Functional blockade of Smad4 leads to a decrease in β-catenin levels and signaling activity in human pancreatic carcinoma cells

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In the last several years, many laboratories have tried to unravel the complex signaling mechanisms activated by TGF-β_1 in transformed cells. Smad proteins are the principal mediators of the transforming growth factor β (TGF-β) response, but this factor can also activate Smad-independent pathways in different cell types. Our previous studies in murine keratinocytes led to the identification of a cooperation between oncogenic *Ras* and *Smad4* inactivation during malignant progression. We further investigated the function of Smad4 in human pancreatic cancer, in which loss-of-function mutations affecting *Smad4* occur with a 50% frequency. Expression of a dominant-negative Smad4 construct in the adenocarcinoma cell line PANC-1 led to increased ubiquitination and proteasomal degradation of β-catenin. Moreover, loss of Smad4 abrogated β-catenin-signaling activity and was associated with a reduction of the tumorigenic potential of PANC-1 cells in *scid* mice. Although the expression of the dominant-negative Smad4 blocked TGF-β_1/Smad2,3-signaling activity, the above-mentioned effects of Smad4 on β-catenin stability were independent of the TGF-β1/Smad2,3-signaling pathway. These findings provide evidence for a cross talk between Smad4 and the Wnt/β-catenin pathway in pancreatic carcinoma cells, suggesting a new role for Smad4 as an attenuator of β-catenin proteasomal degradation.

Introduction

Growth factors from the transforming growth factor β (TGF-β) family regulate multiple biological processes such as cell proliferation, differentiation and apoptosis (1–3). TGF-β_1 binds two surface receptors, named type I and II receptors, which upon activation acquire the ability to phosphorylate the receptor-activated Smads (Smad2,3). Then, these proteins interact with Smad4, a common partner, and form a Smad complex that translocates to the nucleus where it recognizes a specific DNA sequence, the Smad-binding element, in the promoter regions of TGF-β-regulated target genes (1).

TGF-β plays an important role in the control of cell proliferation and in carcinogenesis. Our group and others have extensively demonstrated that TGF-β_1 plays a dual role in mouse skin carcinogenesis, as well as in other human and murine cancer models (4–7). TGF-β_1 acts as a cell growth inhibitor in non-transformed epithelial cells, but at later stages of carcinogenesis it induces an epithelial–mesenchymal transition *in vitro* and the transition from squamous to spindle cell carcinoma associated with increased invasion and metastasis *in vivo*.

β-Catenin mediates the interaction between E-cadherin cell adhesion complexes and the actin cytoskeleton. In addition to this structural role, there is a cytoplasmic pool of β-catenin involved in cell signaling (8,9). The levels of cytoplasmic β-catenin are low in normal epithelial cells and, in the absence of Wnt signals, the protein is incorporated into a complex that includes many proteins, such as adenomatous polyposis coli (APC), axin and glycogen synthetase kinase-3β. β-Catenin is phosphorylated by this protein complex and then it is degraded by the proteasome (10,11). During some physiological processes, like embryonic development, Wnt factors activate signaling mechanisms that lead to the inhibition of glycogen synthetase kinase-3β activity. Then, β-catenin is able to translocate to the nucleus, where it regulates gene transcription in collaboration with factors from the T-cell factor (TCF)/lymphoid enhancer binding factor (Lef) family. In transformed cells, the aberrant secretion of Wnt factors or the presence of mutations in *APC* or *β-catenin* leads to the abnormal activation of β-catenin nuclear signaling (12).

Increasing evidence of cross talk between Smad signaling and other pathways has been reported during the past years, providing a better understanding of TGF-β function (see refs 13,14 for review). Two independent works have described a cooperation between the Smad and Wnt pathways required for the regulation of the transcription of the *Xenopus laevis Xtwn* and *Msx2* genes. This cooperation is possible not only because there is a combination of Smad-binding elements and TCF/Lef recognition sites in the promoter regions of these genes but also because of a physical interaction between the Smads and Lef1 (15,16). It is not known whether this cross talk operates in carcinogenesis. In hereditary colorectal carcinoma, one of the best characterized models of carcinogenesis, a high frequency of mutations occur in *APC* or *β-catenin* and in genes encoding components of the Smad pathway, such as TGF-β receptor (*TβR*)*II* or *Smad4* (17). In pancreatic carcinoma, one of the most aggressive human tumors together with colorectal cancer, *Smad4* is also frequently mutated (18,19). Nevertheless, due to its heterogeneity, there are few data available about mutations in *APC* or *β-catenin* in pancreatic carcinoma.

The objective of this study was to determine the functional consequences of Smad4 inactivation during human pancreatic carcinogenesis. For this purpose, we transfected PANC-1 cells with a dominant-negative Smad4 construct. Our findings suggest that there is a cooperation between Smad and β-catenin signaling that leads to increased malignancy of PANC-1 cells *in vivo*.

