Inhibition of STAT3\textsuperscript{Tyr705} Phosphorylation by Smad4 Suppresses Transforming Growth Factor \(\beta\)-Mediated Invasion and Metastasis in Pancreatic Cancer Cells

Shujie Zhao, Kolaparthi Venkatasubbarao, Jillian W. Lazor, Jane Sperry, Changqing Jin, Lin Cao, and James W. Freeman

Division of Hematology and Medical Oncology, Department of Medicine, University of Texas Health Science Center, San Antonio, Texas

Abstract

The role of Smad4 in transforming growth factor \(\beta\) (TGF\(\beta\))-mediated epithelial-mesenchymal transition (EMT), invasion, and metastasis was investigated using isogenically matched pancreatic cancer cell lines that differed only in expression of Smad4. Cells expressing Smad4 showed an enhanced TGF\(\beta\)-mediated EMT as determined by increased expression of vimentin and decreased expression of \(\beta\)-catenin and E-cadherin. TGF\(\beta\)-mediated invasion was suppressed in Smad4-intact cells as determined by in vitro assays, and these cells showed a reduced metastasis in an orthotopic model of pancreatic cancer. Interestingly, TGF\(\beta\) inhibited STAT3\textsuperscript{Tyr705} phosphorylation in Smad4-intact cells. The decrease in STAT3\textsuperscript{Tyr705} phosphorylation was linked to a TGF\(\beta\)/Smad4-dependent and enhanced activation of extracellular signal-regulated kinases, which caused an increase in serine phosphorylation of STAT3\textsuperscript{Ser727}. Down-regulating signal transducer and activator of transcription 3 (STAT3) expression by short hairpin RNA in Smad4-deficient cells prevented TGF\(\beta\)-induced invasion. Conversely, expressing a constitutively activated form of STAT3 (STAT3-C) in Smad4-intact cells enhanced invasion. This study indicates the requirement of STAT3 activity for TGF\(\beta\)-induced invasion in pancreatic cancer cells and implicates Smad4-dependent signaling in regulating STAT3 activity. These findings further suggest that loss of Smad4, leading to aberrant activation of STAT3, contributes to the switch of TGF\(\beta\) from a tumor-suppressive to a tumor-promoting pathway in pancreatic cancer. [Cancer Res 2008;68(11):4221–8]

Introduction

Transforming growth factor \(\beta\) (TGF\(\beta\)) are multifunctional cytokines that regulate numerous cellular processes, including cell growth, differentiation, and apoptosis. TGF\(\beta\) signal through a heterotrimeric receptor complex composed of type I and type II receptors. The activated TGF\(\beta\) type I receptor serine/threonine kinase phosphorylates downstream signaling molecules Smad2 and Smad3, which complex with Smad4. On phosphorylation, the Smad complex translocates to the nucleus where it binds DNA, associates with additional cofactors, and mediates the transcription or repression of target genes (1, 2). TGF\(\beta\)’s also signal through their activated receptors independent of Smads. Smad-independent signaling by TGF\(\beta\) is known to activate the Ras/extracellular signal-regulated kinase (ERK), TAK1/p38, RhoA/e-Jun NH\(2\)-terminal kinase, and phosphatidylinositol 3-kinase/AKT pathways (3).

The effects of TGF\(\beta\) signaling pathways can be both tumor suppressive and tumor promoting. These opposing effects of TGF\(\beta\) signaling have undergone intense investigation over the past several years (3, 4). The current dogma is that TGF\(\beta\) signaling is tumor suppressive in normal cells, but during tumor progression, TGF\(\beta\) signaling is somehow altered to favor tumor growth and metastasis (3–5). Work by the Moses group (6, 7) recently showed that loss of autonomous TGF\(\beta\) signaling by knockout of the TGF\(\beta\)-type II receptor caused an increase in metastasis in mouse mammary tumor virus transgenic mice, suggesting that TGF\(\beta\) may retain tumor-suppressive activities in some tumor types. In agreement with this, our laboratory showed that restoring an intact TGF\(\beta\) signaling pathway in a pancreatic cancer cell line enhanced the sensitivity of these tumor cells to radiation (8). Similar to other cytokines, TGF\(\beta\) signaling effects are mediated through both autocrine and paracrine pathways. In normal epithelial cells, the tumor-suppressive effects are largely autonomous and mediated through Smad-regulated genes. These effects include TGF\(\beta\)-induced expression of cyclin-dependent kinase inhibitors, including p21waf1/cip1 and p15, transcriptional repression of c-Myc, and regulation of genes that promote apoptosis (9, 10). Tumor cells often show increased expression of TGF\(\beta\), which through paracrine signaling promotes a microenvironment that favors tumor progression. These paracrine-mediated effects include immune suppression, angiogenesis, and fibrosis (4, 11).

