Carcinogenesis vol.29 no.2 pp.252–262, 2008
doi:10.1093/carcin/bgm249
Advance Access publication November 13, 2007

Loss of type III transforming growth factor β receptor expression increases motility and invasiveness associated with epithelial to mesenchymal transition during pancreatic cancer progression

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Epithelial to mesenchymal transitions (EMTs) contribute to increases in cellular motility and invasiveness during embryonic development and tumorigenesis. The transforming growth factor β (TGF-β) signaling pathway is a key regulator of EMT. The TGF-β superfamily coreceptor, the type III TGF-β receptor (TβRIII or betaglycan), is required for EMT during embryonic heart development and palate fusion. Here, we establish that in a pancreatic cancer model of EMT, TβRIII expression is specifically lost during EMT at the mRNA and protein levels, whereas levels of the TGF-β type I and type II receptors are maintained at the mRNA level. Loss of TβRIII expression at the protein level precedes the loss of E-cadherin and cytoskeletal reorganization during early stages of EMT. However, maintaining TβRIII expression does not block these aspects of EMT, but instead suppresses the increased motility and invasiveness associated with EMT. Reciprocally, shRNA-mediated knockdown of endogenous TβRIII increases cellular motility without affecting Snail or E-cadherin levels. The ability of TβRIII to suppress motility and invasiveness does not depend on its cytoplasmic domain or its coreceptor function. Instead, this suppression of invasion is partially mediated by ectodomain shedding of TβRIII, generating soluble TβRIII (sTβRIII). In human pancreatic cancer specimens, TβRIII expression decreases at both the mRNA and protein levels, with the degree of loss correlating with worsening tumor grade. Taken together, these studies support a role for loss of TβRIII expression during the EMT of pancreatic cancer progression, with a specific role for sTβRIII in suppressing EMT-associated increases in motility and invasion.

Introduction

Pancreatic cancer is the fourth leading cause of cancer death in the USA, with a death rate closely matching the incidence rate, a median survival of 4–6 months and a 5-year survival rate of <5% (1). Several factors account for this poor prognosis, including delayed diagnosis, as patients seldom exhibit symptoms until the cancer has become locally invasive or metastatic, lack of effective screening tests and largely ineffective treatment options (1). Clearly, greater understanding of the processes underlying pancreatic invasiveness and metastasis is required to develop effective treatment strategies for this deadly disease.

An important contributor to the metastatic process is epithelial to mesenchymal transition (EMT), in which adherent and non-motile epithelial cells acquire motility and invasiveness (2,3). EMT is associated with the induction of transcription factors including Snail and Slug, the loss of epithelial markers including E-cadherin and cytokeratins, which are important in the maintenance of epithelial cell junctions, and gain of mesenchymal markers including vimentin and N-cadherin. EMT facilitates metastasis, as epithelial-derived cancer cells within the primary tumor undergo this transition to acquire motility and invasiveness, enabling them to penetrate the basement membrane and access the blood stream and then extravasate to form distant metastasis. As metastatic tumors are the cause of death for >95% of cancer patients, defining mechanisms regulating this metastatic cascade remains a priority for cancer therapy (2,3).

The transforming growth factor β (TGF-β) pathway is an important regulator of EMT, as TGF-β initiates and maintains both developmental and carcinogenic EMT in different systems *in vitro* and *in vivo* (4). Cancer cells may utilize these developmental TGF-β-mediated EMT pathways to promote their metastatic capability. In addition, TGF-β potently inhibits the growth of epithelial cells yet promotes the growth of mesenchymal cells (5). Therefore, carcinogenic EMT may provide the mechanism by which TGF-β switches from a tumor suppressor in early stages of tumorigenesis to a tumor promoter at later stages. TGF-β signals through three cell surface receptors, the type III (TβRIII, or betaglycan), type II (TβRII) and type I (TβRI) receptors. TβRIII is a TGF-β superfamily coreceptor that binds all TGF-β isoforms and presents them to TβRII (6–8). Once ligand bound, TβRII recruits and phosphorylates TβRI to activate its kinase activity. TβRI then phosphorylates and activates Smads2/3, which bind to Smad4, and the complex accumulates in the nucleus and interacts with other transcription factors to regulate the expression of a multitude of target genes (6–8).

Recent studies have demonstrated that TβRIII may have additional functions independent of ligand presentation. Indeed, the TβRIII knockout mouse is embryonic lethal due to extensive heart and liver defects (9). In addition, TβRIII is required for the EMT that occurs during cardiac development to form the valves and septa (10) and is required for the EMT responsible for palate fusion (11,12). We have previously demonstrated that alterations in TβRIII expression critically modulate TGF-β signaling; the cytoplasmic domain of TβRIII interacts with the PDZ domain-containing protein GIPC to stabilize TβRIII on the cell surface and increase TGF-β signaling (13) and with the scaffolding protein β-arrestin2 to down-regulate TβRIII and TβRII from the cell surface and decrease TGF-β signaling (14). Most recently, we have demonstrated that TβRIII expression is dramatically lost in breast (15), prostate (16) and ovarian cancers (17). These studies suggest that loss of TβRIII expression has important consequences during carcinogenesis.

TGF-β is a critical regulator of pancreatic cancer homeostasis (18), and components of the TGF-β pathway are often the target of disruption during pancreatic carcinogenesis. In a majority of human pancreatic cancers and cell lines, TβRI, TβRII and TβRIII expression levels are altered at the protein and/or mRNA level (19–21), and the downstream effector Smad4 is mutated in 50% of all human pancreatic cancers (22–24). These mutations suggest that alterations in TGF-β signaling contribute to pancreatic carcinogenesis. Here, we investigate the role of the TGF-β signaling pathway in regulating EMT, specifically motility and invasiveness in a pancreatic cancer model.

Materials and methods

Cell culture

PANC-1 and COS-7 cells obtained from American Type Culture Collection (Manassas, VA) were maintained in Dulbecco's modified eagles media supplemented with 10% fetal bovine serum. EMT was induced by treating with 0–400 pM of TGF-β1 (R&D Systems, Minneapolis, MN) for up to 48 h as described previously (25).

Abbreviations: CM, conditioned media; EMT, epithelial to mesenchymal transition; NTC, non-targeting control; sTβRIII, soluble TβRIII; TGF-β, transforming growth factor β.

Adenoviral constructs

All adenoviral constructs were made using the Becton Dickinson Adeno-X expression system (Becton, Dickinson and Company, Franklin Lakes, NJ) purified using the Adeno-X Virus Purification Kit and titered using the Adeno-X Rapid Titer Kit. The TβRIII and non-targeting control shRNAs were generated by Dharmacon (Lafayette, CO).

Immunofluorescence

For E-cadherin staining, cells were fixed with a 1:1 solution of methanol and acetone at −20°C. Blocking was performed with 1% normal rabbit serum. Cells were incubated with a 1:200 dilution of E-cadherin antibody (BD Biosciences, San Jose, CA) for 45 min, followed by incubation with an anti-mouse antibody conjugated to Alexa 488 (Molecular Probes, Carlsbad, CA) for 45 min. For F-actin staining, cells were fixed in 3.7% formaldehyde and permeabilized with 0.1% Triton X. Blocking was performed with 1% bovine serum albumin and then cells were incubated with a 1:40 dilution of Phalloidin conjugated to Texas Red (Molecular Probes) for 20 min. Nuclei were stained with DAPI and mounted onto glass slides. Immunofluorescence images were obtained using a Nikon inverted microscope. All assays were performed at least three times and results from one experiment are provided.

TGF-β binding and cross-linking

Cells were incubated with Krebs-Ringer-HEPES buffer (50 mM HEPES, pH 7.5, 130 mM NaCl, 5 mM $MgSO_4$, 1 mM $CaCl_2$ and 5 mM KCl) and 0.5% bovine serum albumin for 30 min at 37°C, and then with 100 pM [^{125}I]-TGF-β1 for 3 h at 4°C. For the shedding experiment, PANC-1 cells were serum starved for 24 h, the media was collected and incubated with 25 pM [^{125}I]-TGF-β1 for 3 h at 4°C. [^{125}I]-TGF-β1 was cross-linked with 0.5 mg/ml disuccinimidyl suberate for 15 min and quenched with 20 mM glycine. Cells were rinsed in Krebs-Ringer-HEPES and half of the samples were lysed with radioimmunoprecipitation assay buffer and half were lysed with hot 2× Laemmli buffer. Those samples lysed with radioimmunoprecipitation assay were subjected to overnight immunoprecipitation at 4°C using antibodies directed toward the cytoplasmic domain of TβRIII, TβRII or TβRI bound to protein A sepharose beads. For the soluble TβRIII (sTβRIII) experiments, media was subjected to overnight immunoprecipitation at 4°C using a polyclonal antibody directed toward the extracellular domain of TβRIII (R&D Systems) bound to protein G sepharose beads. All the samples were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and phosphorimaging analysis of dried gels. All assays were performed at least three times and results from one experiment are provided.

