TGF-β regulates invasive behavior of human pancreatic cancer cells by controlling Smad expression

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Abstract

Introduction: To investigate the role of Smads in tumor cell activation, we examined changes in Smad expression as well as changes in proliferative and invasive behaviors in transforming growth factor-β (TGF-β) – stimulated pancreatic cancer cells.

Material and methods: Expression of TGF-β receptor type I (TβR-I) and type II (TβR-II) was determined using RT-PCR and Western blot analysis in the human pancreatic cancer cell lines BxPC-3, Capan-2, and PANC-1. TGF-β-mediated changes in Smad mRNA expression were examined using quantitative real-time RT-PCR. Proliferation of pancreatic cancer cells was monitored using an MTT assay and cell counting. Invasive behavior was examined using a Matrigel double-chamber assay.

Results: TβR-I and TβR-II were expressed in all three cell lines studied here at the mRNA and protein level. Smad2/3 mRNA expression was decreased after TGF-β stimulation in all three cell lines, while Smad4 mRNA expression remained unchanged. Smad6/7 mRNA expression was also attenuated in all three cell lines. TGF-β enhanced the invasive capacity of all three cell lines, but had no effect on the proliferative behavior. Anti-TβR-II antibody inhibited this TGF-β-enhanced invasive potential in pancreatic cancer cells.

Conclusions: The Smad pathway, particularly down-regulation of Smad2/3 and Smad6/7, may be responsible for TGF-β-induced invasion of human pancreatic cancer cells.

Key words: tumor invasion, pancreatic cancer, growth factor.

Introduction

Pancreatic cancer has an extremely poor prognosis, and its incidence has increased in many countries in recent years [1-3]. Less than 5% of patients with pancreatic adenocarcinomas survive more than 5 years. The poor response of advanced pancreatic cancer patients to treatment is attributable to the infiltrative property of pancreatic cancer cells, poor therapeutic options, and the current incomplete knowledge concerning the pathogenesis and biology, particularly the aggressive behavior, of this tumor type [4-7].

Transforming growth factor-β (TGF-β) is a multifunctional protein that regulates a variety of cellular functions including cell proliferation, differentiation, migration, and apoptosis under physiological and pathological conditions [8-10]. In humans, the TGF-β family is represented by three ligands, TGF-β1, TGF-β2, and TGF-β3, which exert their activity through

a family of transmembrane serine/threonine kinase receptors that includes TGF-β receptor type I (TβR-I) and type II (TβR-II) [11-15]. TGF-β plays a complex role in tumorigenesis, as it has both tumor suppressive and oncogenic activities [16-20].

Smad proteins represent a group of molecules that function as intracellular signaling mediators and modulators of TGF-β signaling. Smads can be classified into three groups: receptor-regulated Smads [21-23], common mediator Smads [24, 25], and inhibitory Smads [26, 27]. Activated TβR-I and II induce phosphorylation of Smad2 and Smad3, which form a hetero-oligomeric complex with Smad4. In response to TβR activation, these complexes accumulate in the nucleus, where they regulate transcriptional responses together with additional DNA-binding cofactors [28]. Smad6 and Smad7 are intracellular antagonists for TGF-β signaling. Smad7 associates with activated TβRs and hinders the activation of Smad2 and Smad3 by preventing their interaction with activated TβRs and consequent phosphorylation [29, 30].

In this study, we investigated changes in Smad protein expression as well as changes in Smad messenger RNA (mRNA) levels using quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis to elucidate the role of Smads in TGF-β signaling in pancreatic cancer cell lines. We also examined changes in the proliferative and invasive behavior of pancreatic cancer cells following TGF-β stimulation. Our results suggest that negative feedback elicited by the Smad signaling pathway, particularly that accompanied by marked Smad2/3 and Smad6/7 down-regulation, may be responsible for the TGF-β-mediated tumorigenesis observed in human pancreatic cancer cells.

Material and methods

Cell culture

Pancreatic cell lines BxPC-3, Capan-2, and PANC-1 were derived from the American Type Culture Collection (Rockville, MD, USA). BxPC-3 cells were maintained in RPMI 1640 medium (Gibco BRL, Eggenstein, Germany) supplemented with 10% fetal calf serum (FCS). Capan-2 and PANC-1 cells were maintained in Dulbecco's modified Eagle's medium (Gibco BRL) with high glucose and 10% FCS. All cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

Reagents and antibodies

Recombinant TGF-β1 was provided by Sigma (St. Louis, MO, USA). The following primary antibodies were used: rabbit polyclonal anti-TβR-I (V-22) and anti-TβR-II (H-567), goat polyclonal anti-Smad2, mouse monoclonal anti-Smad4, and goat polyclonal anti-Smad7 antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); rabbit polyclonal anti-Smad3 and anti-Smad6 antibodies were purchased from Zymed Laboratories Inc. (South San Francisco, CA, USA); and mouse monoclonal anti-β-actin antibody was purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA).

