025

Table1 Primers and Probes used for Real-time RT-PCR

Real-time RT-PCR primers and probes.

Gene	Forward	Probe	Reverse
<i>SERPINE 1</i>	GGCTGACTTCACGAGTCTTTCA	ACCAAGAGCCTCTCCACGTCGCG	GTTCACCTCGATCTTCACTTTCTG
<i>JAG1</i>	CTTAACTGGCAATGGTAGTTTCTG	TCGAGTGCCGCATCTCACAGC	GGGTACTGTTGACTAGCTTTTTCGA
<i>ITGB5</i>	CCAGGGCCCCGCTATGAA	CCATTATACAGAAAGCCTATCTCCACGCACACT	ATTTGTTGAAGTTGTTGAAGGTGAAG
<i>ITGAV</i>	GCAAAATGTAATGATGAGCTTGGT	TACCTATGTGCAGCCACTACCCATC	TACAAGCTATCCAAGAATGCAACA
<i>IGF2</i>	CCGTGCTTCCGACAACCTT	CCCAGATACCCGTGGGCAAGTTCTT	GGACTGCTTCCAGGTGTCATATT
<i>F2R</i>	AGGCTATTCTGAGAGCTGCAT	TCCGCCCCCGATGGAGGAC	ATGGCCCTGGCATGTGTCT
<i>CYR61</i>	CTTGAGGAGCATTAAGGTATTTCA	ACTGCCAAGGGTGCTGGTGCG	CGTGGCTGCATTAGTGCCAT
<i>CTGF</i>	TGTGTGACGAGCCCAAGGA	CTGCCCTCGCGGCTTACCGA	TAGTTGGGTCTGGGCCAAAC
<i>COL7A1</i>	Applied Biosystems Assays-on-Demand. Assay ABI_Hs00164310_m1		

Table 2

Genes involved in ECM Remodeling, Cell Motility, and Adhesion

MOL# 29025

Gene ID	TGFβ	TGFβ+SD-208	SD-208	Symbol	Name	Accession
<u>Up-regulated Genes</u>						
P01162_F10	7.9	-6.1	-1.3	NRP2	neuropilin 2	NM_201266
P01105_C12	6.5	-5	-1.1	COL7A1	collagen, type VII, alpha 1 (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_004599
P01085_D06	3.1	-3.2	-1	LAMA4	laminin, alpha 4	NM_002299
P01082_H06	3	-3.2	1.2	COMP	cartilage oligomeric matrix protein	NM_000099
P01100_A09	3	-2.2	-1.1	HOOK2	hook homolog 2 (Drosophila)	NM_013311
P01118_E04	2.9	-3	-1	PLEK2	pleckstrin 2	NM_016444
P01063_D06	2.9	-2.6	-1	ITGA2	integrin, alpha 2 (CD49B, alpha 2 subunit of VLA-2 receptor)	NM_002200
P01063_F09	2.8	-2.6	-1.3	TGM2	transglutaminase 2 (C polypeptide, protein-glutamine-gamma-glutamyltransferase)	NM_004611
P00777_A11	2.6	-2.2	-1.1	CYR61	cysteine-rich, angiogenic inducer, 61	NM_001555
P01162_E08	2.5	-2.5	-1.1	CX3CL1	chemokine (C-X3-C motif) ligand 1	NM_002999
P01155_A07	2.5	-1.8	-1	BGN	biglycan	NM_001711
P01088_A02	2.4	-2.2	1	MFAP2	microfibrillar-associated protein 2	NM_017455
P01109_G02	2.4	-1.7	-1	TRO	trophinin	NM_016155
P01130_H07	2.3	-2	-1.3	CDH5	cadherin 5, type 2, VE-cadherin (vascular epithelium)	NM_001799
P01063_D07	2.3	-1.8	-1.2	ITGB5	integrin, beta 5	NM_002211
P01069_F04	2	-2.2	-1.2	F2R	coagulation factor II (thrombin) receptor	NM_001999
P01076_B07	2	-2.2	-1.1	FAT	FAT tumor suppressor homolog 1 (Drosophila)	NM_005244
P01062_D11	2	-2	-1	SERPINE1	serine (or cysteine) proteinase inhibitor, clade E (nexin, plasminogen activator inhibitor type 1), member 1	NM_000600
P01071_A04	2	-1.7	-1.2	TFPI2	tissue factor pathway inhibitor 2	NM_006524
P01147_F05	1.9	-2.4	-1.1	COL11A1	collagen, type XI, alpha 1	NM_080633
P01138_G10	1.9	-2.1	-1	TNFRSF12A	tumor necrosis factor receptor superfamily, member 12A	NM_016633
P01109_B06	1.9	-1.9	-1	ACTN1	actinin, alpha 1	NM_001100
P01163_B07	1.9	-1.8	-1.1	ITGAV	integrin, alpha V (vitronectin receptor, alpha polypeptide, antigen CD51)	NM_002211
P01093_F03	1.9	-1.5	1.1	COL4A2	collagen, type IV, alpha 2	NM_001844
P01155_D07	1.8	-2.2	-1	ZYX	zyxin	NM_003466
P01118_H01	1.8	-1.5	-1.1	GPR56	G protein-coupled receptor 56	NM_005688
<u>Down-regulated Genes</u>						
P01091_E06	-4.9	4.3	1.4	CLDN4	claudin 4	NM_001306
P01136_D03	-4.1	3.5	1.3	PPL	periplakin	NM_002700
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

