RAS/ERK modulates TGFβ-regulated *PTEN* expression in human pancreatic adenocarcinoma cells

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Phosphatase and tensin homolog deleted on chromosome 10 (*PTEN*) is rarely mutated in pancreatic cancers, but its regulation by transforming growth factor (TGF)-β might mediate growth suppression and other oncogenic actions. Here, we examined the role of TGFβ and the effects of oncogenic K-RAS/ERK upon *PTEN* expression in the absence of *SMAD4*. We utilized two *SMAD4*-null pancreatic cell lines, CAPAN-1 (*K-RAS* mutant) and BxPc-3 (WT-*K-RAS*), both of which express TGFβ surface receptors. Cells were treated with TGFβ1 and separated into cytosolic/nuclear fractions for western blotting with phospho-SMAD2, SMAD 2, 4 phospho-ATP-dependent tyrosine kinases (Akt), Akt and PTEN antibodies. *PTEN* mRNA levels were assessed by reverse transcriptase–polymerase chain reaction. The MEK1 inhibitor, PD98059, was used to block the downstream action of oncogenic K-RAS/ERK, as was a dominant-negative (DN) *K-RAS* construct. TGFβ increased phospho-SMAD2 in both cytosolic and nuclear fractions. PD98059 treatment further increased phospho-SMAD2 in the nucleus of both pancreatic cell lines, and DN-K-RAS further improved SMAD translocation in *K-RAS* mutant CAPAN cells. TGFβ treatment significantly suppressed PTEN protein levels concomitant with activation of Akt by 48 h through transcriptional reduction of *PTEN* mRNA that was evident by 6 h. TGFβ-induced *PTEN* suppression was reversed by PD98059 and DN-K-RAS compared with treatments without TGFβ. TGFβ-induced PTEN expression was inversely related to cellular proliferation. Thus, oncogenic K-RAS/ERK in pancreatic adenocarcinoma facilitates TGFβ-induced transcriptional down-regulation of the tumor suppressor *PTEN* in a *SMAD4*-independent manner and could constitute a signaling switch mechanism from growth suppression to growth promotion in pancreatic cancers.

Introduction

Pancreatic cancer has the highest death rate to incident ratio of cancers in the USA (1). The pathogenesis of pancreatic cancer is complex and seems to involve environmental influences and genetic alterations.

Transforming growth factor (TGF)-β1 over-expression is associated with poor survival in patients with pancreatic cancer (2). It often suppresses early tumorigenesis but later enhances tumor progression (3–5). Although TGFβ1 exerts an inhibitory effect on cancer cell growth *in vitro*, it also stimulates the synthesis of the extracellular matrix and has been implicated in the regulation of cell differentiation, angiogenesis, immunosuppression and fibrosis (6).

Abbreviations: Akt, ATP-dependent tyrosine kinases; DN, dominant negative; *PTEN*, phosphatase and tensin homolog deleted on chromosome 10; PI3K, phosphatidylinositol-3-kinase; PCR, polymerase chain reaction; TGF, transforming growth factor.

TGFβ signals through a heterotetrameric receptor complex comprising type II receptors and type I receptors, both serine/threonine kinases (7), followed by a canonical SMAD-dependent signaling cascade (8). Several studies have shown that expression of dominant-negative (DN) transforming growth factor-β receptor type 2 constructs in mice lead to enhanced mammary tumorigenesis with or without carcinogens (9–11). Moreover, results obtained from conditional ablation of *SMAD4* in mouse mammary epithelium (12) confirm the importance of signaling through the TGFβ/SMAD pathway during tumor initiation and progression as suggested in earlier reports of enhanced colon tumorigenesis in *SMAD4*-null mouse models (13,14). These effects, however, are not limited to the mammary gland and colon since DN transforming growth factor-β receptor type 2 expression in the lung and skin also resulted in enhanced carcinogen-induced tumorigenesis (11,15).

The genetic profile of pancreatic cancer suggests contribution from both oncogenes and tumor suppressor genes. The tumor suppressor *SMAD4* is deleted from >64% of pancreatic cancers (16,17), removing this TGFβ signaling molecule from the majority of these tumors. *K-RAS* is mutated in >90% of pancreatic cancers, although the presence of oncogenic K-RAS is not specific to cancer as it can also be found in benign pancreatic lesions (18,19). Numerous reports have described a correlation between RAS transformation and deficient TGFβ responsiveness, particularly with regards to TGFβ anti-mitogenic responses. RAS transformation of lung, intestinal, liver or mammary epithelial cells confers resistance to growth inhibition by TGFβ (20,21). Kretzschmar *et al.* (22) showed that oncogenic RAS inhibits the TGFβ signal transduction pathway at the level of SMAD by RAS/ERK-induced phosphorylation of clusters of four Ser/Thr-Pro sites in the linker region of SMAD2 and SMAD3 and preventing accumulation of SMAD2 and SMAD3 in the nucleus and inhibiting TGFβ signaling.

Phosphatase and tensin homolog deleted on chromosome 10 (*PTEN*), also known as *MMAC1/TEP1*, is localized to chromosome 10q23 (23,24). It is a dual specificity phosphatase and antagonizes phosphoinositide-3-kinase (PI3K)/ATP-dependent tyrosine kinases (Akt) signaling pathway (25), and thus plays a functional role in cell cycle arrest and apoptosis (26,27). Description of germ line mutations and deletions of *PTEN* in two hereditary cancer predisposition diseases, Cowden Disease and the Bannayan–Riley–Ruvalcaba syndrome (28–31), point to a role of *PTEN* as a tumor suppressor gene in the pathogenesis of both benign and malignant growth. PTEN is one of the most frequently mutated proteins in a variety of cancers (23,24,32), but *PTEN* mutations rarely occur in pancreatic cancer (33). PTEN expression has been shown to be regulated by TGFβ1 in keratinocytes (34), and *PTEN* mRNA levels were also reduced in a model of TGFβ1 over-expressing transgenic mice that develop pancreatic fibrosis (35). Reduction of *PTEN* mRNA levels in pancreatic cancer cells following TGFβ1 treatment has also been reported (35). Although *PTEN* is not found mutated in pancreatic cancers, the reduction of its expression may give pancreatic cells an additional growth advantage.