TGF-β treatment

Cells (plated in flasks) were incubated for 3 days until 70-80% confluency was achieved, and were then washed with phosphate-buffered saline (PBS). The wash medium was replaced with serum-free medium for 1 h prior to treatment with TGF-β1 (10 ng/ml; Sigma).

RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was prepared from pancreatic cancer cells using an Isogen Kit (Nippon Gene, Tokyo, Japan), and RNA quantity was determined spectrophotometrically. Total RNA (1 µg) pretreated with DNaseI (Boehringer Mannheim, Germany) for 20 min at room temperature was denatured at 70°C for 10 min, chilled on ice, and reverse-transcribed into cDNA in a reaction mixture containing 10 mM dithiothreitol, 0.5 mM dNTPs, 1st-standard buffer, and 1U Superscript II (Invitrogen, San Diego, CA, USA) at 42°C for 60 min. The reaction was terminated by heating at 72°C for 10 min. For PCR, 1 µl of the reaction mixture was used. The sequences of sense and antisense primers used in PCR reactions are shown in Table I.

The amplification reactions were performed using a DNA Thermal Cycler (Perkin-Elmer, Norwalk, CT, USA; Model 2400). PCR analysis was performed for 40 cycles as follows: 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min. DNA fragments amplified by PCR were resolved by electrophoresis on 1.0% agarose gels containing ethidium bromide.

Western blot analysis

For detection of TβR-I, TβR-II, and Smad proteins, Western blot analyses were performed following SDS-polyacrylamide gel electrophoresis (PAGE). Pancreatic cancer cells were seeded at a density of 2×10^5 on 60-mm dishes in media containing 10% FCS, and subsequently starved in media with 0.5% FCS for 24 h. Cells were incubated with serum-free media for 2 h, and total cell lysates were prepared. The lysates were separated by 8% SDS-PAGE, transferred to polyvinylidene difluoride membranes (Immobilon PVDF; Nihon Millipore Ltd., Tokyo, Japan) and immunoblotted with each antibody. Specific binding was detected with the Enhanced Chemiluminescence System (ECL; Amersham Life Science Ltd., Buckinghamshire, United Kingdom). Anti-TβR-1 and anti-TβR-II antibodies were diluted 1:500 and anti-Smad antibodies were diluted 1:1000.

Table I. Sense and antisense PCR primers

Target mRNA	Primers	Product size (bp)	GenBank Accession No.
TβR-I	5'-tcgtctgcatctcactcat-3' 5'-gataaatctctgcctcacg-3'	342	NM 004612
TβR-II	5'-gcacgttcagaagtcggtta-3' 5'-gcggtagcagtagaagatga-3'	493	D50683
Smad2	5'-aagaagtcagctggtgggt-3' 5'-gcctgttgtatcccactga-3'	246	AF027964
Smad3	5'-cagaacgtcaacaccaagt-3' 5'-atggaatggctgtagtcgt-3'	308	NM 005902
Smad4	5'-ccaggatcagtaggtggaat-3' 5'-gtctaaaggttgtgggtctg-3'	243	U44378
Smad6	5'-tgaattctcagacgccagca-3' 5'-gctcgaagtcgaacacctt-3'	386	AF035528
Smad7	5'-gccctctctggatatcttct-3' 5'-gctgcataaactcgtggtca-3'	320	AF015261
β-actin	5'-acaatgtggccgaggacttt-3' 5'-gcacgaaggctcatcattca-3'	260	M10277