Reverse transcription and real-time PCR

RNA was isolated from cells using the RNAEasy kit (Qiagen, Germantown, MD), reverse transcribed using an oligodT primer (Invitrogen, Carlsbad, CA) and then PCR was performed using primers specific for GAPDH (27), E-cadherin (28), cytokeratin 8 (29) and cytokeratin 19 (30), vimentin (31) and N-cadherin (forward 5′-GACAATGCCCCTCAAGTGTT-3′, reverse 5′-CCATTAAGCCGAGTGATGGT-3′). Products were analyzed on a 2% agarose gel and images were acquired with a Bio-Rad Gel Doc. Real-time PCR was performed using SYBR Green Supermix (Bio-Rad, Hercules, CA) and primers for TβRI, TβRII and TβRIII (32,33). All assays were performed at least three times and results from one experiment are provided.

Matrigel invasion and motility assays

Invasion assays were performed using 24-well Matrigel-coated transwells (BD Biosciences). Motility assays were performed using 24-well fibronectin (Calbiochem, San Diego, CA)-coated transwells (Corning Costar, Lowell, MA). A total of 100 000 cells were placed in the upper chamber in serum-free media and media containing 10% fetal bovine serum was placed in the lower chamber. After 48 h of incubation in the Matrigel chambers and 18 h in the fibronectin transwells, the cells on the upper surface of the filter were gently scrubbed with a cotton swab. The cells on the underside of the filter were fixed and stained with the Three-Step Stain Set (VWR). The filters were removed and mounted onto glass slides. Each filter was examined using a Nikon inverted microscope at ×10 magnification (×20 for shRNA motility experiment) and the number of cells in the center field was counted. For the sTβRIII invasion assay, conditioned media (CM) was collected from COS-7 cells transiently transfected (Lipofectamine 2000) with 2 μg of a pcDNA3.1 or sTβRIII vector and this media was used in the upper chamber of the transwell. All assays were performed at least three times. Invasion and motility were normalized to untreated PANC-1 in each experiment. Results represent the mean ± SEM from three independent experiments.

Immunohistochemistry of human pancreatic cancer tissue specimens

Studies were performed as previously published (15), using a pancreatic cancer tissue array from Protein Biotechnologies (San Diego, CA). The intensity of staining in each specimen was blindly scored on a scale of 1–5 by a certified pathologist (T.A.F.).

TβRIII gene expression analysis on cDNA filter array

Studies were performed as previously published (15).

TGF-β1-induced Smad2 and p38 phosphorylation

Cells were treated with 400 pM of TGF-β1 for 48 h to induce EMT. Cells were then serum starved for 2 h and treated with either 20 or 200 pM of TGF-β1 for 40 min. Cells were lysed in 2× Laemmli buffer and lysates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Western blotting was performed using Smad2, phospho-specific Smad2 and phospho-specific p38 antibodies (Cell-Signaling Technology, Danvers, MA). Western blotting for HA-tagged TβRIII expression was performed using an αHA antibody (Roche Applied Science, Basel Switzerland) and an αβ-actin antibody (Sigma, St Louis, MO) to control for protein loading. All assays were performed at least three times and results from one experiment are illustrated.

Oncomine microarray data

Gene profiling studies publicly available on the Oncomine Cancer Profiling Database were used to investigate TβRIII mRNA levels in pancreatic cancer (34,35).

Statistical analysis

Significance of results was assessed using the one-tailed Student's *t*-test on the VassarStats Web site (36); $^{*}P < 0.05$, $^{**}P < 0.025$, $^{***}P < 0.01$. The *P*-value for the Oncomine study was generated by the Oncomine database (34). Error bars, were indicated, represent the SEM ($n = 3$).

Results

TβRIII expression decreases during EMT in a pancreatic cancer model

An important contributor to the metastatic process is EMT (2,3). Based on the defined role for the TGF-β signaling pathway in both EMT and pancreatic cancer, we investigated alterations in the TGF-β signaling pathway in a pancreatic cancer model of EMT. The human pancreatic cancer cell line PANC-1 has been reported to undergo EMT in response to TGF-β1, with maximal EMT occurring after 48 h of treatment with 400 pM of TGF-β1 (25). To characterize the TGF-β signaling pathway during EMT, we treated PANC-1 cells with TGF-β1 for up to 48 h and assessed EMT induction by following the loss of cellular junction E-cadherin immunofluorescence staining, a well-established EMT marker. E-cadherin staining at cell–cell junctions and total E-cadherin levels began to decrease at 12 h and continued to decrease until 48 h after TGF-β1 treatment (Figure 1A). Loss of E-cadherin expression also occurred in a dose-dependent manner, with loss observed after 48 h of treatment with 100 pM of TGF-β1 and maximal loss occurring with 400 pM of TGF-β1 (Figure 1B). These dose-dependent and time-dependent reductions in E-cadherin levels also occurred when PANC-1 cells were treated with TGF-β2 (data not shown). EMT was further confirmed by cytoskeletal reorganization as demonstrated by a shift in cortical to diffuse F-actin staining (Figure 1C), as well as by the concomitant decrease in the mRNA levels of the epithelial markers E-cadherin, cytokeratin 8 and cytokeratin 19, and increase in the mRNA levels of the mesenchymal markers vimentin and N-cadherin (Figure 1D).

Having rigorously defined TGF-β-induced EMT in the PANC-1 model, we investigated TGF-β receptor expression during EMT. As endogenous TGF-β receptors could not be detected in PANC-1 cells by western blot or by immunoprecipitation followed by western blot using commercially available antibodies (data not shown), we analyzed the steady-state cell surface levels of the TGF-β receptors expression by [^{125}I]-TGF-β1 binding and cross-linking, which examines both expression and binding affinity. Before EMT, PANC-1 cells express all three TGF-β1 receptors, TβRIII, TβRII and TβRI (Figure 2A). Treatment of PANC-1 cells with TGF-β1 resulted in a rapid

