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TGF- β mediates *PTEN* suppression and cell motility through calcium-dependent PKC- α activation in pancreatic cancer cells

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Abstract

Transforming growth factor- β (TGF- β) suppresses growth via the TGF- β -SMAD pathway but promotes growth in cancer cells with disrupted SMAD signaling and corresponds to an invasive phenotype. TGF- β also downregulates the tumor suppressor *PTEN* that is rarely mutated in sporadic pancreatic cancer; this downregulation may mediate cell proliferation and invasiveness, but the mechanism is unknown. Here, we examined whether TGF- β modulation of *PTEN* was mediated by protein kinase C (PKC). We have previously demonstrated that *SMAD4*-null BxPc-3 pancreatic cancer cells treated with TGF- β 1 (10 ng/ml) suppressed *PTEN* expression and increased cell proliferation. TGF- β -treated cells were examined for PKC activation and its coupling to *PTEN* expression, utilizing pharmacological and knockdown methods. Calcium mobilization and cell migration were also examined. In BxPc-3 cells, only two PKC isoforms were activated by TGF- β , and *PTEN* downregulation by TGF- β was specifically mediated by PKC- α . In parallel, TGF- β rapidly induced an increase in cytoplasmic free calcium from intracellular stores, consistent with subsequent PKC- α activation. The TGF- β -induced increase in cell migration was blocked by knockdown of PKC- α . Thus calcium-dependent PKC- α mediates TGF- β -induced transcriptional downregulation of *PTEN*, and this pathway promotes cell migration in a *SMAD4*-null environment. The TGF- β -PKC- α -*PTEN* cascade may be a key pathway for pancreatic cancer cells to proliferate and metastasize.

Keywords

transforming growth factor- β ; *PTEN*; protein kinase C

Of the ~32,000 new cases of pancreatic cancer that occur every year in the United States (18), more than half will have biallelic loss of *SMAD4*, a key intracellular mediator for transforming growth factor- β (TGF- β) signaling (30). The TGF- β -SMAD signaling cascade is considered to mediate growth suppression in most epithelial cells, and disruption of this pathway is one mode how pancreatic cancer cells escape TGF- β -induced growth suppression. However, TGF- β can also induce cellular proliferation, particularly with disruption of SMAD signaling, suggesting that other TGF- β -mediated pathways may be operative or exposed with loss of intact SMAD signaling (6). These SMAD-independent pathways have not been well characterized, but certain cytoplasmic protein kinases, including PKC and MAPKs, may be involved as a result of TGF- β signaling for induction of certain genes (10,17,20,21,26,31,33,

37). Additionally, increased levels of PKC- α have been associated with pancreatic cancer cell proliferation (38). For instance, when AR4-2J pancreatoma cells were transfected with a PKC- α antisense oligonucleotide, tumor cell growth was reduced in parallel with a reduction in PKC- α expression (38). CAPAN-1 and CAPAN-2 pancreatic cancer cells express elevated amounts of PKC- α and exhibit an aggressive pattern of tumor growth (32).

PTEN mutations or deletions are present in many types of cancer, but its mutation is rarely found in pancreatic cancer (34). *PTEN* expression has been shown to be regulated by TGF- β 1 (6,22). *PTEN* mRNA levels are reduced in a model of TGF- β 1 overexpressing transgenic mice that developed pancreatic fibrosis (9). Reduction of *PTEN* mRNA levels in the pancreatic cancer cell line, PANC-1, following incubation with TGF- β 1, has also been reported (9). In pancreatic cancer, TGF- β 1 is commonly overexpressed, which may be secondary to loss of negative feedback from a disrupted TGF- β -SMAD signaling cascade or other mechanism (11). Although *PTEN* is not found mutated in pancreatic cancers, its reduction in expression gives pancreatic cells an additional growth advantage and may be an instrumental pathway for TGF- β -induced cell proliferation (6). However, the signaling pathway involved in TGF- β -induced *PTEN* suppression has not been characterized.

In this study, we hypothesized that TGF- β -induced *PTEN* suppression in pancreatic cancer cells occurs via the activation of a calcium-dependent PKC. Here, we demonstrate that TGF- β induces calcium influx and PKC- α activity, which mediates TGF- β -induced *PTEN* suppression in *SMAD4*-null pancreatic cancer cells.