Our present study focused on whether TGFβ modulates *PTEN* expression in *SMAD4*-null pancreatic cancer cells and whether that modulation was influenced by oncogenic K-RAS. This investigation may provide clues to the conversion of TGFβ function from that of a tumor suppressor in early carcinogenesis to one of a tumor promoter in more mature cancers. Both *SMAD4* loss and K-RAS activation are typical findings in pancreatic cancers. We found that TGFβ reduces PTEN expression in the absence of *SMAD4*, and oncogenic K-RAS and downstream effectors potentiate TGFβ-suppressed PTEN expression. Inhibition of K-RAS or its downstream effectors functionally 'switched on' PTEN expression where it may promote tumor suppression.

Materials and methods

Materials and reagents

PD98059, a MEK inhibitor, was purchased from PharMingen (San Diego, CA). A solution of PD98059 in dimethyl sulfoxide (Sigma, St Louis, MO) was prepared and used after diluting with medium for each assay. Dimethyl sulfoxide was also used as a blank control. DN construct of K-RAS was a generous gift from Dr Rik Derynck (University of California, San Francisco, CA).

Cell cultures

BxPC-3 and CAPAN-1 cells were obtained from American Type Culture Collection and they were maintained in RPMI and Dulbecco's modified Eagle medium (Gibco BRL, Gaithersburg, MA), respectively, supplemented with 10% fetal calf serum (Gibco BRL), without any antibiotics in an incubator at 37°C and 5% CO_2. To analyze the effect of TGFβ1 on PTEN expression and cell proliferation, pancreatic cancer cell lines were grown to 70–80% confluency in the corresponding medium containing 10% fetal bovine serum. Afterwards, cells were washed twice in phosphate-buffered saline, starved for 30 min in serum-free medium and finally treated for 24 and 48 h with 10 ng/ml TGFβ1 or medium alone.

Cell growth assay

For determination of cell number, 30 000 human pancreatic carcinoma cells per well were seeded onto six-well plates (Nunc, Wiesbaden, Germany), and incubated overnight in complete growth medium (RPMI 1640 + 10% fetal bovine serum). Complete medium was replaced with serum-depleted medium with or without TGFβ and/or inhibitors. Cell numbers from each well were determined every 24 h for 3 days. Up to 12 independent experiments were performed.

Transfection of the CAPAN-1 cells

One day before transfection, exponentially growing cells were trypsinized, and $1–2 \times 10^6$ cells were plated onto 10 cm Petri dishes. Cells were then transfected the next day with the DN K-RAS construct or empty vector. The DN construct is known to suppress activated RAS by blocking RAS guanine nucleotide exchange factors (36). Transfection was carried out using Transfast (Promega, Madison, WI). Briefly, plasmid DNA was mixed with serum-free medium, followed by transfection reagent at the charge ratio of 1:1. The mixture was allowed to react for 10–15 min, and it was transferred to the cells to be transfected. One hour after transfection, complete medium was overlaid on top of the cells and allowed to incubate for 48 h.

Total RNA extraction and semi-quantative reverse transcriptase–polymerase chain reaction

Total RNA extraction from the control and TGFβ-treated cells was conducted using Trizol reagent (Invitrogen Corporation, Carlsbad, CA). Cells grown on six-well plate were lysed with the reagent (1 ml per well). Lysates were combined with chloroform, mixed and the pellets precipitated with isopropanol and 75% ethanol and air-dried. Total RNA was then extracted and re-suspended in water. Two micrograms of total RNA were reversed transcribed into cDNA and amplified by polymerase chain reaction (PCR) for PTEN and SMAD4 expression (SuperScript II, Invitrogen Corporation). Briefly, following inactivation at 65°C for 10 min, 1 μl of the reaction mixture were incubated in buffer containing 0.2 mM concentrations of dATP, dCTP, dGTP, dTTP, 0.2 μM concentrations each of oligonucleotide primers, 3 mM $MgCl_2$ and a 10× buffer consisting of 200 mM Tris–HCl (pH 8.0), 500 mM KCl and 1 U Taq polymerase. PCR primers were designed to amplify a region spanning from nucleotides 117 to 741 of the PTEN gene (forward strand, 5′-CAGAA-AGACTTGAAGGCGTAT-3′ and reverse strand, 5′-AACGGCTGAGGGAA-CTC-3′), as described previously (37). The 624 bp fragment contains an NsiI restriction site allowing to distinguish this region from the PTEN pseudogene (37). PCR primers used to amplify SMAD4 were forward strand, 5′-AACGT-TAGCTGTTGTTTTTCAC-3′ and reverse strand, 5′-AGAGTATGTGAAGA-GATGGAG-3′. The conditions for PCR reactions of PTEN were as follows: denaturation at 95°C for 3 min and 35 cycles of 94°C for 30 s, 55°C for 30 s and 74°C for 4 min. Primers used for GAPDH were used as a loading control (forward strand, 5′-ACCACAGTCCATGCCATCAC-3′ and reverse strand, 5′-TCCACCACCCTGTTGCTGTA-3′).

Nuclear–cytoplasmic fractionation, western blotting analysis and immunoprecipitation

Cytoplasmic- and nuclear-enriched fractions were prepared by scraping 5×10^6 cells in 1.5 ml of hypotonic lysis buffer consisting of 10 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (pH 7.9), 2.5 mM $MgCl_2$, 0.2 mM ethylenediaminetetraacetic acid, 0.5 mM dithiothreitol, 40 mM NaF, 4 mM β-glycerophosphate, 0.2% Nonidet P-40, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM benzamidine HCl and 2 μg/ml each aprotinin and leupeptin.