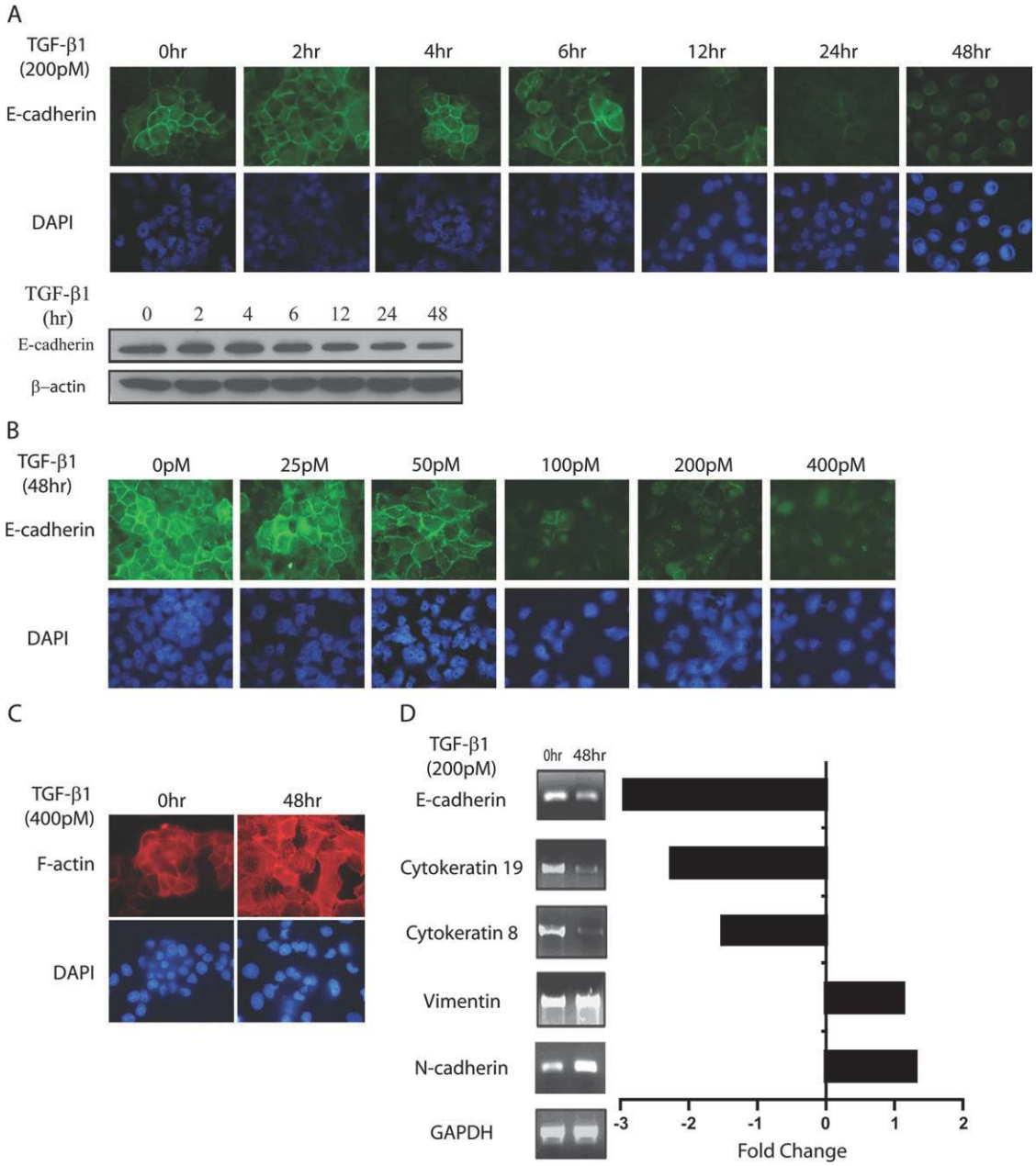

Fig. 1. TGF-β1 induces a time- and dose-dependent EMT in PANC-1. (**A**) PANC-1 cells were incubated with TGF-β1 (200 pM) for the indicated times and then EMT was followed by assessing the relocalization and decrease of the epithelial marker E-cadherin from the cell surface using immunofluorescence microscopy (above, with control DAPI nuclear staining) and loss of the E-cadherin expression using western blot (below). (**B**) PANC-1 cells were incubated with increasing concentrations of TGF-β1 for 48 h and EMT was followed by assessing E-cadherin cell surface levels using immunofluorescence microscopy, with control DAPI nuclear staining. (**C** and **D**) PANC-1 cells were incubated with TGF-β1 at the indicated concentrations for 48 h and EMT was followed by staining for F-actin with Phalloidin conjugated to Texas Red to visualize EMT-associated changes in the actin cytoskeleton (**C**), and assessing expression of the epithelial markers (cytokeratins 8 and 19 and E-cadherin) and the mesenchymal markers (vimentin and N-cadherin) by reverse transcription–PCR (**D**), using GAPDH as a loading control. (**D**) The expression levels were quantified using densitometry and the fold change in expression of each marker is indicated on the right. Assays were performed at least three times with similar results, representative results are shown.

decrease in [^{125}I]-TGF-β1-bound and cross-linked TGF-β receptors, with decreases first detectable at 2 h (Figure 2A). While TβRII expression increased by 48 h, TβRI and TβRIII binding remained low (supplementary Figure 1, available at *Carcinogenesis* Online). As TβRIII presents and increases ligand binding to TβRI and TβRII, loss of TβRIII expression may contribute to the decreases in binding observed for TβRI and TβRII.

TβRIII levels continued to fall to nearly undetectable levels after 12 h of treatment (Figure 2A), when the earliest sign of EMT, loss of E-cadherin peripheral staining, could first be detected (Figure 1A). Loss of TβRIII expression was maintained after 12 h until 72 h after TGF-β1 treatment (Figure 2A, data not shown), similar to loss of E-cadherin expression (Figure 1A). As TβRIII is a proteoglycan that runs as a broad high molecular weight smear between 150 and 200 kDa, loss of TβRIII expression was confirmed by immunoprecipitating TβRIII (Figure 2A). Loss of TβRIII expression during EMT was also dose dependent (Figure 2B), with loss of TβRIII initially observed after 48 h with 100 pM of TGF-β1 and maximal at 400 pM, similar to loss of E-cadherin expression (Figure 1B). We compared the loss of TβRIII and E-cadherin protein during the EMT time course (Figure 2C), and