The observation that TGF\(\beta\) promotes tumor progression in late-stage tumors suggests that blockade of this pathway has therapeutic potential. In this regard, small-molecule kinase inhibitors specific for the TGF\(\beta\) type I receptor (RIK1) and soluble peptides that block TGF\(\beta\) receptor binding have been developed and some of these are undergoing clinical trials (12). As a prerequisite for deciding which patients would most benefit from blockade of TGF\(\beta\) signaling, it seems important to understand how their tumors respond to TGF\(\beta\).

TGF\(\beta\) signaling is altered in human pancreatic cancer cells. More than half of these cancers show allelic deletion or inactivating intragenic mutations of the Smad4 gene DPC4 (13), thus blocking the canonical TGF\(\beta\) signaling pathway. TGF\(\beta\) signaling is known to induce epithelial-mesenchymal transition (EMT), an event that occurs normally during embryonic development and is thought to be a critical event contributing to tumor invasiveness and metastasis (14, 15). A recent report by Levy and Hill (16) indicates that TGF\(\beta\) may activate EMT in tumor cells, including pancreatic cancer cell lines that lack Smad4, although others have reported the
requirement of Smad signaling in EMT (17–19). We recently found that restoring Smad4 in pancreatic cancer cells deficient in Smad4 inhibited TGFβ-mediated invasion by in vitro assays (20). However, this study (20) did not establish whether Smad4 might influence EMT or whether the inhibition of invasion by Smad4 observed by in vitro assays might translate to inhibition of metastasis in vivo.

In the present study, we further examined the role of Smad4 in regulating TGFβ-induced EMT and metastasis using isogenically matched pancreatic cancer cell lines that differed only in Smad4 expression. TGFβ induced EMT in both Smad4-intact and Smad4-deficient cells, although the induction of EMT was more pronounced in cells expressing Smad4. Moreover, analysis from orthotopic mouse model of pancreatic cancer showed that Smad4 reduced metastasis to the liver.

Interestingly, cells that had an intact Smad pathway showed a reduced activation of signal transducer and activator of transcription 3 (STAT3). STAT3 is a member of the STAT family of transcription factors that are activated through various mitogenic signaling pathways, including interleukin-6 (IL-6; ref. 21), and by phosphotyrosine kinase receptors (22, 23). STAT3 is reported to show constitutive activation in different types of cancers (24) where it is involved in regulating genes that function in cell growth, cell survival, and angiogenesis (25). The inhibition of tyrosine phosphorylation of STAT3 Tyr705 was dependent on an enhanced activation of ERKs by Smad4-dependent TGFβ signaling. Furthermore, down-regulating STAT3 expression by short hairpin RNA (shRNA) prevented TGFβ-mediated invasion in Smad4-deficient cells. These studies are the first to establish that cross-talk between Smad4-independent TGFβ signaling and STAT3 activity contributes to TGFβ-mediated tumor progression.