Cells suspension was maintained on ice for 20 min and inverted several times. The nuclear fraction (P1) was isolated by centrifugation of the suspension at 3500g for 5 min, followed by washing in 0.5 ml of hypotonic buffer. The supernatant, representative of cytoplasmic fraction (S1), was added with NaCl to 0.14 M final concentration and clarified by 10 min centrifugation at 10 000g. Nuclear proteins were extracted from nuclear fraction by lysis in 0.3 ml hypertonic lysis buffer (50 mM Tris–HCl, 0.14 M NaCl, 0.25 M KCl, 1 mM ethyleneglycol-bis(aminoethylether)-tetraacetic acid, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 0.5 mM dithiothreitol, plus protease and phosphatase inhibitors), followed by 1 h rotation at 4°C, and clarified by 10 min centrifugation at 10 000g. Respectively, 50 μg of cytoplasmic and 30 μg of nuclear proteins were analyzed by western blotting and probed to specific antibodies. Phospho-SMAD2, phospho-Akt, ERK1/2 and phospho-ERK1/2 1:1000 (Cell Signaling Technology, Danvers, MA); SMAD2 1:1000 (BD Transduction Laboratories, San Diego, CA); SMAD4 (1 μg/ml Upstate Biotechnology, Lake Placid, NY); Akt 1:200 and PTEN 1:500 (Santa Cruz Biotechnology, Santa Cruz, CA); α-tubulin, histone H1 1:500 (Santa Cruz Biotechnology) and GAPDH 1:5000 (Ambion, Austin, TX) antibodies were utilized.

For total lysates, cells were washed three times with ice-cold phosphate-buffered saline, and the cells lysed using total lysis buffer (150 mM NaCl, 10 mM Tris–HCl, pH 7.8, 1 mM ethylenediaminetetraacetic acid, 0.5% Triton X-100, 1 mM sodium orthovanadate) containing protease inhibitors (1 μg/ml leupeptin and 100 μg/ml phenylmethylsulfonyl fluoride). Cells were then incubated at 4°C for 30 min with constant shaking, then scraped into microcentrifuge tubes and centrifuged at 12 000g for 15 min to remove insoluble material. The protein content in each sample was determined and adjusted. For immunoprecipitation, lysates were incubated with the immunoprecipitating antibody for 1 h at 4°C, followed by another 1 h incubation with protein A–agarose again at 4°C. Pellets were treated with 2× loading buffer (50 mM Tris, pH 6.8, 2% sodium dodecyl sulfate, 100 mM dithiothreitol, 0.2% bromophenol blue, 20% glycerol) and boiled for 5 min. Loading buffer supernatants from immunoprecipitation studies or cell lysates were then re-suspended in 2× gel loading buffer, boiled for 5 min and then separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (9% polyacrylamide). Resolved proteins were transferred overnight at 4°C onto a polyvinylidene difluoride membrane (Millipore Corporate, Billerica, MA), blocked with a 5% solution of skim milk for 30 min at room temperature, followed by further incubation with monoclonal antibodies to specific antibodies. After washing with phosphate-buffered saline with 1% Tween, the secondary antibody was applied to the membrane. After washing with PBS with 1% Tween, the membrane was treated with a chemiluminescent solution according to manufacturer's instructions and exposed to X-ray film. Densitometric analysis of the blots was performed using an AlphaImager digital imaging system (Alpha Innotech Corporation, San Leandro, CA).

Data analysis

All data are expressed as the means for a series of experiments ± SEM. Data were analyzed by either one-way analysis of variance followed by Student–Newman–Keul's post hoc test or by Student's t-tests for unpaired samples using Instat 3.0 (San Diego, CA). $P < 0.05$ was considered statistically significant.

Results

TGFβ mediates SMAD2 activation and nuclear translocation in SMAD4-null and ERK activated BxPc-3 and CAPAN-1 cells

TGFβ1 signaling usually requires SMAD4 together with SMAD2 activation, and nuclear translocation to transmit the TGFβ1-SMAD-responsive signaling event. To assess if TGFβ1 could mediate SMAD2 translocation in SMAD4-null cells, we treated BxPc-3 and CAPAN-1 cells with TGFβ, and observed SMAD compartmentalization using western blots after cytoplasmic/nuclear separation. As shown in Figure 1, TGFβ1 (10 ng/ml) caused activation and translocation of SMAD2 in WT-K-RAS BxPc-3 pancreatic cancer and oncogenic K-RAS CAPAN-1 cells. To address if activated RAS/ERK affected TGFβ-SMAD signaling, we inhibited MEK with the pharmacologic inhibitor, PD98059, which is activated downstream of K-RAS, and inhibited K-RAS itself with a DN-K-RAS construct. Pretreatment of CAPAN-1 cells with PD98059 or with transfection with the DN-RAS construct (Figure 2A), or pre-treatment of BxPc-3 cells with PD98059 (Figure 2B) in the presence of TGFβ markedly improved SMAD2 activation and translocation. Note that SMAD4 protein was not detectable which further confirmed that the cells are
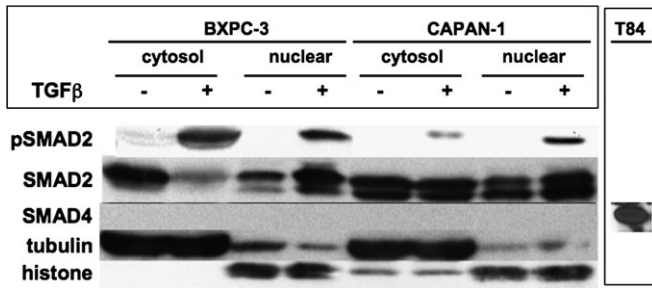
Fig. 1. Effects of TGFβ treatment on subcellular trafficking of SMAD2 in BxPc-3 and CAPAN-1 cells. Cells were treated with or without TGFβ (10 ng/ml) for 24 h. The cells were then lysed and separated into nuclear and cytoplasmic fractions. Note the appearance of phospho-SMAD2 in the nuclear and cytosolic fractions. Tubulin and histone H1 blots indicate purity of the fractions. *SMAD4* is not detectable in either cell line, confirming their *SMAD4*-null status. T84 colon cancer cells were electrophoresed separately as a positive control for SMAD4 expression.
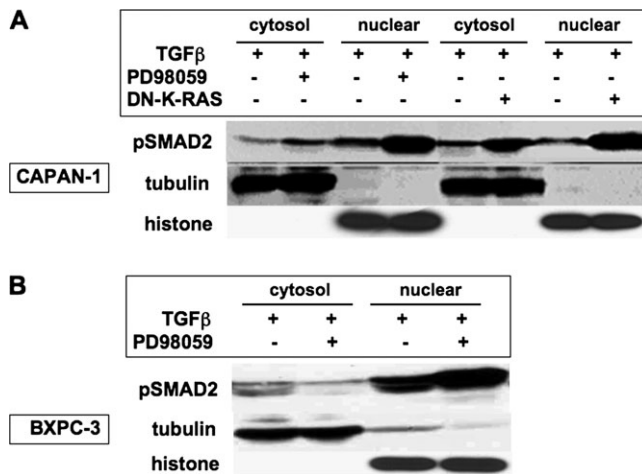