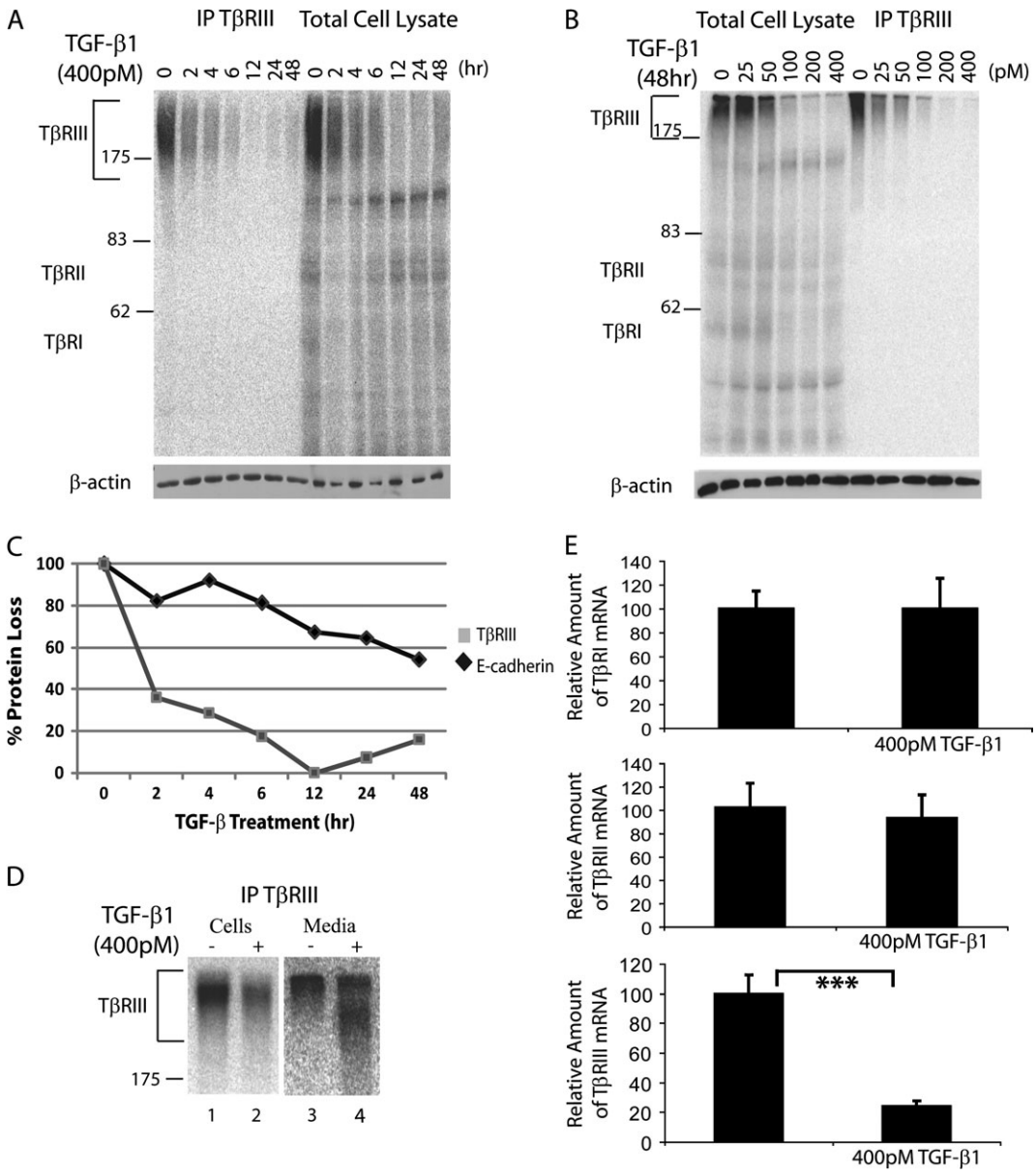

Fig. 2. TβRIII expression decreases during TGF-β1-induced EMT in PANC-1. (**A**) PANC-1 cells were incubated with TGF-β1 (400 pM) to induce EMT for the indicated times and (**B**) with increasing TGF-β1 concentrations for 48 h. Cell surface levels of TGF-β receptors were assessed by [^{125}I]-TGF-β1 binding and cross-linking followed by immunoprecipitation with an αTβRIII cytoplasmic antibody. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and phosphorimaging of dried gels was performed. β-actin is shown as a loading control. (**C**) The levels of TβRIII protein on the cell surface in (**A**), and the levels of E-cadherin in Figure 1A during 48 h of TGF-β treatment were quantified using densitometry and plotted relative to the levels at time 0. (**D**) TβRIII cell surface expression in untreated and treated PANC-1 cells (lanes 1 and 2) and sTβRIII expression in their media (lanes 3 and 4) were assessed by [^{125}I]-TGF-β1 binding and cross-linking, followed by immunoprecipitation using an extracellular αTβRIII antibody. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and phosphorimaging of dried gels were performed. (**A–D**) Assays were performed at least three times with similar results, representative results are shown. (**E**) RNA was isolated from untreated PANC-1 and PANC-1 that had been treated for 48 h with 400 pM TGF-β1. The RNA was reverse transcribed and then real-time PCR was performed with primers specific for TβRI, TβRII and TβRIII to quantify the amount of mRNA before and after EMT. Data are normalized to untreated PANC-1 and represent the mean ± SEM ($n = 3$), $^{***}P < 0.01$.

determined that loss of TβRIII protein expression preceded loss of E-cadherin protein expression. Taken together, these data support time- and dose-dependent loss of TβRIII expression during TGF-β1-induced EMT in this pancreatic cancer model, with loss of TβRIII expression preceding loss of E-cadherin expression during EMT.

Interestingly, during TGF-β treatment, cell surface expression of an ~120 kDa TGF-β-binding protein increased in both a time- and dose-dependent manner (Figure 2A and B). As this was the approximate size of the TβRIII core and the increase in expression mirrored the loss of expression of full-length TβRIII, we investigated whether this was the TβRIII core. Using antibodies to either the extracellular or the cytoplasmic domain of TβRIII, we were unable to immunoprecipitate this TGF-β-binding protein (data not shown). Therefore, the identity of this potentially novel TGF-β-binding protein remains to be elucidated.

Regulation of TβRIII expression at the protein and mRNA level during EMT

Although there was an acute loss of cell surface TβRIII expression, this loss was maintained >3 days after treatment with a single dose of TGF-β1 (Figure 2A, data not shown), suggesting that TβRIII expression might be regulated at several levels, with loss at the protein level

accounting for the initial and rapid loss of TβRIII expression (within hours), and loss at the mRNA level accounting for the sustained loss of TβRIII expression (for days).

To investigate regulation of TβRIII expression at the protein level, we first assessed whether receptor down-regulation was responsible for the acute loss of TβRIII expression. Cell surface receptors are down-regulated after internalization through either lysosomal- or proteasomal-mediated degradation. Accordingly, we assessed the effects of lysosome and proteasome inhibitors on loss of TβRIII expression during TGF-β1-induced EMT. These inhibitors did not significantly affect the loss of TβRIII induced by TGF-β1 during EMT (data not shown), suggesting that receptor down-regulation is not responsible for the dramatic loss of TβRIII cell surface expression during EMT in PANC-1 cells.

As with other coreceptors, TβRIII undergoes ectodomain shedding, with proteolytic cleavage in the extracellular domain near the transmembrane segment releasing the soluble extracellular domain (sTβRIII) into the extracellular space (37). When examining sTβRIII expression by immunoprecipitating [^{125}I]-TGF-β1-bound and cross-linked sTβRIII with a TβRIII-specific antibody, sTβRIII was detected in the CM of untreated PANC-1 cells (Figure 2D, lane 3) suggesting that TβRIII is constitutively shed from the surface. However, in TGF-β1-treated PANC-1 cells undergoing EMT, there was a significant increase in the amount of sTβRIII in the media (Figure 2D, lane 4). These data suggest that TβRIII shedding increases during EMT in PANC-1 cells, resulting in a rapid decrease in cell surface levels of TβRIII.

To investigate regulation of TβRIII expression at the mRNA level, we assessed steady-state mRNA levels for TβRIII, as well as for TβRII and TβRI, using reverse transcription–real-time PCR. There was significant loss of TβRIII expression at the mRNA level during EMT (Figure 2E, $P < 0.01$), with loss of expression beginning at 2 h and persisting out to 48 h (data not shown). In contrast, steady-state mRNA levels for TβRI and TβRII remained similar before and after EMT (Figure 2E). Taken together, these results demonstrate that loss of TβRIII expression during EMT occurs through regulation at both the mRNA and protein levels. They also support specific regulation of TβRIII during EMT among TGF-β receptors, as mRNA levels of TβRII and TβRI are maintained.

Loss of TβRIII expression is required for the increased motility and invasiveness associated with EMT

The specific loss of TβRIII expression at both the mRNA and protein levels during EMT suggested that loss of TβRIII expression might have functional significance. As the loss of TβRIII expression occurred prior to the onset of E-cadherin loss (Figure 2C), the first detectable sign of EMT, we investigated whether loss of TβRIII expression was required for E-cadherin loss to occur in this pancreatic model. To prevent loss of TβRIII expression, we transiently expressed HA-tagged TβRIII from an exogenous promoter in PANC-1 prior to the induction of EMT. This effectively blocked the EMT-induced loss of TβRIII expression, with the TβRIII adenovirally infected TGF-β treated post-EMT PANC-1 cells expressing more TβRIII than the treated non-infected PANC-1 cells (Figure 5A, lanes 3 and 4). Under these conditions, the PANC-1 cells still underwent EMT as judged by loss of cell–cell junction E-cadherin staining (Figure 3A), suggesting that loss of TβRIII was not required for induction of EMT.

To directly investigate a role for loss of TβRIII expression in EMT, we assessed the effects of shRNA-mediated silencing of TβRIII expression on EMT markers in the PANC-1 model. TβRIII shRNA specifically decreased TβRIII expression at the mRNA (Figure 3B) and protein levels (Figure 3E), whereas a non-targeting control shRNA had no effect on TβRIII expression. There were no effects of reducing TβRIII expression on the EMT markers E-cadherin or Snail, or on the TGF-β-mediated reduction in E-cadherin mRNA expression or induction of the mesenchymal marker Snail mRNA expression during EMT (Figure 3B). These studies suggest that loss of TβRIII expression was not sufficient or required for induction of EMT in PANC-1 cells.

EMT is associated not only with altered morphology and altered expression of epithelial and mesenchymal markers but also with altered function, including both increased motility and invasiveness. As expected, TGF-β-induced EMT resulted in increases in both motility (Figure 3C, top panels) and invasiveness (Figure 3D, top panels) in the PANC-1 model. When we assessed the effect of preventing loss of TβRIII expression on TGF-β-mediated increases in motility, the TGF-β-mediated increases in motility were suppressed (Figure 3C, bottom right panel, $P < 0.05$). In addition, when we prevented loss of TβRIII expression in PANC-1 cells, the TGF-β-mediated increases in invasiveness were also suppressed (Figure 3D, bottom right panel, $P < 0.01$). Expression of TβRIII was able to significantly decrease the basal invasiveness of PANC-1, even in the absence of TGF-β1 treatment (Figure 3D, bottom left panel, $P < 0.01$).

To further investigate the role of TβRIII in regulating motility, we used shRNA to specifically silence TβRIII expression in PANC-1 cells and assessed effects on motility before and after EMT. As expected, decreasing TβRIII protein levels by 70% in PANC-1 prior to initiating EMT (Figure 3E, top panel) increased their motility (Figure 3E, bottom panel, $P < 0.05$). Taken together, these studies suggest that loss of TβRIII expression is not required for induction of EMT but is required for the increase in motility and invasiveness associated with EMT during pancreatic cancer progression.