MATERIALS AND METHODS

Materials and reagents

Gö6976, a PKC inhibitor, was purchased from Pharmingen (San Diego, CA). A solution of Gö6976 in DMSO (Sigma, St. Louis, MO) was prepared. The solution was used after diluting in cell culture medium for each assay. All other reagents were purchased from Sigma.

Cell cultures

BxPc-3 and CAPAN-1 cells were obtained from the American Type Culture Center and were maintained in RPMI medium (GIBCO-BRL, Gaithersburg, MD) supplemented with 10% fetal calf serum (Gibco-BRL) without any antibiotics in an incubator at 37°C and 5% CO₂. To analyze the effect of TGF- β 1 on *PTEN* expression, pancreatic cancer cell lines were grown to 70–80% confluence in medium containing 10% FBS. Afterward, cells were washed twice in PBS, incubated for 30 min in serum-free medium and treated for 24 and 48 h with 10 ng/ml TGF- β 1 or medium alone without serum throughout the experiment.

Subcellular fractionation

Cells were lysed and separated into various compartments so as to determine the translocation of PKC isoforms when these enzymes are activated. This was carried out with a Cell Compartment Kit (Qiagen, Valencia, CA), and experimental procedures were based on the manufacturer's instructions. After treatment, cells were lysed in extraction buffer CE1 on ice for 10 min. This step disrupted the plasma membrane without solubilizing the cells. The lysates were then centrifuged at 1,000 g for 10 min at 4°C. The supernatant was removed, and this fraction contained cytosolic proteins. The pellet was resuspended in extraction buffer CE2 that solubilized the plasma membrane, as well as all organelle membranes but not the nuclear membrane, by pipetting up and down using a 1-ml pipette tip. The lysates were then incubated at 4°C for 30 min on a shaker. Lysates were then centrifuged at 6,000 g for 10 min at 4°C. The supernatants, which contained the membrane proteins, were again transferred to new Eppendorf tubes and used for experiments. Pellets were then discarded.

Small interfering RNA transfection

We used validated small interfering (si)RNAs with inhibitory activities to PKC- α and PKC- λ from Ambion (Austin, TX). Approximately 10^6 cells were used for each siRNA transfection reaction. Trypsinized cells were mixed with a transfection reagent and the siRNAs per the manufacturer's instruction, followed by electroporation (Amaxa, Gaithersburg, MD). Transfected cells were then plated in each well of a six-well plate and then incubated at 37°C for over 72 h. Gene knockdown was verified by PKC mRNA and protein expression.

Total RNA extraction and semiquantitative reverse transcriptase-polymerase chain reaction

Total RNA was extracted from control or TGF- β -treated cells with Trizol reagent (Invitrogen, Carlsbad, CA). Cells were grown on six-well plates and lysed. Lysates were combined with chloroform, mixed, and the pellets were precipitated with isopropanol and 75% ethanol and then air dried. Two micrograms of total RNA were reverse transcribed into cDNA and amplified by PCR for PKC- α and PKC- λ expression (SuperScript II, Invitrogen). Briefly, following inactivation at 65°C for 10 min, 1 μ l of the reaction mixture was incubated in buffer containing 0.2 mM of dATP, dCTP, dGTP, dTTP, 0.2 μ M concentrations each of oligonucleotide primers, 3 mM MgCl₂, and a 10 $\times$ buffer consisting of 200 mM Tris-HCl (pH 8.0), 500 mM KCl, and 1 U *Taq* polymerase. PCR primers of the PKC- λ and - α genes were used (PKC- α : forward strand, 5'-CGACTGTCTGTAGAAATCTGG-3' and reverse strand, 5'-CACCATGGTGCACCTCCACGTC-3'; and PKC- λ : forward strand, 5'-TATAATCCTTCAAGTCATG-3' and reverse strand, 5'-TTACACATGCCGTAAGTCAGT-3'), and the conditions for PCR reactions for PKCs were previously described (35). Primers used for *GAPDH* were used as a control (forward strand, 5'-ACCACAGTCCATGCCATCAC-3' and reverse strand, 5'-TCCACCACCCTGTTGCTGTA-3').