Materials and Methods

Cell culture and reagents. Human pancreatic ductal adenocarcinoma cell lines BxPC3, Capan-2, MiaPaCa-2, CFPAC-1, and Panc-1 were obtained from the American Type Culture Collection (ATCC); UK Pan-1 was established in our laboratory (26). Human recombinant TGFβ1 was purchased from R&D Systems. The RIKI and mitogen-activated protein kinase/ERK kinase (MEK) inhibitor PD98059 were purchased from Calbiochem. SureSilencing shRNA-STAT3 plasmid was purchased from SuperArray Bioscience Corp., and the retroviral vectors pBabe/Smad4 and pBabe/STAT3-C were kindly provided by Bernard E. Weissman (University of North Carolina, Chapel Hill, NC) and Jacqueline Bromberg (Memorial Sloan-Kettering Cancer Center, New York, NY), respectively.

Expression of Smad4, STAT3-C, and shRNA-STAT3. pBabe/Smad4 or pBabe/STAT3-C plasmids were transfected into Phoenix packaging cells (ATCC) using Fugene 6 (Roche Applied Science) according to the manufacturer’s protocol. Cells were then infected with retroviral medium from the packaging cells 48 h after transfection. For knocking down STAT3, cells were transfected with shRNA-STAT3 plasmid using Fugene 6. The stable clones were selected with 1 μg/mL puromycin, and the expression levels of Smad4, STAT3-C, or STAT3 were determined by Western blot analysis.

Western blot analysis. Western blot analysis was performed as described previously (27). Primary antibodies used were as follows: Smad2/3 was from BD Biosciences; Smad4, E-cadherin, β-catenin, STAT3, and phosphorylated STAT3 Tyr705 were from Santa Cruz Biotechnology; vimentin was from Oncogene Research Products; and ERK1/2, phosphor-phorylated ERK1/2 Thr202/Tyr204, and STAT3 Ser727 were from Cell Signaling Technology. Phosphorylated Smad2 antibodies were purchased from Chemicon. Horseradish peroxidase–conjugated secondary antibodies were purchased from Amersham Biosciences.

Clonogenic assay. Single-cell suspension was plated at density of 50, 200, and 500 cells per 60-mm dishes and cultured for 2 wk. The cells were then stained with 0.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-

4222 www.aacrjournals.org

Figure 1. Expressing Smad4 reduces the tumorigenic properties of BxPC3 cells. A, expression of Smads in human pancreatic cancer cell lines. Whole-cell lysates (50 μg) from each cell line were analyzed by Western blotting using antibodies to Smad2/3 and Smad4. β-Actin was used as a protein loading control. B, Smad4 cDNA was infected into BxPC3 cells using a retroviral vector (Bx/Smad4). Expression of Smad4 was detected by Western blot. p, parental cell; v, empty vector control cells. Panc-1 cells were used as a Smad4-positive control cell line. C, BxPC3 and Bx/Smad4 cells (pool) were seeded at 50, 200, and 500 per dish in 6 cm² dishes and treated with 5 ng/mL TGFβ1 or vehicle control for 2 wk and colonies were counted. Columns, mean of triplicate experiments; bars, SD. *, P < 0.05; **, P < 0.01.

zolium bromide in culture medium for 4 h and washed with PBS. Colonies were counted by Image-Pro Plus software (Media Cybernetics, Inc.).

Cell invasion assay. The invasive behavior of cells was analyzed by Matrigel invasion assays as described elsewhere (28, 29). Cells (3 × 10⁴ per well) were plated in a 24-well Matrigel invasion chambers (Becton Dickinson Labware) in 0.5 mL of serum-free medium. The outer chambers contained 0.7 mL of medium containing 10% fetal bovine serum. The cells were treated with 5 ng/mL of TGFβ1 or vehicle control for 48 h. The cells on the top surface of the membrane were gently removed with cotton swabs. The cells migrating to the undersurface of the membrane were fixed in 70% ethanol and stained with crystal violet. The invasion values were determined by eluting crystal violet in 10% acetic acid, and the absorbance was taken using a FLUOstar Optima Plate Reader at 584 nm.

Orthotopic pancreatic cancer mouse model. Cells were grown to 80% confluence, trypsinized, and resuspended in PBS. Cells (1 × 10⁶ per mouse) were directly injected into the tail of the pancreas of anesthetized 4-wk-old...
male nude mice (Harlan Corp.). Mice were housed and maintained in accordance with the standards of The University of Texas Health Science Center Animal Care and Use Committee. Six and 8 wk after implantation, mice were sacrificed. During necropsy, mice were evaluated grossly for evidence of tumor invasion and metastasis into the peritoneum and liver.