The cytoplasmic domain of TβRIII and ligand-presenting function are not required for TβRIII-mediated suppression of motility and invasion

TβRIII serves as a TGF-β coreceptor to enhance TGF-β ligand binding to TβRII and increase TGF-β signaling (26). To carry out this coreceptor function, TβRIII must be able to bind ligand through its extracellular domain and interact with TβRII through its cytoplasmic domain (26,38,39). To investigate the mechanism by which TβRIII expression decreased basal invasiveness and suppressed the TGF-β-mediated increases in motility and invasiveness associated with EMT in the PANC-1 cell line, we explored the role of the cytoplasmic domain in mediating the effects of TβRIII on suppressing motility and invasiveness. Surprisingly, TβRIII lacking its cytoplasmic domain, TβRIIIΔcyto, was just as effective as TβRIII in decreasing motility and invasiveness (Figure 4A and B, data not shown). As a TGF-β coreceptor, TβRIII is thought to enhance TGF-β-mediated Smad-dependent signaling. To further investigate whether TβRIII was functioning as a TGF-β coreceptor, we examined the effects of TβRIII expression on TGF-β signaling by assessing Smad2 phosphorylation before and after EMT in the PANC-1 model. Prior to EMT, TGF-β potently induced Smad2 phosphorylation (Figure 4C, lanes 1–3). As TGF-β treatment was used to induce EMT, after EMT induction, the cells were washed and incubated in serum-free media for 2 h prior to restimulation with TGF-β. Intriguingly, after EMT, the ability of TGF-β to induce Smad2 phosphorylation was almost completely abrogated (Figure 4C, lanes 4–6). To determine whether this decrease in TGF-β responsiveness was due to loss of TβRIII expression, we assessed the effect of preventing loss of TβRIII expression on TGF-β-mediated Smad phosphorylation. Preventing loss of TβRIII expression in PANC-1 cells did not rescue the decrease in TGF-β-induced Smad phosphorylation after EMT (Figure 4C, lanes 10–12). Instead, TβRIII decreased Smad phosphorylation before and after EMT. TβRIII also suppressed TGF-β-mediated p38 phosphorylation (Figure 4C, lanes 7–12). Taken together, these results suggest that the TGF-β coreceptor function of TβRIII did not mediate the effects of TβRIII on motility and invasiveness.

TβRIII suppresses EMT-associated increases in invasiveness, in part, through generation of sTβRIII

As we had demonstrated that the ectodomain shedding of TβRIII to produce sTβRIII increased during EMT (Figure 2D), we explored whether sTβRIII was mediating the effects of TβRIII. Initially, we investigated the effects of overexpressing full-length TβRIII in PANC-1 cells on sTβRIII levels and established that there were higher levels of sTβRIII in the media of PANC-1 cells overexpressing

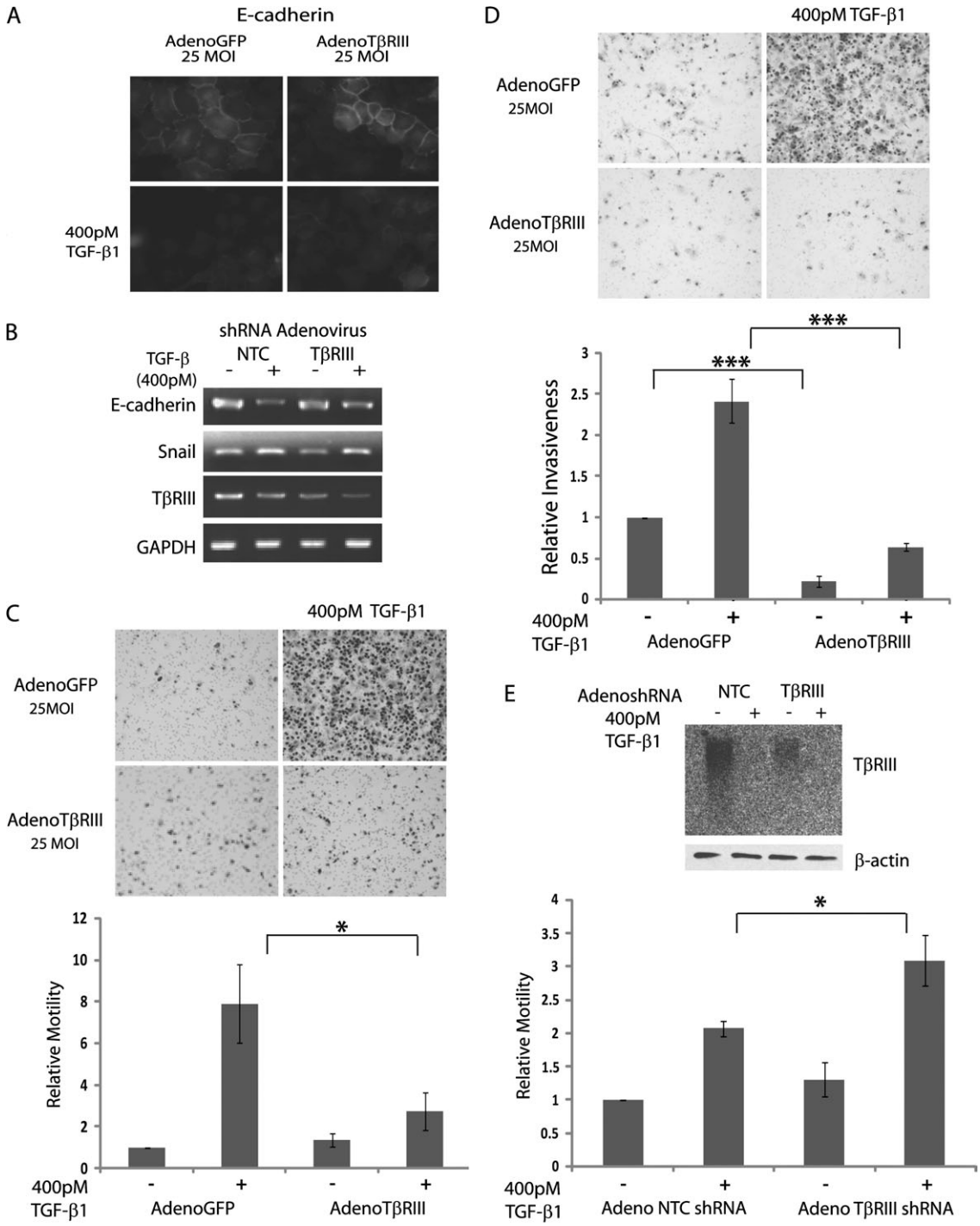

Fig. 3. Overexpression of TβRIII inhibits TGF-β1-induced EMT-associated increases in motility and invasiveness of PANC-1 cells but does not affect E-cadherin loss during EMT. PANC-1 cells adenovirally infected with GFP, and PANC-1 cells adenovirally infected with TβRIII were treated with 400 pM of TGF-β1 for 48 h and then EMT was determined by assessing E-cadherin levels using (**A**) immunofluorescence microscopy. (**B**) PANC-1 cells were infected with an adenovirus-expressing shRNA to TβRIII or a non-targeting control (NTC) shRNA. After 72 h of infection, cells were treated with TGF-β to induce EMT. RNA was isolated from the cells, reverse transcribed and then real-time PCR was performed with primers specific for E-cadherin, Snail, TβRIII and GAPDH. (**C**) Motility of untreated and treated PANC-1 cells, overexpressing either GFP or TβRIII, was assessed using 24-well fibronectin-coated transwells. (**D**) Invasion assays were performed using 24-well Matrigel-coated transwells. (**E**) PANC-1 cells were infected with an adenovirus-expressing shRNA to TβRIII or NTC shRNA. After 72 h of infection, cells were treated with TGF-β to induce EMT. After 48 h, binding and cross-linking were performed to confirm reduction of TβRIII expression and a fibronectin-coated transwell assay was performed to assess migration. All assays were performed at least three times and invasion and motility were normalized to untreated PANC-1 in each experiment. Data represent the mean ± SEM ($n = 3$), $^{*}P < 0.05$ and $^{***}P < 0.01$. Colour version is available as supplementary data at *Carcinogenesis* Online.

full-length TβRIII compared with control cells (Figure 5A, lanes 7 and 8). To determine whether sTβRIII could affect EMT-associated invasiveness, we used CM from COS-7 cells transiently transfected with either empty vector or a sTβRIII-expressing vector (Figure 5B) in Matrigel invasion assays with untreated and TGF-β treated PANC-1 cells. Both basal and TGF-β-mediated increases in invasion were attenuated ($P < 0.025$) when PANC-1 cells were incubated in CM-containing sTβRIII (Figure 5C). These data suggest that TβRIII