Western blotting

BxPc-3 cells were washed three times with ice-cold PBS. Cells were then lysed with total lysis buffer [150 mM NaCl, 10 mM Tris-HCl (pH 7.8), 1 mM EDTA, 0.5% Triton X-100, and 1 mM sodium orthovanadate] containing protease inhibitors (1 μ g/ml leupeptin and 100 μ g/ml PMSF). Cells were then incubated at 4°C for 30 min with constant shaking. The cells were then scraped into microcentrifuge tubes, and the samples were centrifuged at 12,000 g for 15 min to remove insoluble material. The protein content in each sample was determined and adjusted. For immunoprecipitation studies, lysates were incubated with the immunoprecipitating antibody for 1 h at 4°C, followed by another 1-h incubation with protein A-agarose at 4°C. Pellets were then resuspended in 2 $\times$ loading buffer [50 mM Tris (pH 6.8), 2% SDS, 100 mM dithiothreitol, 0.2% bromphenol blue, and 20% glycerol] and boiled for 5 min. Loading buffer supernatants from immunoprecipitation studies or cell lysates were then resuspended in 2 $\times$ gel loading buffer, boiled for 5 min, and then separated by SDS-polyacrylamide gel electrophoresis (9% polyacrylamide). Resolved proteins were transferred overnight at 4°C onto a PVDF membrane (Millipore Corporate, Billerica, MA). Membranes were then blocked with a 5% solution of skim milk for 30 min at room temperature, followed by further incubation with specific monoclonal antibodies [PKCs, 1:1,000 (BD Transduction Laboratories, San Diego, CA), PTEN 1:500 (Santa Cruz Biotechnology, Santa Cruz, CA), and GAPDH 1:5,000 (Ambion)]. After being washed with PBS with 1% Tween (PBST), the secondary antibody was applied to the membrane. After being washed with PBST, 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 with the use of an AlphaImager digital imaging system (Alpha Innotech, San Leandro, CA).

Measurement of cytoplasmic free calcium ion concentration by digital Ca^{2+} imaging

Cytoplasmic free calcium ion concentration ($[\text{Ca}^{2+}]_{\text{cyt}}$) in human BxPc-3 cells were measured by fura-2 fluorescence ratio digital imaging. Briefly, BxPc-3 cells, grown on coverslips, were loaded with 5 μM fura-2 AM [dissolved in 0.01% Pluronic F-127 plus 0.1% DMSO in physiological salt solution (PSS, described below)] at room temperature for 50 min and then washed in PSS for 30 min. Thereafter, the coverslips with BxPc-3 cells were mounted in a perfusion chamber on a Nikon microscope stage. The ratio of fura-2 fluorescence with excitation at 340 or 380 nm ($F_{340/380}$) was followed over time and captured with an intensified CCD camera (ICCD200) and a MetaFluor Imaging System (Universal Imaging, Downingtown, PA). The PSS solution used in digital Ca^{2+} measurement contained the following (in mmol/l): 140, K^+ 5.0 K^+ , 2 Ca^{2+} , 147 Cl^- , 10 HEPES, and 10 glucose. For the Ca^{2+} -free solution, Ca^{2+} was omitted and 0.5 mM EGTA was added to prevent possible Ca^{2+} contamination. BAPTA-AM, a chelator of calcium, was used in some experiments at concentrations of 2 and 20 μM before TGF- β treatment. The osmolalities for all solutions were ~ 284 mOsm/kg.

Wound-healing cell motility assay

For determination of cell motility, human pancreatic carcinoma cells were seeded in six-well plates (Nunc, Wiesbaden, Germany), incubated in complete growth medium (RPMI1640 + 10% FBS). Complete medium was replaced with blank medium or medium that contained inhibitor or ligand. Cell layers were then scratched with a pipette tip and the gap distance immediately measured and recorded. The cells were then allowed to incubate with ligand and/or inhibitor for 24 h, and the gap distance was measured and recorded again.