Histopathology and immunohistochemistry. Paraffin-embedded primary tumor and liver sections were stained with H&E by standard methods (Histology and Pathology Laboratory, University of Texas Health Science Center, San Antonio, TX). The primary tumor and liver metastasis were examined under the light microscopy. For immunohistochemistry, paraffin-embedded tissue sections were deparaffinized and antigen retrieval was done in 10 mmol/L sodium citrate buffer (pH 6.0). Immunohistochemistry was performed using the LSAB2 System-HRP (DakoCytomation) according to the manufacturer’s protocol.

Statistical analysis. Statistical analysis was performed using InStat software (GraphPad Software, Inc.). The significance of differences between groups was determined by one-way ANOVA followed by Bonferroni multiple comparison test or Fisher’s exact test.

Results

Restoring Smad4 in BxPC3 cells reduces clonogenicity and metastasis. The DPC4 gene, which codes for Smad4, is lost due to allelic deletion or intragenic mutation in more than half of pancreatic cancers (13). Smad4 was not expressed in three of six cell lines examined in this study (Fig. 1A). All six cell lines express Smad2 and Smad3, except that MIA PaCa-2 expressed a very low level of Smad3.

To determine the effects of Smad4 on TGFβ1-induced tumorigenicity, we introduced Smad4 into BxPC3 cells by infecting cells with a retroviral vector that possesses a Smad4 cDNA. Expression of Smad4 was confirmed by Western blot analysis (Fig. 1B). We compared BxPC3 (vector control cells; Fig. 1B, lane 3) with BxPC3/Smad4 (Fig. 1B, pool) cells for their clonogenic potential. BxPC3/Smad4 cells showed a decrease in their capacity to form colonies compared with control BxPC3 cells, suggesting that expression of Smad4 restored the growth-inhibitory response of TGFβ (Fig. 1C).

An orthotopic mouse pancreatic cancer model was used to determine whether intact Smad signaling could suppress metastatic potential in vivo. Both BxPC3 and BxPC3/Smad4 cells formed primary tumors in the pancreas of mice. Primary tumors from mice were analyzed by weight and histology at 6 and 8 weeks after implantation (Fig. 2A and B). Although not significantly different, tumors from BxPC3 mice were slightly larger compared with BxPC3/Smad4 mice (Fig. 2A and B). Fifteen mice from each group were sacrificed at week 8 and examined macroscopically for peritoneal metastasis and microscopically for liver metastasis (Fig. 2C and D). Mice implanted with BxPC3 cells compared with mice implanted with BxPC3/Smad4 cells showed an increase in numbers of peritoneal metastasis (12 of 15 versus 4 of 15) and liver metastasis (9 of 15 versus 2 of 15; Fig. 2C).

Smad4 signaling enhances TGFβ-induced markers of EMT and ERK activation but suppresses tyrosine phosphorylation of STAT3. TGFβ induces EMT, which is associated with cancer cell...
invasiveness and metastasis (19). TGFβ-induced EMT is reported to require Smad signaling, although a recent report suggests that TGFβ may induce EMT in Smad4-deficient cells (16). Two sets of isogenically matched cell lines that differed only in Smad4 expression (BxPC3 and BxPC3/Smad4; Capan-2 and Cap/Smad4 cells) were treated with exogenous TGFβ1 and examined for molecular changes indicative of EMT. The induction of EMT by TGFβ requires activation of ERKs (30). As shown in Fig. 3A, BxPC3 cells that expressed Smad4 showed a greater level of phosphorylation of ERKs at 24 h after stimulation with TGFβ1 compared with the Smad4-deficient parental cells. The steady-state level of ERK phosphorylation was higher in Cap/Smad4 cells compared with the parental Smad4-deficient cell line Capan-2 possibly due to autocrine TGFβ signaling.