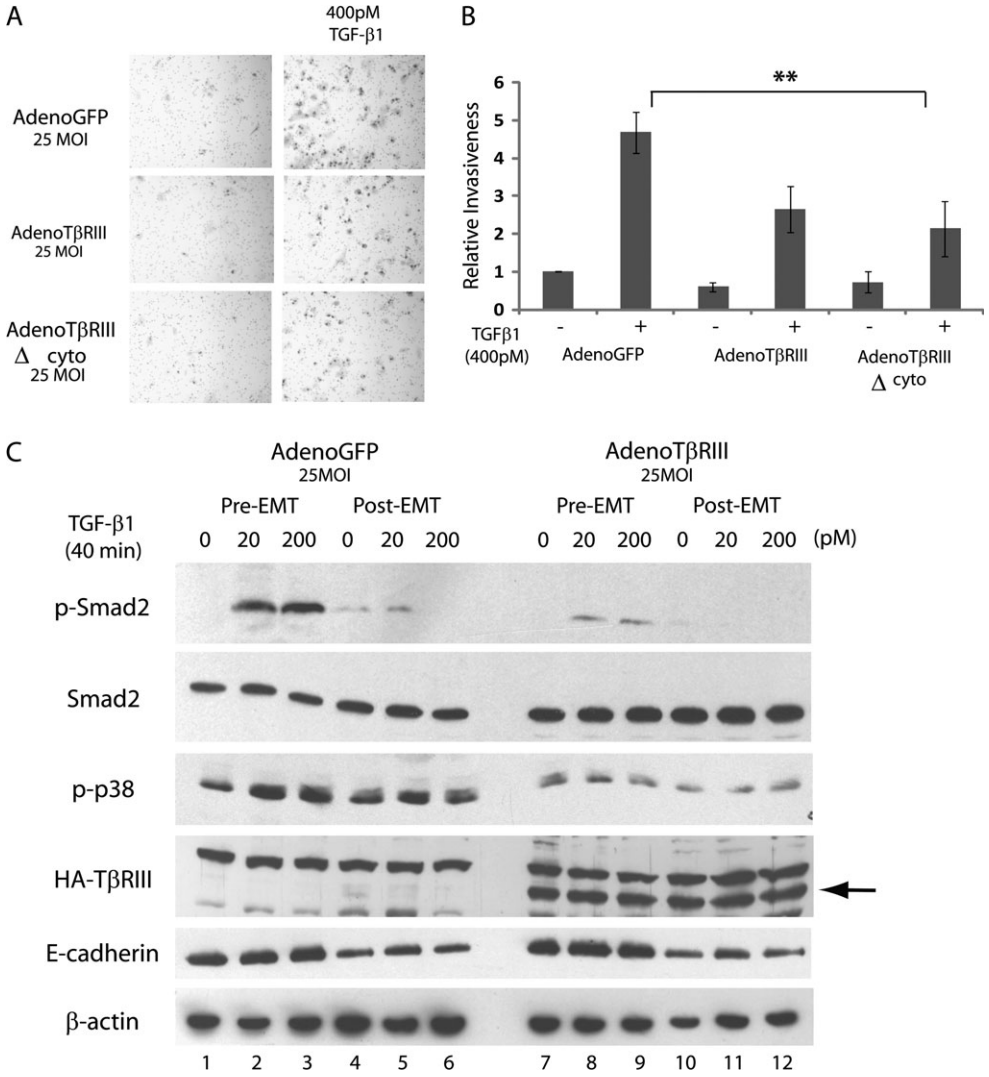

Fig. 4. The coreceptor function of TβRIII is not required to suppress invasion associated with EMT in PANC-1. (**A**) PANC-1 cells adenovirally infected with GFP and PANC-1 cells adenovirally infected with full-length TβRIII or TβRIII lacking its cytoplasmic domain were treated with 400 pM of TGF-β1 for 48 h, and then invasiveness was assessed using the Matrigel invasion transwell assay. (**B**) Pre-EMT PANC-1 cells and post-EMT PANC-1 cells (TGF-β treated for 48 h) and TβRIII overexpressing pre-EMT and post-EMT PANC-1 cells were serum starved for 2 h and then restimulated with 20 or 200 pM TGF-β for 40 min. The cells were lysed and western blotting was performed to detect the levels of phospho-Smad2, phospho-p38 and total protein levels of Smad2. Western blotting was performed to control the overexpression of TβRIII using an αHA antibody, EMT with an αE-cadherin antibody, and protein loading was controlled using an αβ-actin antibody. The arrow indicates the core of TβRIII. All assays were performed at least three times and invasion and motility were normalized to untreated PANC-1 in each experiment. Data represent the mean ± SEM ($n = 3$), $^{**}P < 0.025$. Colour version is available as supplementary data at *Carcinogenesis* Online.

functions to suppress EMT-associated increases in invasiveness, in part, through the generation of sTβRIII. As sTβRIII did not fully recapitulate the effect of full-length TβRIII, there are probably additional mechanisms that TβRIII utilizes to suppress motility and invasiveness associated with EMT.

TβRIII expression is lost in human pancreatic cancer

The dramatic loss of TβRIII expression during EMT in this human pancreatic cancer cell model suggested that TβRIII expression might be altered during human pancreatic cancer progression. To assess TβRIII expression and investigate its association with tumor grade, we performed immunohistochemical analysis of TβRIII expression on a pancreatic cancer tissue array containing 71 pancreatic cancer specimens and 9 matched normal pancreatic tissue specimens. Pancreatic adenocarcinoma is thought to arise from pancreatic ductal cells (40). Immunohistochemical staining of normal human pancreatic specimens revealed that TβRIII is highly expressed in pancreatic ductal cells (Figure 6A, left two panels, arrows). However, in pancreatic cancer specimens, TβRIII expression is significantly reduced or absent (Figure 6A, right two panels, arrows indicate remnant ductal cells). The proportion with abundant TβRIII expression decreased from 67% in normal pancreatic cancer specimens to 14% in Grade 1 specimens, to 5% in Grades 2 and 3 specimens. At the same time, the proportion with low or no TβRIII expression increased from 11% in normal pancreatic specimens to 14% in Grade 1 specimens, to 40% in Grade 2 specimens and to 50% in Grade 3 specimens (Figure 6A). Importantly, the normal pancreatic specimen with low TβRIII expression represents a specimen with no pancreatic ducts present to assess. The progressive loss of TβRIII expression with increasing pancreatic tumor grade suggests that loss of TβRIII expression may be an important contributor to pancreatic carcinogenesis.

To evaluate whether there was progressive loss of TβRIII expression in matched patient specimens, we compared the expression of TβRIII in all nine matched tumor and normal specimens. In six of these nine (67%) cases, there was reduced TβRIII expression in the tumor relative to matched normal tissue (supplementary Figure 2A,