Table 2 (continued)

Genes involved in Cell cycle, Proliferation, and Apoptosis**MOL# 29025**

Gene ID	TGFβ	TGFβ+SD-208	SD-208	Symbol	Name	Accession
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 alpha polypeptide	NM_033023
P01076_A11	4	-3.1	1	SPOCK	sparc/osteonectin, cwcw and kazal-like domains proteoglycan (testican)	NM_004598
P01072_F03	3.8	-3.2	-1	LTBP2	latent transforming growth factor beta binding protein 2	NM_000422
P01071_H06	3.3	-2.5	-1.2	PDGFB	platelet-derived growth factor beta polypeptide (simian sarcoma viral (v-sis) oncogene homolog)	NM_002600
P00777_A11	2.6	-2.2	-1.1	CYR61	cysteine-rich, angiogenic inducer, 61	NM_001555
P01162_A11	2.4	-2.1	-1.4	PGF	placental growth factor, vascular endothelial growth factor-related protein	NM_002633
P01110_E09	2.4	-1.8	-1.2	PPP1R13L	protein phosphatase 1, regulatory (inhibitor) subunit 13 like	NM_006666
P01074_B06	2.2	-1.7	-1.1	NOLC1	nucleolar and coiled-body phosphoprotein 1	NM_004744
P01102_D05	2.1	-2.1	-1.1	TNFRSF11B	tumor necrosis factor receptor superfamily, member 11b (osteoprotegerin)	NM_002544
P01104_C09	2.1	-1.9	-1.1	CKLF	chemokine-like factor	NM_016955
P01099_G10	2.1	-1.5	-1.1	DAB2	disabled homolog 2, mitogen-responsive phosphoprotein (Drosophila)	NM_001344
P01069_F04	2	-2.2	-1.2	F2R	coagulation factor II (thrombin) receptor	NM_001999
P01068_C05	2	-1.6	-1.2	IER3	immediate early response 3	NM_003899
P01106_G06	2	-1.5	-1	PTH1H	parathyroid hormone-like hormone	NM_198966
P01069_E03	1.9	-2.2	-1	CDK6	cyclin-dependent kinase 6	NM_001255
P01138_G10	1.9	-2.1	-1	TNFRSF12A	tumor necrosis factor receptor superfamily, member 12A	NM_016633
P01081_E11	1.9	-1.8	-1	MYC	v-myc myelocytomatosis viral oncogene homolog (avian)	NM_002466
P01063_F06	1.9	-1.6	-1.1	VEGFC	vascular endothelial growth factor C	NM_005422
P01065_B07	1.8	-1.6	-1.1	ARHGEF6	Rac/Cdc42 guanine nucleotide exchange factor (GEF) 6	NM_004844
Down-regulated Genes						
P01078_F08	-3.9	3	1.1	TP53I11	tumor protein p53 inducible protein 11	NM_006033
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_005744
P01136_C06	-2.4	2.2	1.1	TSPAN1	tetraspanin 1	NM_005722
P01064_F03	-2.2	2.1	1.1	TOB1	transducer of ERBB2, 1	NM_005744
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_002466
P01091_G06	-2.2	1.5	1.1	PARD6A	par-6 partitioning defective 6 homolog alpha (C.elegans)	NM_016944
P01100_F10	-2.1	2.3	1.1	FLT1	fms-related tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor)	NM_002016
P01065_F11	-2.1	2.1	-1	PPP1CA	protein phosphatase 1, catalytic subunit, alpha isoform	NM_206875
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, alpha	NM_000417
P01067_D03	-1.8	2.2	-1	BNIP3	BCL2/adenovirus E1B 19kDa 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