Transwell migration assay

After coating the Corning Costar insert chambers as well as Transwell 24-well plates (8- μm pores; Corning, Corning, NY) with 0.1% fibronectin (Sigma) and blocking with 1% bovine serum albumin in $1\times$ PBS for 1 h at 37°C , we seeded BxPc-3 cells in triplicate at 50,000 cells per well in serum-free media containing 1% bovine serum albumin with or without TGF- β (10 ng/ml) and/or siRNA treatment. Cells were then allowed to migrate for 3 h. After removal of medium from both the chamber and the Transwell followed by 3 washes with $1\times$ PBS, the chamber was gently wiped with a cotton swab. Migrated cells were fixed in 100% methanol for 1 h and then allowed to air dry overnight. Cell staining was performed with a modified Giemsa stain (Sigma) at 1:10 for 1 h. After carefully rinsing the Transwell and the chamber with water, we captured the images by using an Axiovert 2000 microscope with an AxioCAM HRC Camera (both Zeiss Microimaging, Thornwood, NY). Images were taken from six microscopic fields at the center of each well, and the stained cells on the bottom of the chamber were counted.

Statistical analysis

All data are expressed as the means for a series of n experiments $\pm$ SE. Data were analyzed by one-way ANOVA followed by Tukey's post hoc test or by Student's t -tests for unpaired samples with GraphPad Prism 3.0 (San Diego, CA). $P < 0.05$ was considered statistically significant.

RESULTS

Expression of protein kinase isoforms in BxPc-3 cells

There are more than 11 isoforms of PKC that have been identified (26). To further our understanding of the functions of different isoforms of PKC in BxPc-3 cells, we screened the PKC isoforms by Western blot analysis of whole cell extracts. BxPc-3 cells contained proteins immunoreactive with antibodies to PKC- α (82 kDa), - β (80 kDa), and - γ (80 kDa) in the

conventional family, PKC- ϵ (90 kDa), - μ (115 kDa), and - δ (82 kDa) in the novel family, and PKC- λ/ι (74 kDa/74 kDa) and - ζ (72 kDa) in the atypical family (Fig. 1). PKC- θ expression was not detected in BxPc-3 cells. On the basis of these data, we examined whether the PKCs that were expressed could be activated by TGF- β treatment.

TGF- β activates PKC- α and - λ/ι isoforms in BxPc-3 cells

Because almost all of the isoforms of PKC were expressed in BxPc-3 cells, we studied whether TGF- β could activate specific isoforms of PKC in the cells. BxPc-3 cell monolayers were incubated with a single dose of TGF- β (10 ng/ml) for various time points as shown in Fig. 2. Of all isoforms tested, TGF- β only activated PKC- α and - λ/ι in the cells as early as 30 min after addition of the ligand, as determined by translocation of the kinase to the membrane fraction (Fig. 2). PKC- λ/ι showed high basal levels expressed, suggesting partial activation at baseline. All other PKC isoforms were not activated by TGF- β , such as PKC- γ (Fig. 2). Both PKC- α and PKC- λ/ι were activated maximally at 30 min and maintained for up to 24 h. Forty-eight hours after TGF- β treatment, most of the PKC returned to the basal level by either shuffling back to the cytosolic fraction or were degraded and cytosolic PKCs were replaced by the new ones. These data indicate that TGF- β stimulates activation of PKC- α and - λ/ι in BxPc-3 pancreatic ductal cancer cells.

TGF- β treatment is able to rapidly mobilize intracellular $[Ca^{2+}]_{cyt}$ in BxPc-3 cells