TGFβ caused a decrease in the expression of β-catenin and up-regulated the expression of vimentin in both BxPC3 and BxPC3/ Smad4 cells (Fig. 3B). The development of an EMT phenotype in Smad4-deficient parental BxPC3 cells was further confirmed by loss of β-catenin and E-cadherin from cell membranes and the induction of vimentin as determined by immunofluorescent staining (Supplementary Fig. S1). However, the induction of vimentin, a marker for EMT, was greater in Smad4 cells, suggesting that Smad-dependent signaling enhances TGFβ-induced EMT (Fig. 3B).

We recently reported that STAT3 activity was constitutively activated in many pancreatic cancer cells (31). Interestingly, TGFβ caused a decrease in the STAT3 phosphorylation in Smad4-restored BxPC3 and Capan-2 cells but not in the parental Smad4-deficient cells (Fig. 3B). IL-6 is a potent activator of STAT3. We determined whether TGFβ signaling could block IL-6-mediated activation of STAT3 in cells where Smad4 was restored. IL-6-mediated phosphorylation of STAT3 Tyr705 was inhibited in BxPC3/ Smad4-expressing cells but not in BxPC3 parental cells (Fig. 3C). The lack of detection of any tyrosine-phosphorylated STAT3 in cells not treated with IL-6 was due to the short exposure time required to detect STAT3 Tyr705 phosphorylation in IL-6–stimulated cells (Fig. 3C). To further determine the requirement of Smad4-dependent TGFβ signaling in suppressing STAT3 Tyr705 phosphorylation, we used a kinase inhibitor, RIKI, to block TGFβ signaling. The efficacy of RIKI to block TGFβ signaling was indicated by detecting a loss of phosphorylation of Stat3 following treatment of cells with TGFβ (Fig. 3D). Blockade of TGFβ signaling with RIKI prevented TGFβ-mediated suppression of phosphorylation of STAT3 Tyr705 in Smad4-restored cells (Fig. 3D), suggesting that suppressing STAT3 tyrosine phosphorylation by TGFβ acts through the TGFβ-receptor/Smad pathway.

### Inhibition of phosphorylation of STAT3 Tyr705 by Smad4-dependent signaling requires activation of ERK1/2

We further investigated the mechanism by which TGFβ suppressed tyrosine phosphorylation of STAT3 Tyr705 in cells that express Smad4. TGFβ is known to activate ERKs (32, 33). Previously, we showed that sustained ERK phosphorylation suppressed STAT3 Tyr705 phosphorylation while increasing the STAT3 Ser727 phosphorylation in PDAC cells (34). Based on these previous findings, we investigated whether the sustained activation of ERKs induced by TGFβ in pancreatic cancer cells might mediate the suppression of STAT3 Tyr705 phosphorylation. TGFβ treatment caused an increase in the