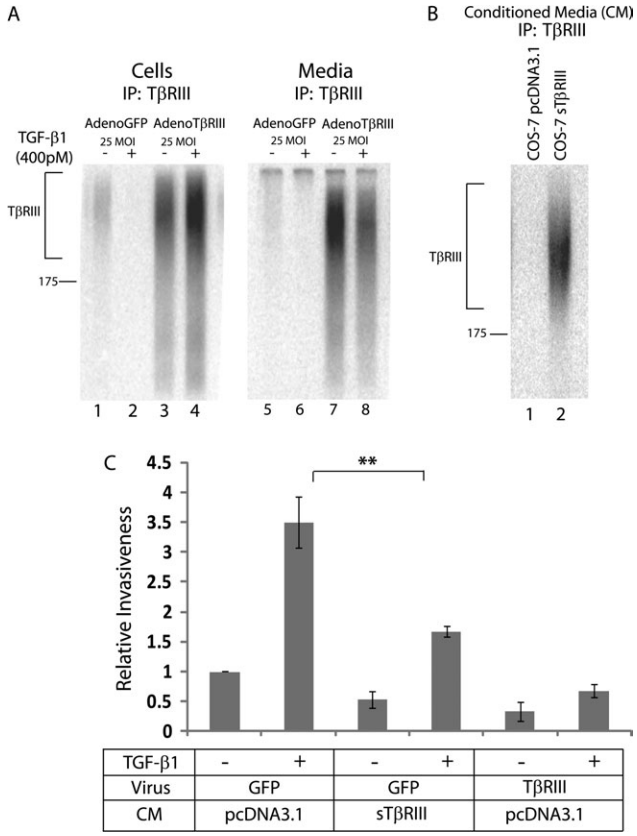

Fig. 5. TβRIII suppresses EMT-associated increases in invasiveness partially through generation of sTβRIII. (**A**) PANC-1 cells adenovirally infected with GFP and PANC-1 cells adenovirally infected with TβRIII were treated with 400 pM of TGF-β1 for 48 h. Cell surface and media levels of TβRIII were assessed by [^{125}I]-TGF-β1 binding and cross-linking followed by immunoprecipitation with an αTβRIII extracellular antibody. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and phosphorimaging of dried gels were performed. (**B**) COS-7 cells were transiently transfected with either empty vector or a sTβRIII construct. The amount of sTβRIII present in CM from these cells was assessed by [^{125}I]-TGF-β1 binding and cross-linking. (**C**) PANC-1 cells adenovirally infected with GFP were treated with 400 pM of TGF-β1 for 48 h. These and untreated PANC-1 GFP-expressing cells were incubated in COS-7 control CM or CM-containing sTβRIII in the top chamber of the Matrigel transwell, and invasiveness was assessed after 48 h. PANC-1 cells adenovirally overexpressing TβRIII were treated with 400 pM of TGF-β1 for 48 h and then incubated in the control CM to assess their invasiveness in parallel. All assays were performed at least three times and invasion and motility were normalized to untreated PANC-1 in each experiment. Data represent the mean ± SEM ($n = 3$), $^{**}P < 0.025$.

available at *Carcinogenesis* Online). One additional case exhibiting no change in TβRIII expression already exhibited little to no TβRIII expression in the normal pancreatic specimen, as there were no ducts present to assess (supplementary Figure 2A, available at *Carcinogenesis* Online). In the remaining two cases, TβRIII expression in tumor and normal tissue remained the same (supplementary Figure 2A, available at *Carcinogenesis* Online). Overall, these data suggest that there is loss of TβRIII protein expression during pancreatic carcinogenesis.

To investigate whether there was also loss of TβRIII expression at the mRNA level, we analyzed a cDNA array containing seven human pancreatic cancer samples with matched normal controls (supplementary Figure 2B, available at *Carcinogenesis* Online). TβRIII mRNA levels were reduced in pancreatic cancer tissue, an average of 2.64 ± 0.49-fold relative to matched normal control tissue, with six out of seven (86%) of the pancreatic adenocarcinoma specimens exhibiting loss of expression. To confirm and extend these results, we analyzed the previously published gene profiling studies, publicly available through the Oncomine Gene Profiling Database (34,35), for relative TβRIII expression in normal and tumor specimens. This analysis confirmed that TβRIII mRNA levels were decreased in pancreatic tumors, as the median expression value for TβRIII mRNA was significantly lower ($P < 0.05$) in pancreatic adenocarcinomas compared with normal pancreatic tissue (supplementary Figure 2C, available at *Carcinogenesis* Online). Taken together, our studies support the loss of TβRIII expression during pancreatic cancer progression, with specific effects on EMT-associated motility and invasiveness.

Discussion

Here we demonstrate, for the first time, that loss of TβRIII expression occurs during pancreatic carcinogenesis and during TGF-β-induced EMT in a pancreatic cancer model. Loss of TβRIII expression does not appear to be essential for induction of EMT, but instead appears to be required for the increased motility and invasiveness associated with EMT (Figure 3). Before EMT, PANC-1 cells have high cell surface TβRIII expression (Figure 2) and produce abundant amounts of sTβRIII (Figures 2 and 5). Although sTβRIII has been best characterized as an antagonist of TGF-β signaling, this does not appear to be the mechanism by which sTβRIII partially contributes to decreased motility and invasiveness, as PANC-1 cells lose TGF-β responsiveness independently of loss of TβRIII/sTβRIII expression (Figure 4). sTβRIII could potentially suppress EMT-associated invasiveness through sequestration of other ligands, as most coreceptors are promiscuous and TβRIII has been demonstrated to bind inhibin (41) and basic fibroblast growth factor (42) (Figure 6B). Early during TGF-β-induced EMT (i.e. hours), shedding of TβRIII increases, which reduces TβRIII cell surface levels and transiently increases sTβRIII levels (Figure 2D). Reduction of TβRIII and ultimately sTβRIII expression is then maintained (over days) by repression of TβRIII mRNA expression by TGF-β (Figure 2A and E). After EMT, in the absence of cell surface TβRIII or sTβRIII, the PANC-1 cells exhibit increased motility and invasiveness (Figure 3). This increase in motility and invasiveness can be suppressed by restoring expression of TβRIII without suppressing TGF-β-induced EMT (Figure 3). In a reciprocal manner, shRNA-mediated decreases in TβRIII expression increase motility, without altering EMT (Figure 3). Importantly, these studies dissociate some of the markers of the EMT phenotype (i.e. induction of Snail and loss of E-cadherin) from the functional consequences of EMT, namely increased motility and invasiveness (Figure 6B). Indeed, we have observed that TβRIII can also suppress the migration of L3.6p pancreatic cancer cells, which do not undergo EMT (data not shown), and that TβRIII can suppress the migration and invasiveness of breast (15) and prostate cancer cells (16) in the absence of EMT. Although sTβRIII does partially mediate the effects of TβRIII on motility and invasiveness, the mechanisms by which TβRIII and sTβRIII inhibit migration and invasion in these diverse cancer models are currently being investigated.

TβRIII has previously been implicated as an important EMT regulator during developmental processes. During chick-heart development, TβRIII is required for the EMT necessary for formation of the valves and septa, and exogenous TβRIII expression is sufficient to induce TGF-β-responsive EMT in non-competent cardiac ventricular cells (10). Conversely, specifically blocking TGF-β binding to TβRIII prevents the TGF-β-induced EMT in cardiac endothelial cells (10). In addition, TβRIII is required for the EMT during palatal fusion, as siRNA-mediated reduction of TβRIII levels in palatal shelve cultures abrogates their ability to undergo EMT and fuse (11,12). Several studies have suggested a role for TβRIII in regulating carcinogenic EMT, with down-regulation of TβRIII mRNA expression occurring during EMT in breast and skin cancer cell line models (43,44). The current study provides the first evidence for a functional role of TβRIII in carcinogenic EMT; specifically regulation of EMT-associated motility and invasiveness, without altering loss of E-cadherin expression and localization. Interestingly, a recent study demonstrated