Fig. 2. Effect of RAS/ERK inhibition and TGFβ treatment on subcellular trafficking of SMAD2 in CAPAN-1 and BxPc-3 cells. Cells were either pretreated with or without PD98059 (50 μM) for 1 h (**A** and **B**), or transiently transfected with a DN construct of K-RAS (**A**) 48 h before treatment with TGFβ. Cell lysates were blotted with anti-phospho-SMAD2, tubulin and histone antibodies. Note that PD98059 and DN-K-RAS transfection improved TGFβ-induced p-SMAD2 nuclear translocation. Tubulin served as a loading control and indicated purity of the cytoplasmic fractions, whereas histone served as a loading control and purity of the nuclear fractions.

SMAD4 null (Figure 1). Thus, inhibition of the RAS/ERK pathway markedly improved translocation of SMADs in pancreatic cancer cells, indicating a modulatory role by RAS on SMAD2 translocation, even in the absence of *SMAD4*.

TGFβ treatment suppresses PTEN expression in SMAD4-null pancreatic cells

We examined the effects of TGFβ upon expression of *PTEN* in *SMAD4*-null pancreatic cancer cells. Both BxPC-3 and CAPAN-1 cells expressed abundant *PTEN* mRNA as determined by semi-quantitative reverse transcriptase–PCR (Figure 3). We observed minimal reduction of *PTEN* mRNA levels after 3 and 6 h of TGFβ1 treatment as compared with the untreated control. However, beginning at 6 h in BxPC-3 cells and by 24 h incubation with TGFβ1, a significant reduction of amplifiable *PTEN* mRNA was observed in both pancreatic cancer cell lines that recovered to some degree by 48 h after treatment (Figure 3). To verify the *PTEN* mRNA findings, we then examined the steady-state levels of PTEN protein. At the protein level in BxPc-3 cells, PTEN protein was also present and unchanged from controls through
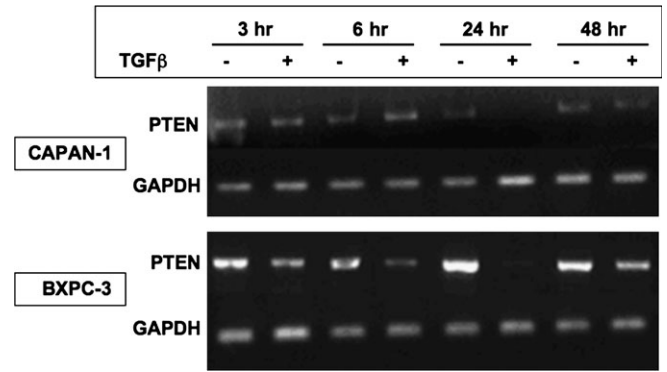

Fig. 3. TGFβ treatment down-regulates *PTEN* transcription. CAPAN-1 and BxPc-3 cells were treated with a single dose of TGFβ (10 ng/ml), and transcription of *PTEN* was assessed by semi-quantitative reverse transcriptase–PCR. Cells were lysed by Trizol reagents and mRNA was extracted. *PTEN* mRNA is markedly reduced by 24 h, with subsequent recovery of transcription by 48 h after treatment with TGFβ. Thus, *PTEN* is down-regulated transiently at the transcriptional level by 24 h after a single TGFβ treatment. GAPDH was used as loading control.

24 h after TGFβ1 treatment (Figure 4A). However, PTEN protein expression was reduced by 48 h after treatment in BxPc-3 (Figure 4A) and CAPAN-1 cells (not shown, but 48 h data shown in Figure 6B), following the reduced *PTEN* mRNA expression at 24 h. Therefore, TGFβ1 appears to mediate suppression of *PTEN* mRNA and protein in a *SMAD4*-independent manner. PTEN function antagonizes PI3K function, and one of the effectors of PI3K, Akt (25). To assess whether the TGFβ-induced reduction in PTEN expression coincides with an imbalance in function of PI3K, we determined the activation of phospho-Akt at the same time PTEN protein is suppressed. As shown in Figure 4B, the suppression of PTEN by TGFβ is simultaneous with Akt activation, demonstrating functional loss of PTEN.