TβR-I, TGF-β receptor type I; TβR-II, TGF-β receptor type II

Quantitative real-time RT-PCR

Real-time quantitative RT-PCR analyses were performed using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). In a Microamp Optical tube (ABI), 50 µl of solution containing SYBR Green PCR Master Mix (ABI) (25 µl), Multiscribe Reverse Transcriptase (ABI) (0.25 µl), RNase Inhibitor (ABI) (1.0 µl), total RNA (3.0 µl), sense primer (1.0 µl), antisense primer (1.0 µl) and sterilized distilled water (18.75 µl) were mixed. The sequences of sense and antisense primers used in PCR reactions are shown in Table I. Standard curves for GADPH (internal control) and RNA were obtained using their respective control kits (ABI). Amplifications were performed as follows: 48°C for 30 min, 95°C for 10 min, and 40 cycles at 95°C for 15 sec, 60°C for 60 sec, and 72°C for 30 sec. Real-time RT-PCR analysis was performed as described previously with minor modifications [8]. All RT-PCR data were captured using the Sequence Detection System version 1.7 software (PE, Applied Biosystems). For each cell line, an amplification plot was generated, from which a threshold cycle value was calculated, representing the PCR cycle number at which fluorescence was detectable above an arbitrary threshold [31, 32].

Pancreatic cancer cell proliferation assay following TGF-β treatment

MTT assay. Pancreatic cancer cells were seeded at a density of 5×10^3 cells/100 µl into 96-well plates and allowed to adhere overnight. Culture media were replaced, and cells were then cultured in medium alone (control) or in medium containing different concentrations of TGF-β. After 24 h, cells were cultured for 4 h with the metabolic substrate tetrazolium salt MTT reagent (10 µL 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) at a final concentration of 0.5 mg/ml. Formazan was detected spectorphotometically by a microplate reader (Molecular Devices, Sunnyvale, CA, USA) with a test wavelength of 570 nm and a reference wavelength of 630 nm.

Cell counting. Pancreatic cancer cells were seeded at a density of 1×10^5 cells/2 ml into 35-mm culture dishes. After a 24 h-incubation, cells were starved with 0.5% FCS for another 24 hours. Culture media was replaced to the fresh serum free media, and added TGF-β at concentration of 10 ng/ml. After 24 h incubation, cells were washed once with PBS, trypsinized, and centrifuged for 3 min at 1,500 rpm. The cell pellet was resuspended in 2 ml of PBS and viable cells were counted using a light microscope.

In vitro invasion assay

In vitro invasion assays were performed using a Matrigel invasion chamber (BD Biosciences Discovery Labware, Bedford, MA, USA). Pancreatic cancer cells (2×10^5) were seeded in the upper chamber in serum-free media in the presence or absence of TGF-β1 (10 ng/ml). After a 24 h-incubation, the filter membrane fitting onto the upper chamber was removed, and cells that were lightly attached to the upper surface were wiped off with a cotton swab. Cells that invaded the reverse surface of the upper chamber membrane through the small pores were fixed with 80% ethanol, stained with Giemsa solution, and counted in five random microscope fields (×200). In some blocking experiments, 1.0 µg/ml anti-TβR-II antibody was added to the cancer cells for 30 min prior to TGF-β1 addition. Assays were run in triplicates using the same lot of Matrigel invasion assay kits.