Gene ID	TGFβ	TGFβ+SD-208	SD-208	Symbol	Name	Accession
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 alpha polypeptide	NM_033023
P01071_H06	3.3	-2.5	-1.2	PDGFB	platelet-derived growth factor beta polypeptide (simian sarcoma viral (v-sis) oncogene homolog)	NM_002608
P01096_H11	2.9	-2.2	1.4	EPAS1	endothelial PAS domain protein 1	NM_001436
P00777_A11	2.6	-2.2	-1.1	CYR61	cysteine-rich, angiogenic inducer, 61	NM_001558
P01162_A11	2.4	-2.1	-1.4	PGF	placental growth factor, vascular endothelial growth factor-related protein	NM_002633
P01138_G10	1.9	-2.1	-1	TNFRSF12A	tumor necrosis factor receptor superfamily, member 12A	NM_016633
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, beta receptor III (betaglycan, 300kDa)	NM_003249
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, alpha-induced protein 2	NM_006299

The functional gene clusters identify genes that are regulated by TGFβ and affected by SD-208 in PANC-1: ECM remodeling, Cell Motility, and Adhesion; Cell Cycle, Proliferation, and Apoptosis ; Angiogenesis. Mean fold expression of TGFβ regulated genes in PANC-1 from quadruplicate hybridizations: control vs. TGFβ, 24 hours (TGFβ); TGFβ vs. TGFβ + SD-208, 24 hours (TGFβ + SD-208); control vs. SD-208, 24 hours (SD-208). Gene expression profiles were determined from cDNA microarrays as previously described (Kapoun et al., 2006). Functional groups were classified using Gene Ontology terms and published functions.

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Table 3 Reduced Incidence of Metastasis with SD-208 Treatment

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

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.