Modulation of TGFβ-induced PTEN suppression by RAS/ERK constitutes an 'on/off' switch

As the RAS/ERK pathway is activated in pancreatic cancers, we assessed if RAS/ERK modulates the TGFβ-induced *PTEN* suppression observed in the *SMAD4*-null cell lines. PD98059, the MEK inhibitor, had no effect on amplifiable, semi-quantitative *PTEN* mRNA alone in CAPAN-1 (Figure 5A) and had no statistical significance to the small observed increase in *PTEN* mRNA in BxPc-3 cells with 50 μM PD98059 (Figure 5B). Treatment with PD98059, however, rescued TGFβ-induced *PTEN* mRNA suppression (Figure 5A and B). Similarly at the protein level, PD98059 had no effect on PTEN protein alone (Figure 6A). However, PD98059 reversed TGFβ-induced PTEN protein suppression (Figure 6A). Additionally, CAPAN-1 cells transfected with DN-K-RAS and treated with TGFβ for 48 h reversed the TGFβ-induced PTEN suppression (Figure 6B). Thus, RAS/ERK appears to facilitate TGFβ-induced PTEN suppression as blockage of ERK or RAS restores *PTEN* expression to pre-treatment levels. Here, RAS/ERK can 'switch' *PTEN* off through TGFβ signaling when activated and restore PTEN to 'on' through TGFβ signaling when RAS/ERK is inactivated.

TGFβ-induced pancreatic cancer cell proliferation parallels PTEN expression and modulation by RAS/ERK

We assessed the biological consequences of modulation of TGFβ-induced *PTEN* suppression in the *SMAD4*-null pancreatic cancer cell lines. Cells were incubated with TGFβ at concentrations shown to suppress *PTEN* expression both at protein and mRNA levels. As shown in Figure 7, addition of TGFβ for 72 h significantly affects cell growth. TGFβ treatment causes pancreatic cell proliferation when compared with cells that were not treated with TGFβ. We then examined the effect of ERK by inhibiting its upstream inducer MEK1 using
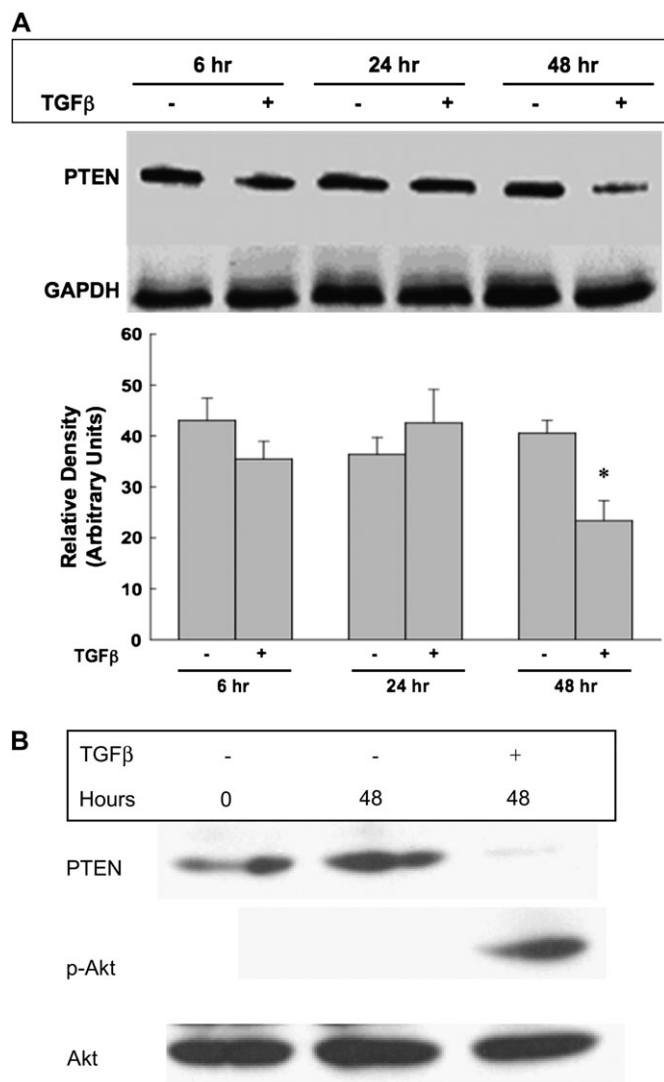
A

B

Fig. 4. TGFβ treatment reduces PTEN protein and activates Akt in *SMAD4*-null BxPc-3 cells. (**A**) Cell lysates were blotted with an anti-PTEN antibody. Note that PTEN protein is reduced by ∼50% at 48 h after a single TGFβ dose (10 ng/ml). Data shown are mean ± SEM of four experiments. *$P < 0.05$ versus control of 48 h. (**B**) The identical treatment condition in (**A**) also activated Akt at 48 h in BxPc3 cells. The data shown are representative of three experiments.

PD98059, using three increasing concentrations for inhibition. As shown in Figure 7, PD98059 (12.5 μM to 50 μM) caused a dose-dependent reduction in cell growth with TGFβ treatment. With increasing blockade of MEK1/ERK, TGFβ-induced proliferation was converted to TGFβ-induced growth suppression. Thus, the RAS/ERK pathway contributes to the ability of *SMAD4*-null pancreatic cancer cells to proliferate. RAS/ERK also modulates TGFβ-induced proliferation, which is paralleled by modulation of TGFβ-induced PTEN expression.