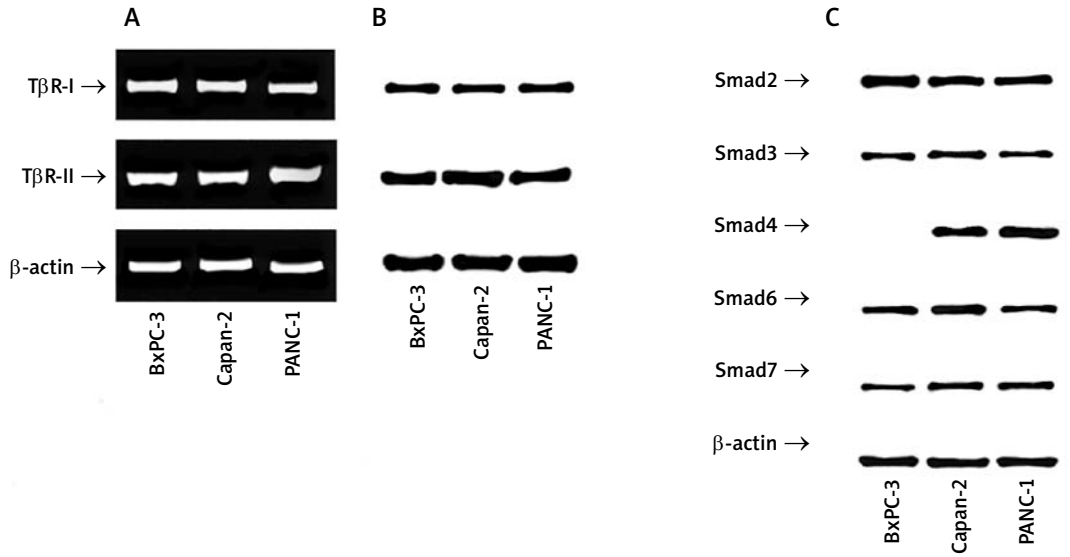

Figure 1. Expression of TGF-β receptor type I (TβR-I) and type II (TβR-II) in pancreatic cancer cells. **A** – Specific primers were used during RT-PCR to detect TβR-I and TβR-II mRNA. PCR products were separated on 1.0% agarose gels; **B** – TβR-I and TβR-II proteins were detected in pancreatic cancer cells; β-actin served as a loading control; **C** – expression of Smads in pancreatic cancer cells. Smad2, Smad3, Smad4, Smad6, and Smad7 proteins were detected in pancreatic cancer cells; β-actin served as a loading control

Statistical analysis

Mean values and standard deviations were calculated from three independent experiments, each performed in triplicates. For statistical analysis, Student's t test or one-way analysis of variance and post hoc test (Dunnett multiple comparison) were used p values <0.05 were considered to be significant.

Results

Expression of TGF-β receptors and Smads

To confirm mRNA and protein expression of TβR-I and II in pancreatic cancer cell lines, RT-PCR and Western blot analysis were performed. As shown in Figure 1A and 1B, all three cell lines expressed TβR-I and II at both the mRNA and protein level. Western blot analysis was used to compare the expression of the different Smad proteins in pancreatic cancer cells. Smad2, Smad3, Smad6, and Smad7 proteins were expressed in all three cell lines (Figure 1C). In contrast, Smad4 protein was expressed in Capan-2 and PANC-1 cells, but not in BxPC-3 cells.

Quantitative analysis of Smad expression

To investigate whether TGF-β had any effect on Smad mRNA expression, we performed real-time RT-PCR. The expression of Smad2 and Smad3 mRNA in all three pancreatic cancer cell lines significantly decreased following TGF-β treatment (Figure 2A and 2B). However, TGF-β had no effect on the expression of Smad4 mRNA in any of the cell lines (Figure 2C). The expression of Smad6 and Smad7 mRNA significantly decreased in all three cell lines (Figure 2D and 2E).

Effect of TGF-β on proliferation and invasion of pancreatic cancer cells

We used an MTT assay and cell counting to examine the influence of TGF-β on pancreatic cancer cell proliferation. No significant difference was observed in the proliferation of pancreatic cancer cells treated with TGF-β (data not shown).

Next, the effect of TGF-β and anti-TβR-II antibody on the invasion of pancreatic cancer cells through a reconstituted basement membrane coated by Matrigel was evaluated. Pancreatic cancer cells treated with TGF-β showed an enhanced ability to invade through the Matrigel matrix (Figure 3). Addition of 1.0 μg/ml anti-TβR-II antibody diminished this TGF-β-stimulated invasive ability.

Discussion

Cancer cell proliferation is regulated by both positive and negative signaling, and loss of negative growth constraints may contribute to oncogenic processes both in normal and cancer cells [33]. TGF-β acts as a tumor suppressor at early stages, predominantly through its ability to induce growth arrest and apoptosis in the epithelial cells from which most human cancers originate [18, 19]. At late stages, tumor cells acquire resistance to the antiproliferative effects of TGF-β, but often remain responsive to TGF-β signaling, and frequently secrete TGF-β. For example, pancreatic tumors overexpress all known TGF-β isoforms, and this overexpression is correlated with decreased patient survival [34]. Furthermore, in late-stage tumors, TGF-β acts directly on tumor cells to increase motility, invasiveness, and metastasis, and