Figure 1

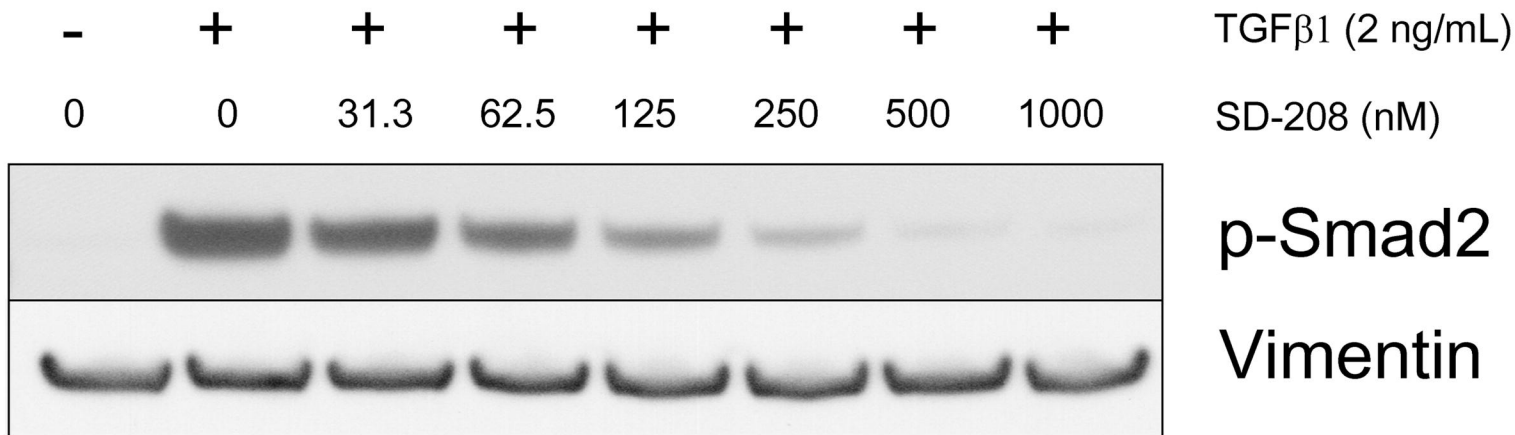
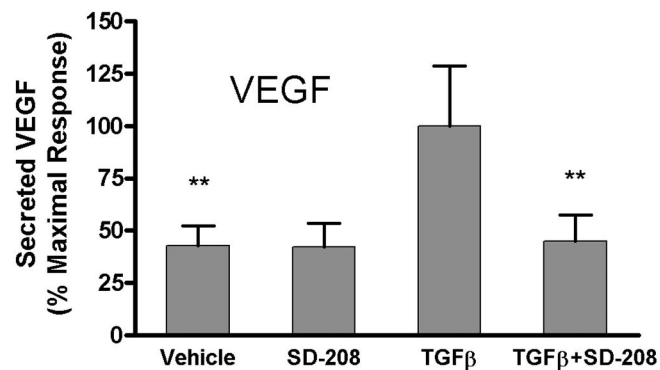
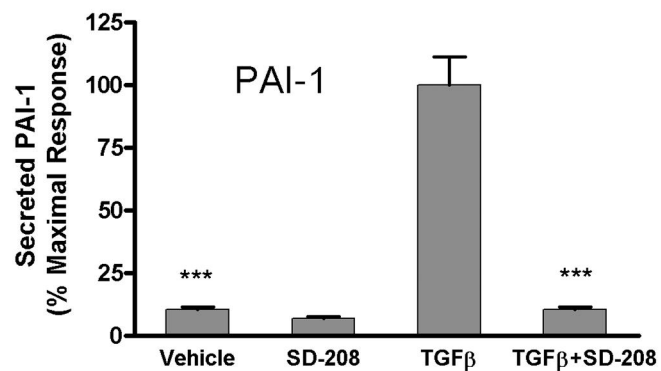
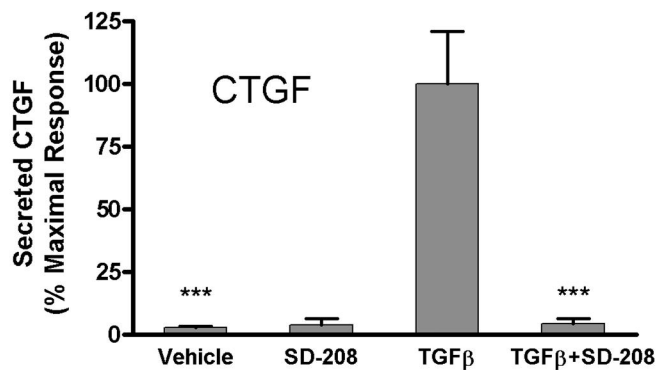
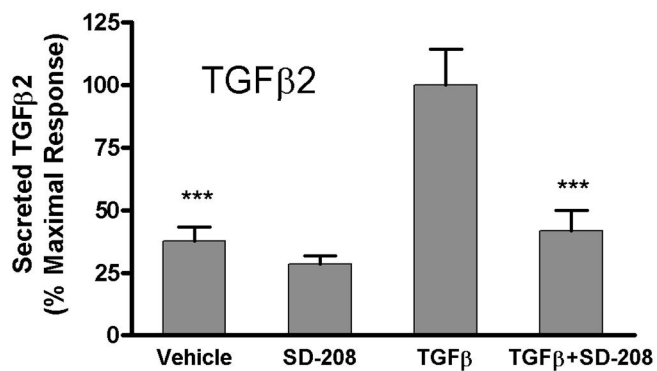
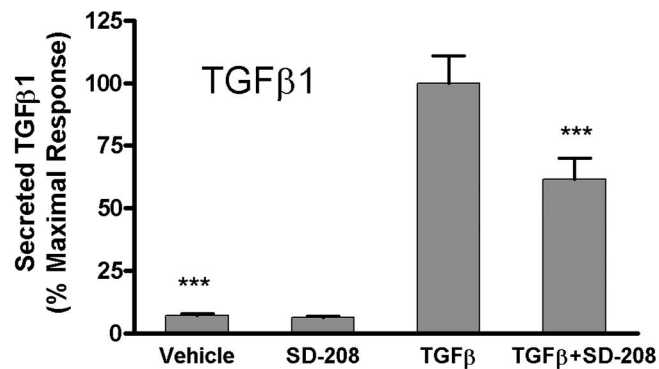