Discussion

Our investigation links two commonly affected signaling pathways in pancreatic cancer in the regulation of the tumor suppressor *PTEN*. *PTEN* mRNA suppression by TGFβ was first described in keratinocytes when *PTEN* was originally cloned (34), but the mechanisms for TGFβ-induced *PTEN* regulation had not been examined. On the other hand, PTEN expression blocked TGFβ-mediated cell invasion whereas PTEN absence increases TGFβ-induced invasion (38). The
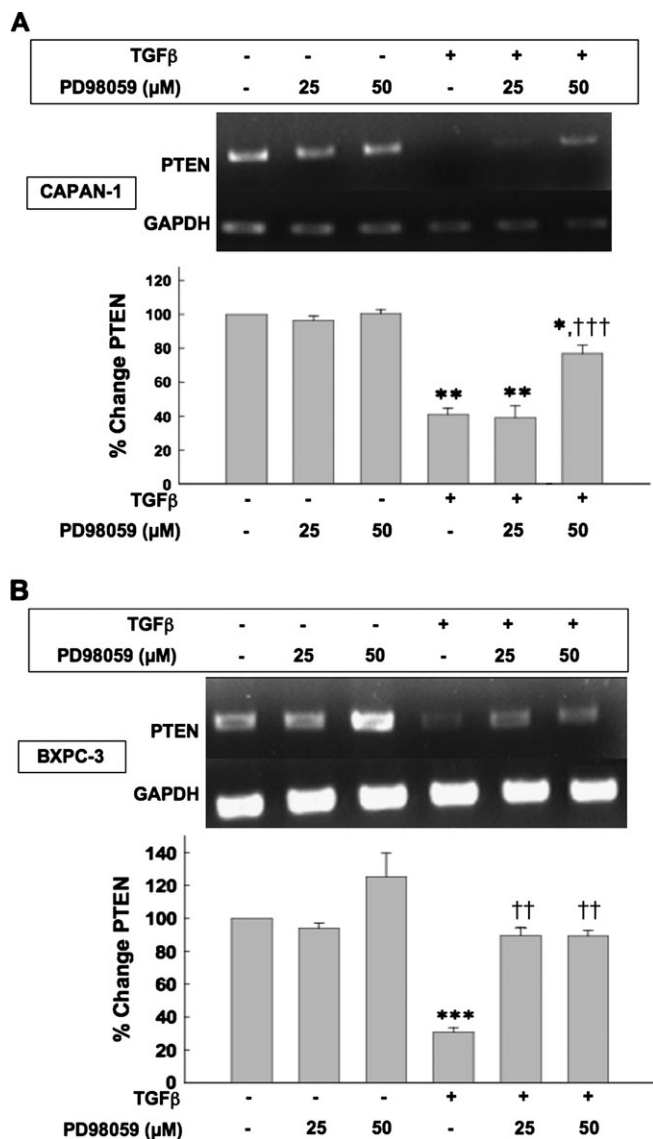
A

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Fig. 5. PD98059 reverses TGFβ-induced *PTEN* suppression at the transcriptional level. We treated both *SMAD4*-null CAPAN-1 (**A**) and BxPc-3 (**B**) cells with the MEK1 inhibitor PD98059 and performed semi-quantitative reverse transcriptase–PCR for *PTEN* mRNA at 24 h after TGFβ treatment. PD98059 had little effect on the basal levels of *PTEN* mRNA (**A** and **B**, lanes 2 and 3). However, MEK inhibition rescued TGFβ-induced *PTEN* transcriptional suppression in a dose-dependent manner (**A** and **B**, lanes 5 and 6). Thus, by blocking MEK/ERK activation, downstream of K-RAS activation, *PTEN* transcription can be switched back 'on' in the presence of TGFβ treatment. Data shown are means ± SEM of three to four experiments. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus control; ††$P < 0.01$ and †††$P < 0.001$ versus TGFβ treatment alone.

effects might be mediated by SMAD3, as this intracellular signaling molecule inducibly interacts with PTEN (38). *PTEN* is commonly affected in multiple cancers, often inactivated by genetic (23,24,33) or epigenetic (39) events during the pathogenesis of a particular cancer. This does not appear to be the case in pancreatic adenocarcinoma. PTEN protein expression in pancreatic cancers has previously been shown via immunohistochemistry to be reduced (35), but not due to genetic events (33). Here, we demonstrate that (i) TGFβ suppresses *PTEN* expression in *SMAD4*-null cells at the transcriptional level with subsequent reduction in protein expression and simultaneous increase in phospho-Akt; (ii) RAS/ERK signaling interferes with TGFβ-SMAD nuclear translocation, and SMAD2 translocation is improved
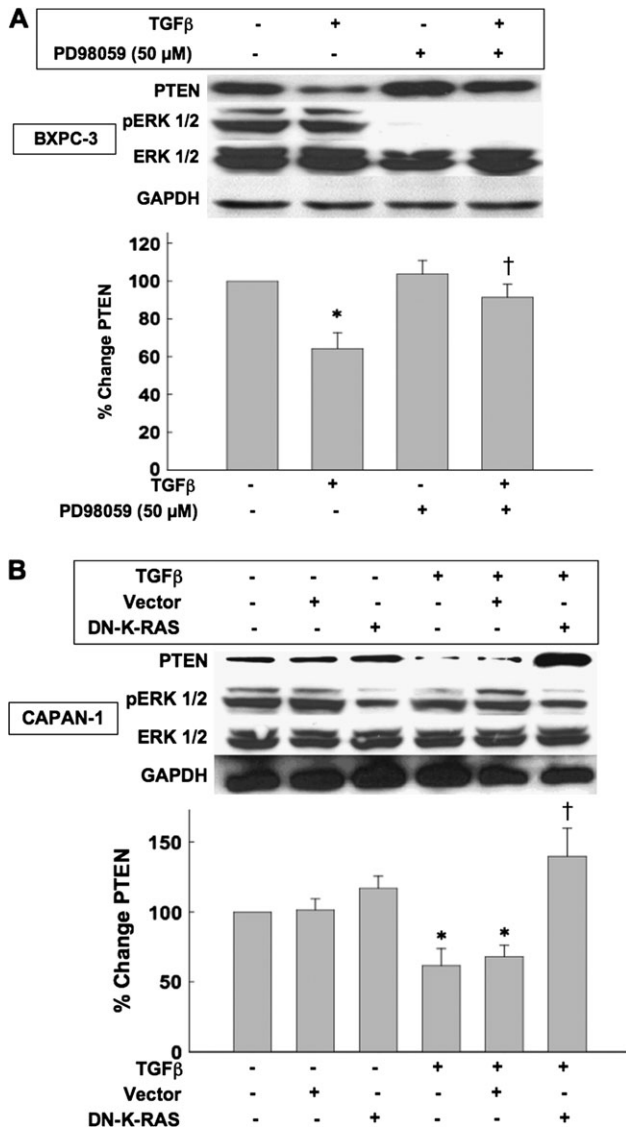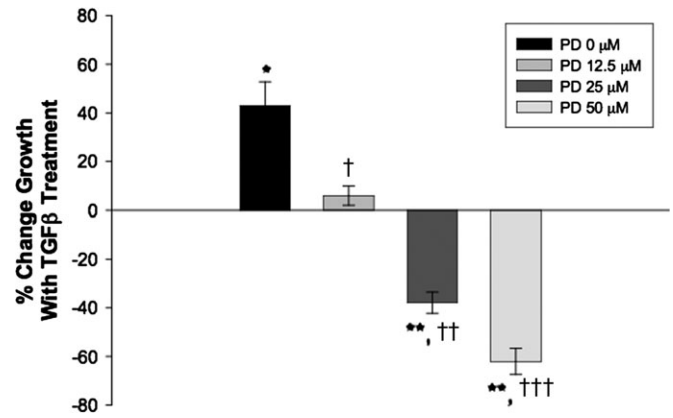
Fig. 7. Effect of TGFβ and RAS/ERK inhibition on BxPc-3 cell growth. BxPC-3 cells were plated in complete medium (supplemented with 10% fetal bovine serum). Cells were then grown in serum-free medium (serum depleted) in control medium, in medium containing 10 ng/ml TGFβ alone, or TGFβ in the presence of 12.5–50 μM PD98059. Cell number was determined on day 3. Data shown are means ± SEM of 12 independent assays and normalized to controls. $^*P < 0.05$ and $^{**}P < 0.01$ versus cells without TGFβ treatment; $†P < 0.05$, $††P < 0.01$ and $†††P < 0.001$ versus cells with TGFβ but no PD98059 inhibitor treatment.