Depending on the isoform of PKC, the classical means of activation of the conventional PKCs is via hydrolysis of phosphatidylinositol with concomitant release of diacylglycerol (DAG) and 1,4,5 inositol trisphosphate (IP_3) followed by an increase in $[Ca^{2+}]_{cyt}$ levels from the intracellular organelles, in particular, the endoplasmic reticulum (ER). Here we show that PKC- α , one of Ca^{2+} -dependent PKC isoforms, is activated by TGF- β , which directed us to test whether TGF- β can mobilize intracellular Ca^{2+} in our system. As shown in Fig. 3A, when BxPc-3 cells were initially perfused with a normal PSS solution containing 2 mM Ca^{2+} for 3 min and then perfused with a Ca^{2+} -free solution containing TGF- β (10 ng/ml), a large transient Ca^{2+} signal was observed. After that, TGF- β was washed out with normal PSS solution for another 5 min and followed by a Ca^{2+} -free solution containing ATP (10 μ M) as a positive control, which produced a large transient signal Ca^{2+} as expected. These results suggest that TGF- β is able to induce Ca^{2+} release from the intracellular organelles, in particular, the ER. To further test whether TGF- β -induced Ca^{2+} release is via the IP_3 pathway in the ER, BxPc-3 cells were pretreated with 2-aminoethoxydiphenyl borate (2-APB) (100 μ M), a cell-permeable selective IP_3 receptor antagonist. When the cells were perfused with a Ca^{2+} -free solution containing TGF- β (10 ng/ml), no obvious Ca^{2+} signal was observed (Fig. 3B). These results clearly indicate that TGF- β is able to mobilize Ca^{2+} release from the ER via the IP_3 pathway. These findings not only show that $[Ca^{2+}]_{cyt}$ is likely involved in conventional PKC activation in response to TGF- β but also suggests that TGF- β utilizes pathways other than the canonical SMAD pathway in pancreatic cells.

Chelation of intracellular calcium by BAPTA-AM reverses TGF- β -induced activation of PKC- α

To determine whether PKC- α activation is directly affected by calcium released by TGF- β inside of the cells, we pretreated the cells with BAPTA-AM (2 and 20 μ M) followed by TGF- β treatment for 60 min. This inhibitor is able to cross the lipid bilayer of the cell membrane to exert its calcium chelating effect. As shown in Fig. 4, TGF- β is able to activate PKC- α translocation, but pretreatment of the cells at 20 μ M of BAPTA-AM blocked membrane translocation of PKC- α . This finding indicates that TGF- β -induced activation of PKC- α is calcium dependent.

siRNAs and pharmacological inhibitors against PKC- α , but not PKC- λ , reverses TGF- β -induced PTEN suppression

We made use of a conventional PKC inhibitor, Gö6976, which is selective in inhibiting PKC- α ($IC_{50} = 2.3$ nM) and PKC- β_1 ($IC_{50} = 6.2$ nM) (29). However, this inhibitor does not affect the kinase activity of other calcium-independent isoforms even at the micromolar concentrations (13,24,36). As shown in Fig. 5, *lane 2*, and similar to what we reported previously (6), TGF- β inhibits PTEN protein expression. Inhibition of PKC- α alone by Gö6976 did not affect PTEN expression. However, Gö6976 reversed TGF- β -induced PTEN suppression (Fig. 5). This result indicates that the conventional PKC- α mediates the regulation of PTEN expression induced by TGF- β .

Additionally, we utilized specific siRNAs to PKC- α and PKC- λ and knocked down mRNA and protein expression of these PKC isoforms by more than 80% (Fig. 6, A and C). We then examined the effects of siRNA transfection of PKC- α and PKC- λ on PTEN expression. One day after siRNA transfection, cells were treated with TGF- β for 48 h, and Western blotting was performed with anti-PTEN antibody to observe any change in steady-state PTEN protein levels after treatment. As shown in Fig. 6, B and D (*lane 2*), TGF- β suppressed PTEN expression in BxPc-3 cells 48 h after ligand treatment. siRNAs against either PKC isoforms alone did not affect total PTEN protein expression (Fig. 6, A and B, *lane 3*). However, only siRNA against PKC- α but not that of PKC- λ reversed the PTEN protein suppressed by TGF- β treatment (Fig. 6, B and D, *lane 4*). Therefore, only PKC- α mediates TGF- β -induced PTEN suppression. To assess these findings in another pancreatic cancer cell line, we determined that PKC- α is also expressed in CAPAN-1 cells, and we were able to nearly completely abolish PKC- α expression utilizing siRNA to PKC- α (Fig. 6E). CAPAN-1 cells treated with TGF- β suppressed PTEN protein expression similar to BxPc-3 cells, and siRNA to PKC- α prevented the TGF- β -induced PTEN suppression (Fig. 6F). These data indicate that TGF- β mediates PTEN suppression through PKC- α activation.