---

**Figure 3.** Smad4 signaling enhances TGFβ-induced markers of EMT and ERK activation but suppresses tyrosine phosphorylation of STAT3. A, Smad4 restoration causes enhanced activation of ERK1/2. BxPC3, Bx/Smad4, Capan-2, and Cap/Smad4 cells were treated with 5 ng/mL TGFβ1 for 24 h. Total cell lysates were analyzed by Western blotting for phosphorylated (pERK1/2) and total ERK1/2 and for Smads. B, Smad4 restoration enhances TGFβ-induced EMT but suppresses phosphorylation of STAT3 Tyr705. BxPC3 and Bx/Smad4 cells were treated with 5 ng/mL TGFβ1 or vehicle for 72 h. Left, expression of E-cadherin, β-catenin, vimentin, STAT3, pSTAT3 Tyr705, and Smad4 was analyzed by Western blotting; right, phosphorylation of STAT3 Tyr705 was also inhibited by restoring Smad4 expression in Capan-2 cells (Cap/Smad4). β-Actin was detected as a protein loading control. C, Smad4 restoration inhibited IL-6–mediated activation of STAT3. BxPC3 and Bx/Smad4 cells were treated with 5 ng/mL TGFβ1 for 24 h and then stimulated with 10 ng/mL IL-6 for 20 min. Activation of STAT3 Tyr705 was determined by Western blotting. Phosphorylation of Smad2 was used as a control for activation of TGFβ signaling. D, blockade of TGFβ signaling by RIKI reversed TGFβ-mediated suppression of phosphorylation of STAT3 Tyr705. Cells were pretreated with 1 μM RIKI for 6 h followed by 5 ng/mL TGFβ1 or vehicle for 48 h. Cell lysates were analyzed by Western blotting with the indicated antibodies. The efficacy of RIKI to block TGFβ signaling was assessed by the inhibition of phosphorylation of Smad2.
phosphorylation of ERK1/2 as well as an increase of STAT3Ser727 phosphorylation in Bx/Smad4 and Cap/Smad4 cells while inhibiting STAT3Tyr705 phosphorylation (Fig. 4A). To determine whether activation of ERKs is involved in Smad4-dependent suppression of STAT3Tyr705 phosphorylation, cells were treated with TGFβ and the MEK inhibitor PD98059. As expected, PD98059 treatment inhibited the phosphorylation of ERK1/2 (Fig. 4A). The inhibition of ERK1/2 phosphorylation was associated with a decrease of STAT3Ser727 phosphorylation while increasing STAT3Tyr705 phosphorylation. Moreover, TGFβ-induced inhibition of STAT3Tyr705 phosphorylation was reversed by PD98059 treatment (Fig. 4A). We further linked TGFβ signaling with suppression of tyrosine phosphorylation of STAT3Tyr705 using a kinase inhibitor, RIKI. RIKI treatment of these cells inhibited Smad2 phosphorylation, reversed TGFβ-mediated suppression of STAT3Tyr705, and reduced the phosphorylation of ERK1/2 in Smad4-expressing cells (Fig. 4B). Taken together, these observations suggest that TGFβ-mediated inhibition of STAT3Tyr705 phosphorylation requires Smad4-dependent activation of ERK1/2 in PDAC cells.

**STAT3Tyr705 phosphorylation is required for TGFβ-mediated invasion.** To examine the requirement of STAT3 in TGFβ-induced invasion, STAT3 expression was knocked down using a RNA interference approach (Fig. 5A). Knockdown of STAT3 in parental BxPC3 cells prevented TGFβ-induced invasion (Fig. 5A). The role of STAT3 in TGFβ-induced invasion was also determined in the Capan-2 cell model (Fig. 5B and C). As observed for BxPC3 cells (Fig. 5A), knockdown of STAT3 in Capan-2 cells inhibited cell invasion (Fig. 5B and C). Restoration of Smad4 in Capan-2 cells significantly reduced cell invasion, although it did not completely block exogenous TGFβ-mediated invasion. Conversely, expression of a constitutively active form of STAT3 (STAT3-C) in cells where Smad4 was restored dramatically increased the invasive properties of these cells (Fig. 5B and C). We further compared the phosphorylation status of STAT3Tyr705 in the orthotopic mouse models of BxPC3 and Bx/Smad4 cells by immunohistochemistry. BxPC3 tumors showed both strong cytoplasmic and nuclear staining of phosphorylation of STAT3Tyr705; in contrast, only cytoplasmic staining was detected in Bx/Smad4 tumors (Fig. 5D). These studies suggest that invasion may be suppressed in Smad4-intact cells by Smad4-dependent regulation of STAT3 activity. Therefore, cells that lose Smad4 show a constitutive activation of STAT3, which cooperates with Smad-independent TGFβ signaling to promote invasion and metastasis.

**Discussion**

In this study, we investigated the role of Smad4 in TGFβ-induced EMT, invasion, and metastasis of pancreatic cancer cells. This was accomplished by comparing isogenically matched pancreatic cancer cell lines that differ only in expression of Smad4. These...
studies indicate that Smad4 signaling was not required for developing an EMT phenotype, although the development on an EMT was enhanced in cells expressing Smad4. We previously showed that restoring Smad4 signaling inhibits TGFβ-mediated invasion in vitro (20), and here, we show that restoring Smad4 expression in Smad4-deficient pancreatic cancer cells reduces metastases in vivo. The mechanism by which Smad4 inhibited invasion was further investigated. In this regard, we found that Smad4 signaling down-regulated the tyrosine phosphorylation of STAT3 (STAT3Tyr705). This Smad4-dependent inhibition of tyrosine phosphorylation required a prolonged and Smad4-dependent activation of ERKs. Moreover, STAT3Tyr705 phosphorylation was required for TGFβ-mediated invasion in pancreatic cancer cells that are Smad4 deficient.