Fig. 6. RAS/ERK inhibition reverses TGFβ-induced PTEN protein suppression in pancreatic cancer cells. (**A**) We treated ERK-activated BxPc-3 cells with PD98059 in the presence or absence of TGFβ and performed western blotting. Note that pretreatment of cells with PD98059 1 h before TGFβ treatment significantly reversed the suppressive effect of TGFβ on PTEN protein expression (lane 4 versus lane 2). GAPDH was used as a loading control. (**B**) RAS/ERK-activated CAPAN-1 cells were transfected with 3 μg of DN-K-RAS followed by TGFβ treatment. Cells were then lysed 48 h after TGFβ treatment and analyzed by western blotting for PTEN protein and GAPDH protein as a loading control. Note that direct K-RAS inhibition coupled with TGFβ treatment yielded the greatest expression of PTEN protein, as K-RAS inhibition reversed TGFβ-induced PTEN protein suppression (lane 6 versus lane 4). Data shown are means ± SEM of five experiments. $^*P < 0.05$ versus control and $†P < 0.05$ versus TGFβ treatment alone.

with inhibition of RAS/ERK activity; (iii) RAS/ERK signaling facilitates TGFβ-induced *PTEN* suppression and *PTEN* expression is restored with inhibition of RAS/ERK; and (iv) proliferation events are inversely related to expression of the tumor suppressor *PTEN* as regulated by TGFβ and modulated by RAS/ERK.

Taken together, our findings have a number of implications in pancreatic adenocarcinoma. First, we implicate PTEN, as regulated by TGFβ, as a controller of cellular proliferation in pancreatic cancer. Thus, PTEN through ligand regulation, in addition to genetic and epigenetic events, contributes to cancer growth. Regulation of PTEN

by TGFβ is complex as this regulation occurs in *SMAD4*-null cells. As depicted in Figure 8, we speculate that there are at least two intracellular pathways by which TGFβ regulates PTEN. With TGFβ-mediated SMAD-dependent signaling, a well-characterized suppressor pathway in epithelial cells, we suggest that this cascade enhances PTEN expression. In our pancreatic cell models studied, *PTEN* expression is lessened by (i) loss of *SMAD4*, which is well-described to facilitate translocation of SMAD2 into the nucleus; and (ii) interference of SMAD2 translocation by activated RAS/ERK, which has been suggested to phosphorylate the linker region of SMAD2/3 and prohibits its translocation to the nucleus (22). Because SMAD2 translocation was improved with blockage of RAS/ERK, as well as restoration of TGFβ-induced *PTEN* expression, SMAD2 may have nuclear effects that are independent of the presence of SMAD4, and this has been suggested in the literature (40,41).

Second, we also speculate the presence of a TGFβ-mediated SMAD-independent pathway, which down-regulates *PTEN* and facilitates cellular growth. Our data strongly suggest the existence of such a pathway because TGFβ mediates PTEN suppression in the absence of *SMAD4* and in the presence of activated RAS/ERK, both of which attenuate SMAD2 nuclear translocation. Thus, SMAD-dependent signaling is blocked, which is the case in most human pancreatic cancers, while TGFβ still has cellular effects. There are a number of reports that indicate that TGFβ can have dual roles: growth suppression and, in the right scenario, induce growth proliferation. In many cases, TGFβ's role in cellular proliferation is demonstrated in metastasis, which represents mature cancers with the accumulated pathway derangements. In pancreatic cancers, TGFβ ligand is often up-regulated, and this is believed to be due to loss of negative feedback regulation by SMAD-dependent signaling (35). The increased amounts of ligand could help increasingly signal through a proliferative-driven SMAD-independent pathway through down-regulation of *PTEN* favoring unbalanced mitogenic PI3K signaling. Recently, a second mechanism has been demonstrated that reduces PTEN levels after TGFβ stimulation. TGFβ signaling induces dephosphorylation of PTEN, reducing its stability in the cytoplasm (42). Thus, there appears to be at least two mechanisms, reduced *PTEN* expression and reduced PTEN stability, to decrease PTEN protein levels to increase the pancreatic cancer cell's ability to proliferate with TGFβ signaling.