PKC- α mediates TGF- β -induced cell motility in BxPc-3 cells

TGF- β -induced proliferation is accompanied by increased cell motility and is characteristic of advanced cancers. We assessed whether PKC- α mediates stimulation of cell motility by TGF- β through the use of a wound-healing assay. As depicted in Fig. 7A, *lane 2*, TGF- β significantly induces cell mobility by closing the wound gap within 24 h after treatment. Inhibition of PKC- α with Gö6976 alone did not significantly affect the gap distance compared with control cells. Inhibition of PKC- α simultaneously with TGF- β stimulation significantly reduced TGF- β -induced cell motility (Fig. 7A, *lane 4*). To further confirm the role of PKC- α in mediating TGF- β -induced PTEN suppression and consequently increased cell mobility, a Transwell migration assay was performed with the use of cells that were transfected with siRNA to PKC- α . As shown in Fig. 7, B and C, cells transfected with PKC- α siRNA showed similar motility compared with control cells that were transfected with scrambled siRNA in the absence of TGF- β treatment. However, TGF- β -treated cells had its increased Transwell migration reduced by PKC- α siRNA inhibition (Fig. 7C, *lane 4*). Therefore, PKC- α is indeed a mediator for TGF- β -induced cell motility, in addition to its ability to suppress PTEN.

DISCUSSION

TGF- β appears to have dual and opposing functions on cell growth, which may be dependent on the maturity of the tumor as well as disruption of suppressive signaling, causing an imbalance between the potential proliferation and suppressive roles of TGF- β . Pancreatic cancers commonly possess disruption of TGF- β -SMAD signaling, and this pathway is considered growth suppressive in various epithelial cells. Interruption of the suppressive pathway would leave TGF- β proliferative pathways unchecked. We have previously shown

that TGF- β suppresses *PTEN* expression in pancreatic cancer cells that are *SMAD4*-null (6), but TGF- β -induced proliferative pathways have not been clearly elucidated in pancreatic cancer cells. In this report, we demonstrate that TGF- β -induced PKC- α activation is responsible for mediating the reduced expression of the tumor suppressor PTEN. Specifically, we show that 1) TGF- β induces rapid release of intracellular calcium ions, 2) TGF- β induces activation of the specific PKC isoform, PKC- α , and 3) TGF- β -induced PTEN suppression and cell motility is mediated by activation of PKC- α . Our findings indicate a TGF- β proliferative pathway that reduces PTEN expression, which is mediated by an increase of cytosolic calcium concentration and activation of PKC- α .

Our study demonstrates TGF- β -induced elevation of intracellular calcium levels in pancreatic cancer cells. The effect of TGF- β on intracellular calcium trafficking is not conclusive. Previous reports in other nonepithelial and epithelial cell lines suggested that TGF- β might induce changes in intracellular calcium (3), but in A549 lung carcinoma cells, TGF- β induces intracellular DAG concentration increases, but not calcium (17). In our study using pancreatic cancer cells, we demonstrate that TGF- β stimulates signaling events via the induction of a calcium-dependent pathway, but this may be cell type specific.

We have also demonstrated that a conventional PKC mediates TGF- β -induced PTEN suppression. Prior studies have suggested that TGF- β could activate PKCs in keratinocytes (33) and gastric cancer cells (21), but this has not been previously elucidated in pancreatic cancer cells. Additionally in keratinocytes, TGF- β -induced PKC activation involved growth suppression (33), the opposite functional effect noted in these nontransformed cells compared with mature pancreatic cancer cells. Conventional PKC isoforms are ubiquitous calcium and phospholipid dependent protein kinases involved in transmembrane signal transduction (26). They also play a role in growth control and carcinogenesis in the colon (28). At least 11 PKC isoforms have been identified and characterized (4,6,19). The functional significance of individual isoforms, however, is not well understood. Alterations in PKC expression are suggested to play a role in tumor progression and in the maintenance of the malignant phenotype (8,23,26–28). In addition, PKCs are implicated to play a functional role in multidrug resistance (MDR) (1). Specifically, PKC- α activates the MDR-1 gene product, gp170, by phosphorylation and thus increases efflux of drugs from the cell (12). Recent work identifies the PKC- α isoform in controlling the adhesion response (induction of adhesion molecules and receptors for these molecules) of TGF- β , and TGF- β stimulates a rapid rise in PKC phosphotransferase activity (5). PKC- λ , on the other hand, is an atypical PKC, and it is not dependent on calcium and DAG. Although we cannot connect activated PKC- λ to alterations in TGF- β -induced PTEN expression, it is apparent that TGF- β induces other SMAD-independent pathways inside of cells that may regulate other genes or functions. One possibility is the PI₃ kinase pathway that has been shown to induce atypical PKCs (25).