Figure 2



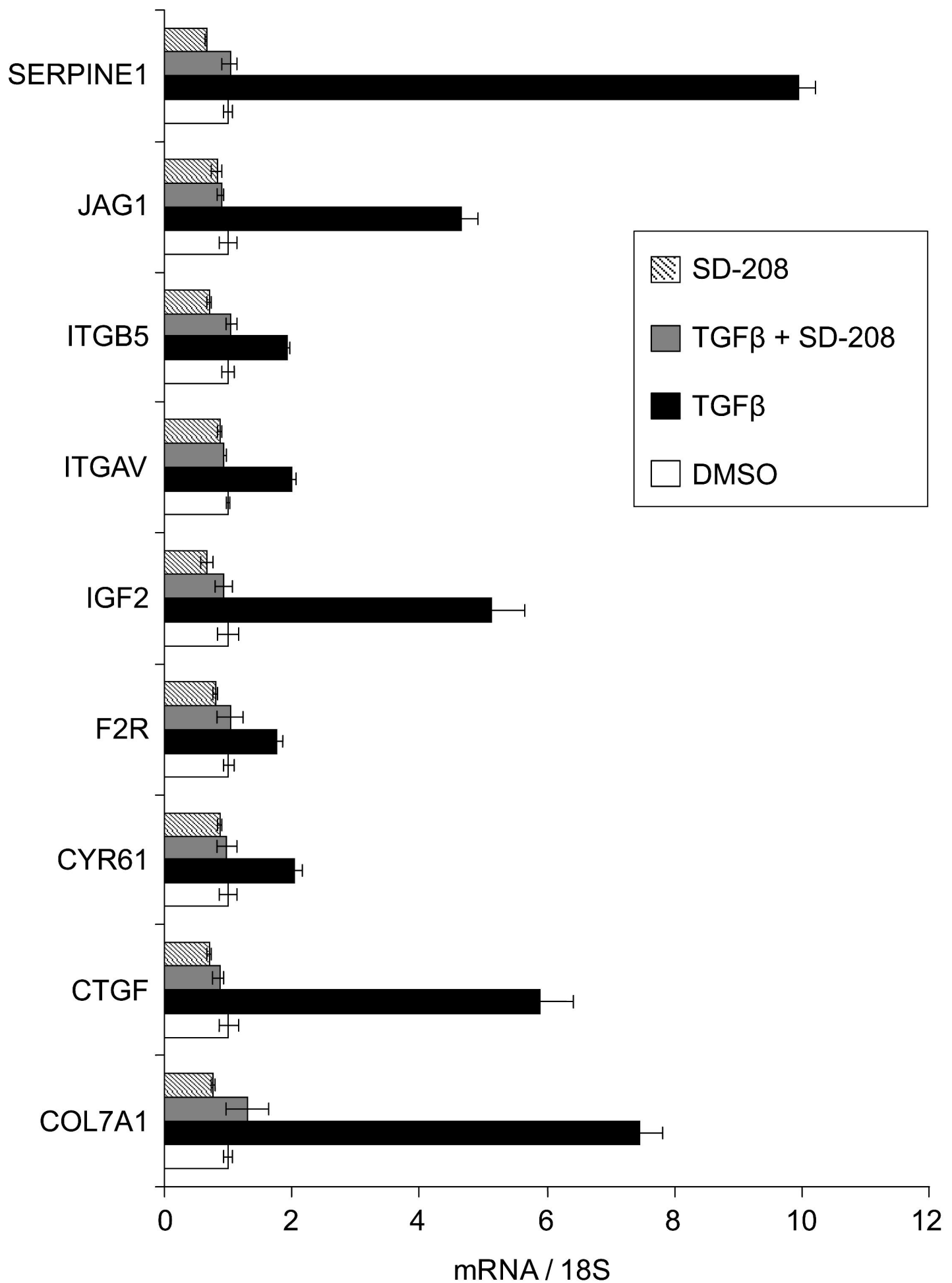


Figure 4