Third, RAS/ERK, which is activated in over 90% of pancreatic cancers, modulates *PTEN* expression through TGFβ signaling. The regulation appears to be through blocking SMAD translocation, as
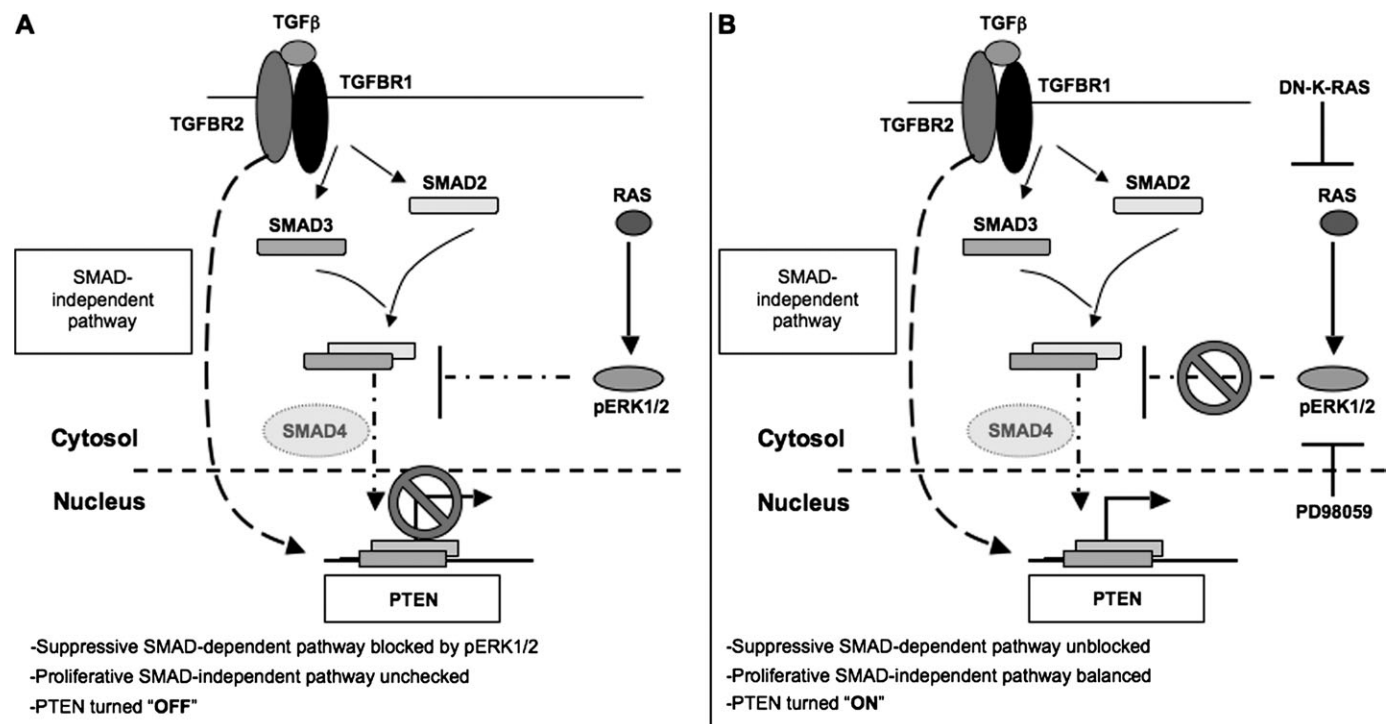
Fig. 8. Schematic summarizing our working model. Pancreatic cancer cells are often *SMAD4* null, and thus TGFβ utilizes signaling pathways to regulate PTEN expression in a *SMAD4*-independent manner. We hypothesize that TGFβ-induced SMAD-dependent signaling restores *PTEN* expression, and speculate that *PTEN* is suppressed via non-SMAD pathways. As most pancreatic cells have activated RAS/ERK because of oncogenic mutations, TGFβ-SMAD-induced *PTEN* transcription is inhibited (**A**). When activated RAS/ERK is blocked, TGFβ-induced *PTEN* transcription is resumed (**B**). Thus, in a *SMAD4*-null environment, activated RAS/ERK acts as a 'switch' to convert TGFβ signaling functionally from a tumor suppressor to tumor promoter via modulation of *PTEN* expression.

previously suggested in the literature (22). However, K-RAS mutations, in particular, are one of the earliest events in pancreatic cancer pathogenesis, and oncogenic mutations are seen in benign pancreatic conditions such as pancreatitis (18,19). Because RAS/ERK activation is a potent inhibitor of SMAD signaling, TGFβ-induced *PTEN* regulation is likely affected at a very early stage in pancreatic carcinogenesis.

In summary, we indicate that PTEN may be a key tumor suppressor involved in pancreatic tumorigenesis. Unlike other tumors in which PTEN is inactivated by a variety of genetic or epigenetic mechanisms, *PTEN* expression is ligand regulated by TGFβ signaling. The TGFβ-induced *PTEN* expression may be determined by either or both of SMAD-dependent and SMAD-independent signaling pathways. When SMAD-dependent signaling is blocked by oncogenic K-RAS or activated ERK, TGFβ induces down-regulation of *PTEN* coupled with growth proliferation of pancreatic cells. When RAS/ERK activation is blocked, SMAD2 nuclear translocation is restored (even without SMAD4), TGFβ-induced *PTEN* down-regulation is reversed and cellular proliferation is reduced to non-TGFβ-induced levels. We suggest that RAS/ERK activation 'switches' the balance of the dual roles of TGFβ in favor of proliferation when *PTEN* is turned off, and the switch can be reversed with blockage of RAS/ERK to turn PTEN expression on reducing cellular proliferation. This regulatory mechanism could potentially be controlled pharmacologically.

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