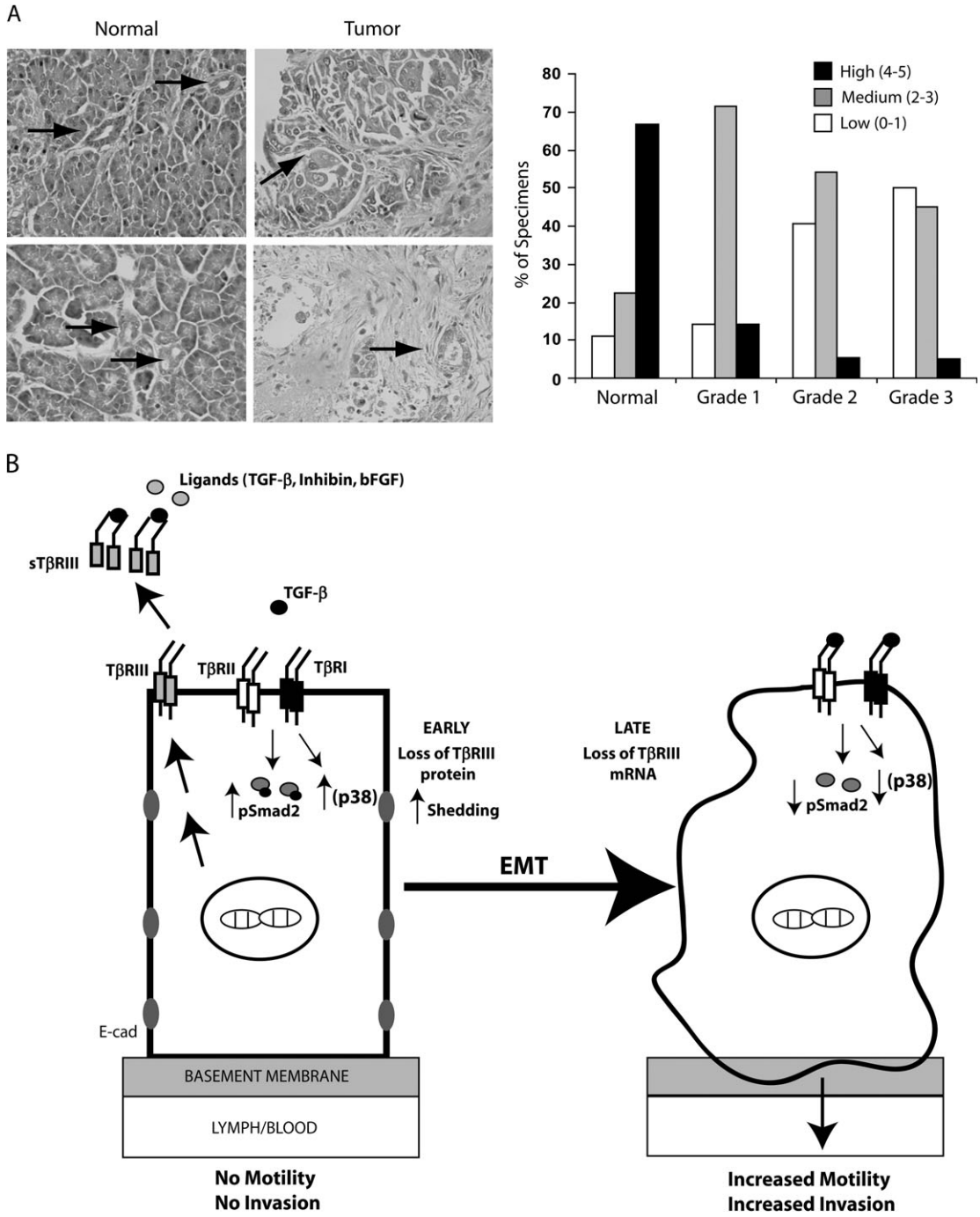

Fig. 6. TβRIII protein expression decreases during pancreatic carcinogenesis. (**A**) Immunohistochemical analysis of TβRIII was performed on a pancreatic cancer tissue array containing 71 tumor specimens and nine normal specimens. The left panels are two normal specimens, and the arrows indicate that the strongest brown staining, correlating to TβRIII, is in the pancreatic ductal cells. The right panels are two tumor specimens, and the arrows indicate remnant pancreatic ducts, which exhibit no brown staining, and therefore, express no TβRIII. Staining in all 80 specimens was blindly scored by a pathologist (T.A.F) and this analysis was quantified. The tumor grade of each pancreatic specimen was provided with the tissue array. (Colour version is available as supplementary data at *Carcinogenesis* Online). (**B**) Mechanism of TβRIII suppression of EMT-associated motility and invasiveness. Cell surface and sTβRIII levels are high before EMT. sTβRIII may bind and sequester ligands to suppress motility and invasiveness, whereas cell surface TβRIII inhibits motility and invasiveness through mechanisms yet to be defined. During EMT, shedding of TβRIII increases which decreases cell surface TβRIII and sTβRIII levels, and this loss is maintained via repression of TβRIII at the mRNA level. After EMT, in the absence of TβRIII and sTβRIII, the pancreatic cancer cells exhibit increased motility and invasiveness.

that shRNA-mediated decreases in TβRIII expression in the normal murine mammary epithelial cell line, NMuMG, induced loss of E-cadherin and increased motility and invasiveness (45). Whether TβRIII also specifically regulates motility and invasion in breast and skin cancer models of EMT, as well as in developmental models of EMT is currently under investigation.

TGF-β has dichotomous effects on epithelial and mesenchymal cells, inhibiting the proliferation of epithelial cells while stimulating the proliferation of mesenchymal cells. TGF-β also has dichotomous tumor suppressor and tumor promoter effects in most human cancers, and altered responsiveness during carcinogenic EMT has been suggested as a potential mechanism for this paradoxical role (4). Here, we

demonstrate that there are opposing effects of TGF-β on Smad2 and p38 phosphorylation before and after EMT in the PANC-1 pancreatic cancer model, with PANC-1 cells being TGF-β responsive prior to EMT, but TGF-β unresponsive after EMT (Figure 4C). In addition, when examining TGF-β-mediated inhibition of proliferation, we observed a similar phenomenon: PANC-1 cells were TGF-β responsive prior to EMT, but TGF-β unresponsive after EMT (data not shown). Both before and after EMT, expression of exogenous TβRIII resulted in further suppression of Smad2 and p38 phosphorylation, an effect which could be due to increased sTβRIII production (Figure 5A). However, as restoring TβRIII expression was unable to restore TGF-β responsiveness in either case, the effects of TβRIII on motility and invasiveness appear to be independent of effects on TGF-β signaling. These studies support a fundamental alteration in TGF-β responsiveness during carcinogenic EMT, which may help explain the dichotomous roles of TGF-β during cancer progression. The mechanism of altered TGF-β responsiveness during EMT in the PANC-1 model is currently under investigation.

In the current study, we demonstrate that sTβRIII can partially suppress motility and invasion, indicating that ectodomain shedding is a critical regulator of TβRIII function. We have previously demonstrated that sTβRIII is important in suppressing the tumorigenic effects of TβRIII during breast cancer progression (15). As sTβRIII exerts important anti-motility and -invasive effects, insight into mechanisms regulating the ectodomain shedding and generation of sTβRIII is needed, including identification of the proteases responsible for TβRIII shedding. Overexpression of membrane tethered matrix metalloproteinase 1 is able to increase shedding of TβRIII (46). As expression of matrix metalloproteinases increase during EMT (3), matrix metalloproteinases are prime candidates for mediating TβRIII shedding. We are currently investigating the mechanisms regulating the ectodomain shedding of TβRIII.

TβRIII expression has been examined in both pancreatic cancer cell lines and human adenocarcinomas at the mRNA level with conflicting results. Friess *et al.* (47) initially reported no loss of TβRIII expression in human pancreatic cancer specimens as assessed by northern blot analysis. In contrast, Venkatasubbarao *et al.* (48) analyzed mRNA levels by RT–PCR and found trace or no TβRIII expression in four of six pancreatic cancer cell lines, including PANC-1, and in 10 of 26 (38%) pancreatic adenocarcinomas. In neither study were comparisons made between matched normal pancreas and pancreatic tumor to evaluate whether there was a decrease in expression and neither study examined protein expression. In the present study, we provide evidence that there is loss of TβRIII expression in pancreatic adenocarcinoma specimens compared with their normal matched counterparts at both the mRNA and protein levels. We also demonstrate that loss of TβRIII increases as the tumor grade increases, suggesting loss of TβRIII expression as a critical component of pancreatic carcinogenesis. In addition, we demonstrate that TβRIII is highly expressed in normal pancreatic ductal cells, where pancreatic adenocarcinoma is thought to arise (40). Our studies suggest that one function of TβRIII in these ductal cells may be to maintain their epithelial character and suppress motility and invasion, with loss of TβRIII expression during pancreatic cancer progression facilitating the increased invasion and metastasis observed in human pancreatic cancer. As 90% of pancreatic cancer patients die from metastases, manipulating TβRIII or sTβRIII levels in pancreatic cancer patients may provide an important therapeutic tool to suppress metastases in these patients.

The current study suggests that TβRIII may function as a tumor suppressor in pancreatic cancer. Consistent with this role as a tumor suppressor, the genomic locus for TβRIII on chromosome 1p is deleted in 49% of human pancreatic cancer specimens (49–52). Our laboratory has previously determined that TβRIII exerts a tumor-suppressing role in breast, prostate, and ovarian cancer (15–17). In addition, Copland *et al.* (53) demonstrated significant loss of TβRIII expression in renal cell carcinoma. These studies, in addition to the present study, suggest a broad role for TβRIII as a tumor suppressor in epithelial-derived malignancies. Whether the effects of TβRIII on EMT-induced motility and invasion are operational in these other cancers warrants further investigation.

Supplementary material

Supplementary Figures 1 and 2 and the colour version of Figures 3, 4 and 6 can be found at http://carcin.oxfordjournals.org/.

Funding

National Institutes of Health/National Cancer Institute (R01-CA106307) to G.C.B.

Acknowledgements

The authors would like to thank Tam How for her technical assistance in generating the TβRIII adenovirus constructs, Dr P.J.Casey's laboratory for generously providing the Green Fluorescent Protein adenovirus construct and Dr F.Lopez-Casillas for generously providing the sTβRIII vector.

Conflict of Interest Statement: None declared.

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Received July 30, 2007; revised October 30, 2007; accepted November 3, 2007