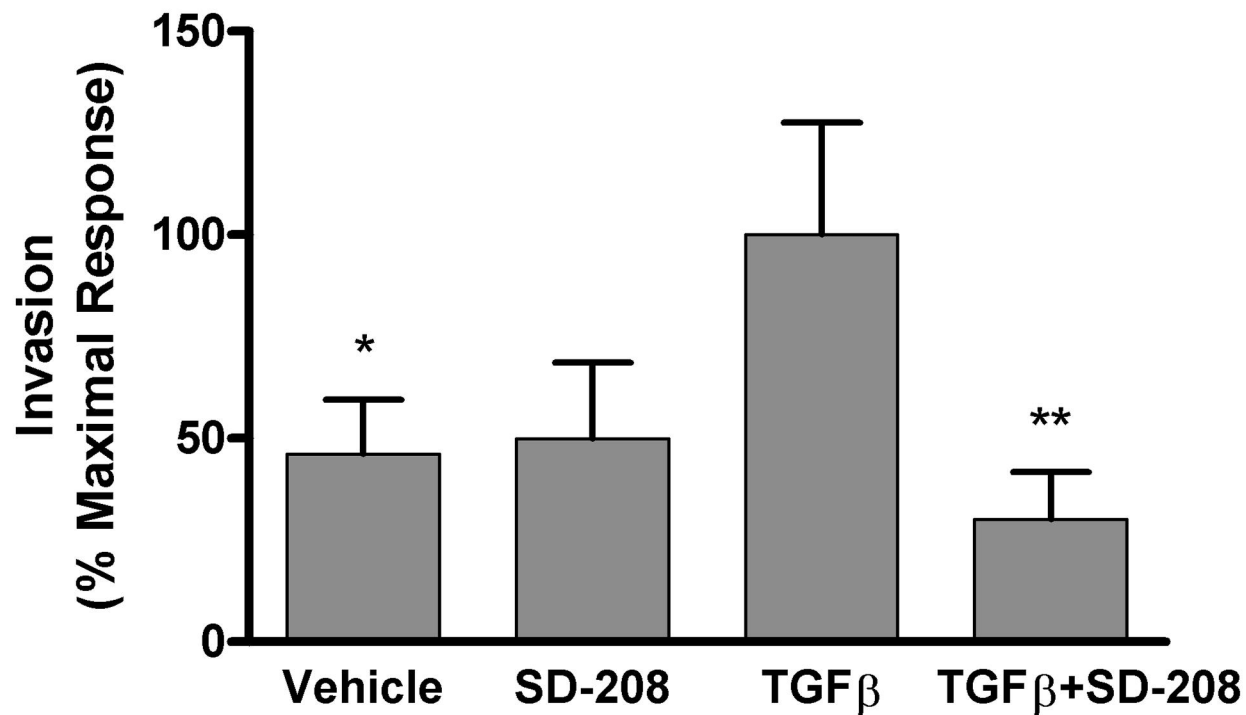
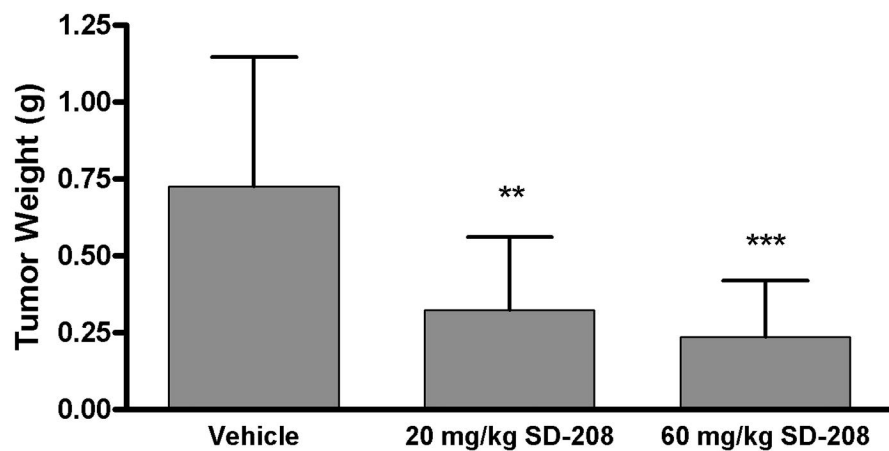


Figure 5

A



B