In normal cells, TGFβ signaling acts as a tumor-suppressive pathway by regulating cell growth and by maintaining sensitivity to apoptosis (3). During tumor development and progression, TGFβ signaling often switches from being tumor suppressive to promoting tumor invasion and metastasis (11). Alterations in TGFβ signaling in tumor cells is caused by several mechanisms, including allelic deletion or mutation of DPC4 (35), inhibition of Smad activation by expression of anti-Smads through inhibitory phosphorylation of Smads by Ras (36), and epigenetic silencing of TGFβ receptor genes or mutations of these genes (5, 37). Of these mechanisms, loss of DPC4 by allelic deletion or mutation occurs in >50% of pancreatic cancers (38, 39). We recently showed that TGFβ promotes tumor invasion in pancreatic cancer cells that are deficient in Smad4 expression (20). One of the mechanisms by which TGFβ signaling mediates tumor progression is by inducing an EMT (14, 15). EMT occurs normally during embryonic development and is thought to be a critical event in tumor progression by enhancing cell motility, invasion, and metastasis (9, 11). TGFβ-induced EMT is reported to require Smad signaling (18, 19), although a recent study by Levy and Hill

Figure 5. Knockdown of STAT3 inhibits TGFβ-mediated invasion. A, BxPC3 and BxPC3 clones where STAT3 was knocked down by small interfering RNA (siRNA; Bx/siSTAT3) were seeded in Matrigel invasion chambers and treated with vehicle or 5 ng/mL TGFβ1 for 48 h. Invasion values were measured by eluting the dye from the underside of inserts. Left, representatives of light microscopy photos (4× objective) of Matrigel invasion assay; right, quantification of the Matrigel invasion assays. Columns, mean of experiments performed in triplicate; bars, SD. **, P < 0.01; ***, P < 0.001. Inset, expression levels of STAT3 in Bx/siSTAT3 clones were determined by Western blotting. 

B, Capan-2 cells were infected with siRNA-STAT3 (Cap/siSTAT3) and Cap/Smad4 cells were infected with Flag-tagged STAT3-C (Cap/Smad4/STAT3-C), respectively. Expression levels of STAT3 or Flag-tagged STAT3-C were detected by Western blotting. C, Matrigel invasion assays were performed in Capan-2, Cap/siSTAT3, Cap/Smad4, and Cap/Smad4/STAT3-C cells. Left, quantitative data of invasion assays. **, P < 0.01, ***, P < 0.001, compared with control Capan-2 cells; **, P < 0.01, ***, P < 0.001, compared with TGFβ-treated Capan-2 cells; ***, P < 0.001, compared with control Cap/Smad4 cells; ***, P < 0.001, compared with TGFβ-treated Cap/Smad4 cells. Right, a representative of light microscopy photos (4× objective) of invasion assays from each cell line treated with vehicle control or 5 ng/mL TGFβ1 for 48 h. D, representative photos of immunohistochemistry (20× objective) of the orthotopic tumor sections from BxPC3 and Bx/Smad4 cells. Top, negative control stained with normal rabbit IgG; bottom, stained with pSTAT3Tyr705 antibody.
(16) suggests that TGFβ can induce EMT in cells that are deficient for Smad4. Our study confirms this latter report (16); however, Smad4-expressing cells show an enhanced TGFβ-induced EMT response. Contrary to the role of EMT in promoting invasion, cells expressing Smad4 showed an inhibition of invasion while exhibiting an enhanced EMT. Thus, these studies suggest that one mechanism that contributes to TGFβ-mediated tumor progression in pancreatic cancer cells is loss of Smad4 and that TGFβ-mediated EMT is not by itself sufficient to promote invasion. In agreement with our findings, a report by Bardeesy et al. (40) using transgenic mouse models of pancreatic cancer showed that Smad4 deficiency promoted tumor progression. Interestingly, tumors from these mice showed a more differentiated phenotype and did not show a pronounced EMT phenotype.