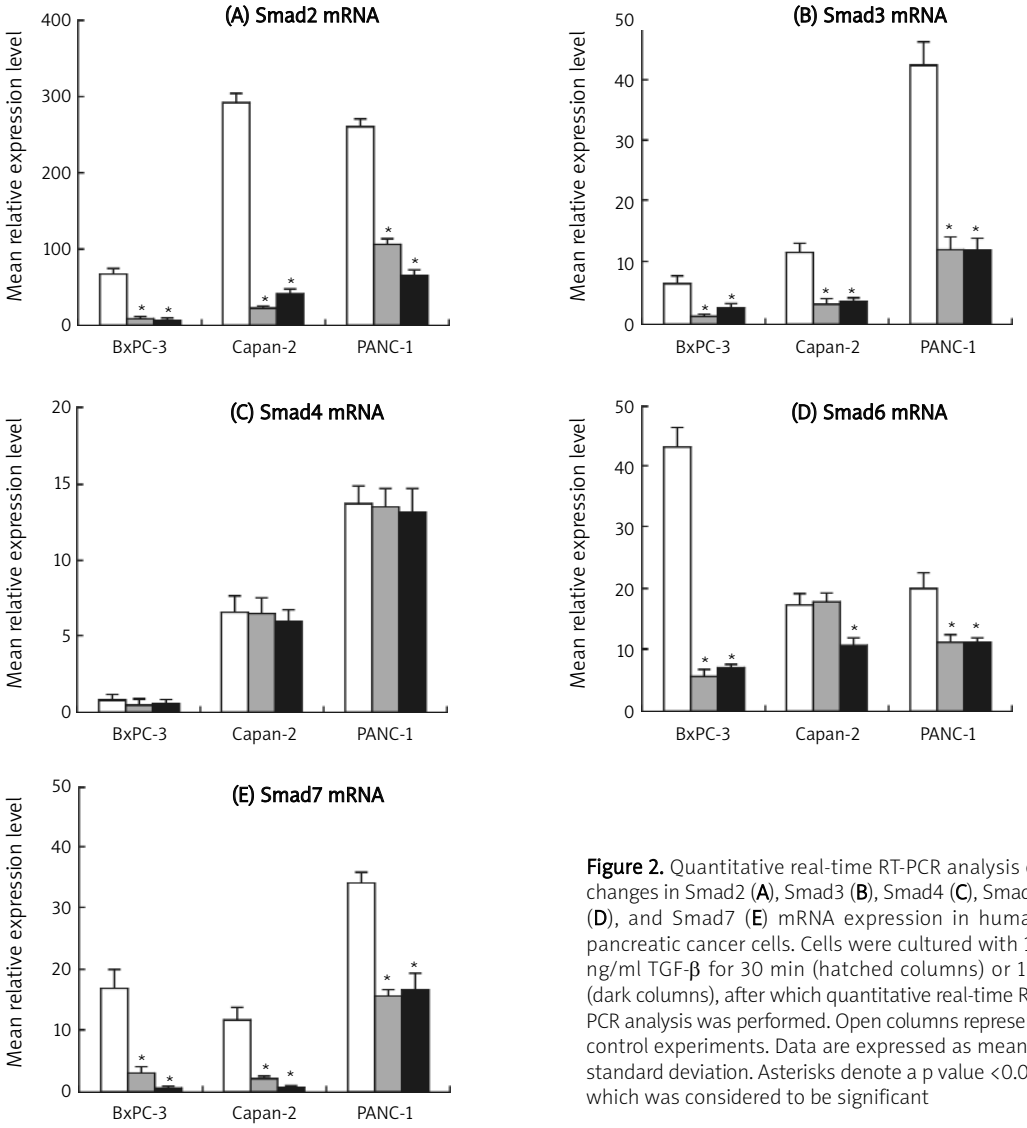

Figure 2. Quantitative real-time RT-PCR analysis of changes in Smad2 (**A**), Smad3 (**B**), Smad4 (**C**), Smad6 (**D**), and Smad7 (**E**) mRNA expression in human pancreatic cancer cells. Cells were cultured with 10 ng/ml TGF-β for 30 min (hatched columns) or 1 h (dark columns), after which quantitative real-time RT-PCR analysis was performed. Open columns represent control experiments. Data are expressed as mean ± standard deviation. Asterisks denote a p value <0.05, which was considered to be significant

on the surrounding stroma to suppress immune surveillance and increase angiogenesis [16, 17, 35]. In this study, TGF-β treatment significantly enhanced the invasive ability of pancreatic cancer cells, while it had no effect on proliferative behavior. These results suggest that TGF-β acts to promote the invasive ability of pancreatic cancer cells.