Studies have shown that somatic mutations in components of the TGF- β signaling pathway are associated with the loss of proliferation control, malignant progression, invasion, and metastasis formation both in vitro and in vivo (2). In pancreatic cancers, both alleles of *SMAD4* are lost from two-thirds of tumors (14), muting the TGF- β -SMAD growth suppressive signaling. Although *PTEN* is rarely mutated in pancreatic cancers, its regulation by TGF- β can confer changes in cell growth behavior. Regulation of PTEN expression by TGF- β is likely complicated, particularly as this regulation is unmasked in *SMAD4*-null cells.

In mature pancreatic tumors, TGF- β induces cell proliferation and increases cell motility, functions akin to the behavior of metastasis. We demonstrate here that PKC- α mediates the increased cell motility in addition to its ability to suppress PTEN. Alternatively, PTEN directly may dictate cell motility (16). The direct mechanism for cell motility induced by TGF- β needs to be addressed and suggests a potential pathway to control metastasis.

In summary, a TGF- β -induced proliferative pathway is mediated by PKC- α to reduce PTEN expression and enhance cell motility of pancreatic cancer cells. The pathway may be an important mechanism that mediates the opposing effects of TGF- β compared with the TGF- β -SMAD suppressive signaling in pancreatic cancer. This cascade may be a key pathway for pancreatic cancer cells to proliferate and metastasize.

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Fig. 1.

Western blot analysis of conventional, atypical, and novel PKC isoforms in BxPc-3 pancreatic ductal adenocarcinoma cells. Cell lysates were subjected to electrophoresis through 9% SDS-polyacrylamide gels and transferred to PVDF membranes. Membranes were probed with monoclonal antibodies specific for the PKC isoforms (α , β , γ , ϵ , δ , μ , η , θ , ζ , λ , and ι). All but PKC- θ are expressed in BxPc-3 cells (Bx) as assessed by molecular weight standards and positive control preparations [rabbit brain (Br) T₈₄ cells, Jurkat cells (J), and THP1 cells (T)]. PKC isoforms were detected through the use of an enhanced chemiluminescent methodology as described under MATERIALS AND METHODS. The diamonds denote the predicted molecular masses of different PKC isoforms. These Western blots are representative of 3 similar experiments.

Fig. 2.

Transforming growth factor- β (TGF- β) treatment induces membrane translocation of PKC- α and - λ but not other PKC isoforms in human BxPc-3 cells. Cells were stimulated by TGF- β for various times as shown. Cells were then lysed and fractionated into cytosolic and membrane fractions and separated by SDS-PAGE. The presence of PKCs in these fractions were determined as described under MATERIALS AND METHODS. The data are representative of 3 similar experiments.

Fig. 3.

TGF- β treatment increases the cytoplasmic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_{\text{cyt}}$) and inhibition of the 1,4,5 inositol trisphosphate (IP_3) receptor prevents the TGF- β -induced increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ in human pancreatic BxPc-3 cells. A: cells were grown on a coverslip 1 day before the experiment. During the day of experiment, the cells were loaded with 5 μM fura-2 AM for 50 min followed by 30 min washout in physiological salt solution; the coverslip was then mounted into a perfusion chamber so that cells were perfused with normal physiological solution at the start of experiments. Afterward, the perfusion was switched to Ca^{2+} -free solution containing TGF- β (10 ng/ml), normal physiological solution, and then a Ca^{2+} -free solution containing ATP (10 μM). The time course of $[\text{Ca}^{2+}]_{\text{cyt}}$ changes in human BxPc-3 cells is the

mean of 40 individual cell measurements. *B*: cells were pretreated with 2-aminoethoxydiphenyl borate (2-APB) (100 μ M), an IP₃ receptor antagonist, and throughout the experiment. Note that the TGF- β -induced increase in $[Ca^{2+}]_{cyt}$ was abolished by 2-APB. The time course of $[Ca^{2+}]_{cyt}$ changes in human BxPc-3 cells is the mean of 50 individual cell measurements. F, fluorescence.