In contrast to our study, there is evidence in some tumor models that signaling by TGFβ in Smad4-intact cells enhances tumor progression. Deckers et al. (18) reported that Smad4 enhances bone metastasis in a mammary cancer model and studies by Ueda et al. (41) indicate that cross-talk of TGFβ/Smad4 with Her2 induces TGFβ-mediated cell motility and cancer progression. There are several different possibilities that may account for these seemingly different outcomes. These include that the effect of TGFβ/Smad signaling may influence bone metastases differently than that seen for liver metastasis generally observed in pancreatic cancer. It is also possible that the increased activity of Her2 seen in some breast cancers may effectively suppress Smad activity.

In the present study, we further compared the downstream pathways of TGFβ signaling in Smad4-deficient cells to the cells where Smad4 expression was restored. We show here for the first time that Smad4 is required for TGFβ-mediated suppression of the phosphorylation of STAT3Tyr705. STAT3 is a transcriptional activator and is reported to be constitutively activated in several tumor types, including in pancreatic cancer (31). STAT3 is reported to play a role in tumorigenesis by causing resistance to apoptosis and by promoting invasion (21, 42). Tyrosine phosphorylation (STAT3Tyr705) is implicated in STAT3 activation and DNA binding (34). In previous studies, we (34) and others (43) found that sustained activation of ERKs increases the serine phosphorylation of STAT3Tyr705, causing a subsequent decrease in the level of tyrosine phosphorylation of STAT3Tyr705. We found in this study that restoring Smad4 expression in pancreatic cancer cells BxPC3 and Capan-2 caused an enhanced phosphorylation of ERKs. We further linked TGFβ-dependent activation of ERKs with inhibition of tyrosine phosphorylation of STAT3Tyr705 by inhibiting ERK activation using a pharmacologic inhibitor.

One implication from this study relates to the use of TGFβ inhibitors for the treatment of pancreatic cancers. TGFβ type I receptor kinase inhibitors are entering clinical trials (4). The data presented in this article suggest that the presence or absence of an intact Smad pathway may play a role in the therapeutic efficacy of these inhibitors. Moreover, our studies suggest that loss of Smad4 that occurs in most pancreatic cancers contributes to a loss of autonomous TGFβ-mediated tumor suppressor activities and allows for TGFβ-mediated invasiveness. Specifically, these studies suggest that Smad4-dependent regulation of STAT3 activity plays a role in the tumor suppressor function of TGFβ in Smad4-intact cells and conversely that TGFβ/Smad4-independent signaling in Smad4-deficient cells cooperates with STAT3 to promote invasiveness. Future studies are necessary to understand the mechanism by which STAT3 and Smad4-independent TGFβ signaling cooperate to promote invasion and metastasis.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
Received 8/31/2007; revised 3/6/2008; accepted 3/7/2008.
Grant support: Veterans Affairs Merit Award, NIH grant CA09622, San Antonio Area Foundation, and San Antonio Cancer Institute (J.W. Freeman).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

References

www.aacrjournals.org 4227 Cancer Res 2008; 68: (11). June 1, 2008


Inhibition of STAT3$^{\text{Tyr705}}$ Phosphorylation by Smad4 Suppresses Transforming Growth Factor $\beta$–Mediated Invasion and Metastasis in Pancreatic Cancer Cells

Shujie Zhao, Kolaparthi Venkatasubbarao, Jillian W. Lazor, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/68/11/4221

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2008/05/20/68.11.4221.DC1

Cited articles
This article cites 42 articles, 20 of which you can access for free at:
http://cancerres.aacrjournals.org/content/68/11/4221.full#ref-list-1

Citing articles
This article has been cited by 10 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/68/11/4221.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.