TGF-β-mediated signaling via the Smad pathway involves nuclear translocation of Smad2-Smad4 and Smad3-Smad4 complexes from the cytoplasm [23]. Smad4 is a tumor suppressor gene that is functionally inactivated in approximately 50% of pancreatic cancers [36]. In a recent report, use of RNA interference (RNAi)-mediated gene silencing revealed a novel TGF-β signaling pathway independent of Smad4 [31]. Smad6 and Smad7 are inhibitory Smad proteins that can downregulate TGF-β signaling [26, 27]. Dong et al. reported that both Smad7 deficiency and Smad3 up-regulation may be responsible for TGF-β hyper-responsiveness [37]. Moreover, Kleeff et

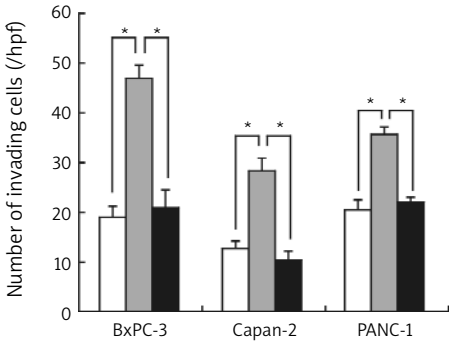

Figure 3. Effect of TGF-β on the invasive capacity of pancreatic cancer cells in Matrigel matrix and the inhibitory effect of a specific anti-TβR-II antibody. Following a 30-min incubation with (hatched columns) or without (dark columns) anti-TβR-II antibody, cells were cultured with 10 ng/ml TGF-β for 24 h in the inner chamber coated with Matrigel. Columns, mean number of invading cells per high power field (/hpf). Open columns represent control experiments. Asterisks denote a p value <0.05, which was considered to be significant

al. demonstrated that Smad6 and Smad7 enhanced tumorigenecity by rendering pancreatic cancer cells resistant to TGF-β-induced growth inhibition [38, 39]. However, the existence of an alternate TGF-β signaling pathway that is independent of all known Smads has also been reported [40]. Recently, there are some reports concerning Smad4 as a tumor suppressor, or in a subset of advanced tumors, Smad4 has an important role as tumor growth factor [41, 42]. Bardeesy et al. reported that Smad4 deficiency led to rapid progression of pancreatic tumors [43]. In this study, we investigated the influence of TGF-β on Smad mRNA expression. Expression of Smad2/3 and Smad6/7 mRNA was decreased in all three pancreatic cancer cell lines following TGF-β treatment, while Smad4 mRNA expression was unchanged. Furthermore, TGF-β enhanced the invasive capacity of all three cell lines even in BxPC-3 cells which don't have Smad4 expression. Based on these results, we propose that TGF-β enhances pancreatic cancer cell invasion by down-regulating the expression of Smad2/3 and Smad6/7 mRNA in pancreatic cancer cells.

TGF-β signaling can also be modulated by other factors. Ellenrieder et al. reported that the TGF-β--induced invasiveness of pancreatic cancer cells is mediated by matrix metalloproteinase-2 (MMP2) and the urokinase plasminogen activator (uPA) system [43]. These authors recently demonstrated that KFL11, an early-response transcription factor that mediates TGF-β-induced growth inhibition, also mediates a critical mechanism in TGF-β signaling that is inactivated by Erk-mitogen-activated protein kinase (MAPK) in pancreatic cancer cells [32]. p38 MAPK has also been reported as a key modulator of Smad phosphorylation and activation. Further, TGF-β-mediated activation of p38 MAPK has been shown to be necessary for Smad activation [44]. Interestingly, activation of MAPK signaling has been reported to have both positive and negative regulatory effects on Smads [45]. Also, there are informative reports that the blockade of TGF-β signaling and activated Ras signaling cooperate to promote pancreatic ductal adenocarcinoma progression [46] and the involvement of TGF-β in epithelial-mesenchymal transition in pancreatic carcinoma [47]. Broad evidence has been reported for a tight integration of Smad signaling within a complex network of crosstalks with other signaling pathways, such as phosphatidylinositol-3-kinase (PI3-K)/Akt and Rho pathways, that largely contributed to modify the initial Smad signals and allow the pleiotropic activities of TGF-β [48, 49]. Together, these reports indicate that TGF-β may exert both tumor suppressive and oncogenic effects by controlling Smad activation. We also speculate that these biological molecules may play a critical role in the activation of pancreatic cancer cell proliferation and invasion. Further studies concerning the relationship between these biological molecules and the TGF-β signaling pathway are necessary to confirm our results.

Conclusions

In summary, we have demonstrated that TGF-β induces changes in Smad mRNA expression and enhancement of invasive behavior in pancreatic cancer cells. Our results suggest that negative feedback elicited by the Smad signaling pathway, particularly marked Smad2/3 and Smad6/7 down-regulation, may be responsible for the TGF-β-mediated tumorigenesis observed in human pancreatic cancer cell lines.

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