Fig. 4.

Depletion of intracellular calcium using the calcium chelator BAPTA-AM inhibits TGF- β -induced compartmental translocation of PKC- α . Cells were pretreated with BAPTA-AM (2 and 20 μ M) followed by TGF- β treatment for 60 min. Note that TGF- β induces membrane translocation of PKC- α as in Fig. 2; however, BAPTA (20 μ M) significantly abolished the TGF- β -induced PKC- α activation, indicating that chelation of intracellular calcium prevented the activation of PKC- α .

Fig. 5.

Inhibition of PKC- α by Gö6976 reverses TGF- β -induced PTEN suppression in BxPc-3 cells. Representative Western blot showing inhibition of PKC- α with the use of Gö6976 reverses TGF- β -induced PTEN suppression. A summation bar graph with means $\pm$ SE of 3 experiments is shown. ** $P < 0.01$ vs. control (lane 1); ++ $P < 0.01$ vs. TGF- β treatment (lane 2).

Fig. 6.

Small interfering (si)RNA against PKC- α but not PKC- λ , reverses TGF- β -induced PTEN suppression in human pancreatic BxPc-3 and CAPAN-1 cells. **A**: representative agarose and Western blots showing that PKC- α siRNA knocks down PKC- α mRNA and protein by more than 80%. **B**: representative Western blot showing the presence of PKC- α siRNA prevents TGF- β -induced PTEN suppression. The bar graph summarizes the results of 5 independent experiments. Data are means $\pm$ SE. GAPDH was used as a loading control. *** P < 0.001 vs. control; +++ P < 0.001 vs. TGF- β treatment alone. **C**: representative agarose and Western blots showing that PKC- λ siRNA knocks down PKC- λ mRNA and protein by more than 80%. **D**: representative Western blot showing the presence of PKC- λ siRNA fails to alter TGF- β -

induced PTEN suppression. The bar graph summarizes the results of 5 independent experiments. Data are means $\pm$ SE. GAPDH was used as a loading control. *E*: similar to the results in *A*, transfection of PKC- α siRNA in CAPAN-1 cells knocked down PKC- α protein expression by more than 80%. *F*: similar to BxPc-3 cells shown in *B*, a representative Western blot showing transfection of PKC- α siRNA into CAPAN-1 cells prevents TGF- β -induced PTEN suppression.

Fig. 7.

Inhibition of PKC- α reverses TGF- β -induced cell motility and invasion in BxPc-3 cells. **A:** PKC- α pharmacological inhibitor Gö6976 blocks TGF- β -induced cell motility in a wound-healing assay. After the creation of a wound in a monolayer of BxPc-3 cells with a 200- μ l pipette tip, cells were treated with DMSO (control), TGF- β (10 ng/ml), and/or Gö6976 (1 μ M) for 24 h. Shown is a bar graph summarizing the data representing the means $\pm$ SE of 5 experiments. ** P < 0.01 vs. control; ++ P < 0.01 vs. TGF- β treatment alone. **B:** photomicrographs ($\times 40$) of BxPc-3 cells transfected with either scrambled or siRNA to PKC- α and treated with TGF- β in Transwell chambers. BxPc-3 cells that had migrated to the outside of the lower surface of the polycarbonate filter were fixed and stained with Giemsa for

evaluation of cell migration. C: bar graph representing the average number of cells per field of 6 fields in each chamber $\pm$ SE. HPF, high-powered field. *** $P < 0.001$ vs. cells transfected with scramble RNA alone; +++ $P < 0.001$ vs. cells transfected with scramble RNA then treated with TGF- β .