Material and methods

Cell culture, transfection procedures and treatment conditions

PANC-1 cells (American Type Culture Collection, Rockville, MD) were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum and antibiotics, 2.5 μg/ml amphotericin B, 100 μg/ml ampicillin and 32 μg/ml gentamicin (Invitrogen, Carlsbad, CA) at 37°C in a 5% CO_2 humidified atmosphere.

PANC-1 cells were co-transfected with an empty pcDNA3 expression vector (Promega, Madison, WI) containing a neomycin resistance gene and with a pCMV5 vector containing a flag-tagged construct, including amino acids 1–514 of full-length Smad4 [(20), kindly provided by Dr Joan Massague, Memorial Sloan-Kettering Cancer Center, New York, NY]. Cells were transfected with Lipofectamine Plus (Invitrogen) according to the manufacturer's instructions and selected in complete medium containing 1 mg/ml G418 (Calbiochem, Darmstad, Germany) for 2 weeks. G418-resistant cell clones were isolated with cloning rings.

For transient co-transfection assays, 3×10^4 cells were seeded in 24-well plates. 3TPlux activity assay was performed using 500 ng of the reporter construct [(21), kindly provided by Dr Carmelo Bernabeu, Centro de Investigaciones Biologicas, Madrid, Spain] and 200 ng of the TK–renilla construct (Promega) as a control for transfection efficiency. For TOP-FLASH and FOP-FLASH activity assay, we used 200 ng of the reporters [(22), kindly provided by Dr Hans Clevers, Hubrecht Laboratory, Utrecht, The Netherlands] and 100 ng of TK–renilla, as well as different amounts of pQE32 β-cat S33Y [(23), kindly provided by Dr Avri Ben-Ze'ev, Weizmann Institute, Rehovot, Israel], and full-length Smad4 [(20), kindly provided by Dr Joan Massague, Memorial Sloan-Kettering Cancer Center]. Luciferase and renilla activities were measured using the Dual-Luciferase Reporter assay kit (Promega).

Abbreviations: APC, adenomatous polyposis coli; TGF-β, transforming growth factor β.

TGF-β_1 treatments were performed with human recombinant TGF-β_1 (R&D Systems, Minneapolis, MN). The growth factor was added to subconfluent cell cultures at the indicated final concentrations. For cell proliferation assay, 2 × 10^5 cells were seeded in 24-well plates, allowed to settle overnight and treated with TGF-β_1 at different concentrations for 48 h. Cell numbers were determined with a hemocytometer. For each concentration, the number of cells at the beginning of the treatment was subtracted from the final cell number, and the ratio of TGF-β_1 treated versus untreated cells was calculated as a percentage.

The TβRI kinase inhibitor SB431542 (Sigma-Aldrich, St Louis, MO) was added to subconfluent cell cultures at different concentrations for 24 h. For inhibition of protein synthesis, 10 μg/ml cycloheximide (Sigma-Aldrich) was added to subconfluent cell cultures at different times. The proteasome inhibitors N-acetyl-Leu-Leu-Norleucinal (ALLN) and MG132 and the calpain inhibitor calpeptin (Calbiochem) were added to subconfluent cell cultures at different concentrations for 9 h.

Immunofluorescence

Immunofluorescence staining for Smad2,3 was performed in subconfluent cells grown on glass coverslips. The cells were fixed in 3.7% formaldehyde, permeabilized in 0.3% Triton X-100 and blocked in 0.3% bovine serum albumin–0.3% Triton X-100. The rabbit polyclonal antibody specific for Smad2,3 (Upstate, Lake Placid, NY) was used at a 1:50 dilution, and a rhodamine-conjugated anti-rabbit IgG (Jackson Immunochemicals, West Grove, PA) was used as secondary antibody at a 1:100 dilution.

Western blot analysis

For total cell protein extraction, cells were lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris–HCl, pH 7.5, and 1 mM sodium orthovanadate) containing a cocktail of protease inhibitors (2 mg/ml aprotinin, 2 mg/ml leupeptin and 1 mM phenylmethylsulphonyl fluoride). Lysates were centrifuged at 12 000*g* for 15 min at 4°C. The supernatants, containing the protein extracts, were aliquoted and conserved at −20°C. Fractions enriched in polyubiquitinated proteins were obtained from these lysates using the ubiquitin enrichment kit (Pierce, Rockford, IL), according to the manufacturer's instructions.

To fractionate cell proteins for its affinity with the cytoskeleton, cells were lysed in a buffer containing 100 mM NaCl, 2 mM $CaCl_2$, 1% Triton X-100, 1% NP-40, 50 mM Tris–HCl, pH 7.4, 1 mM sodium orthovanadate and a cocktail of protease inhibitors. After centrifugation at 12 000*g* for 15 min at 4°C, the pellets, containing the proteins strongly associated with the cytoskeleton (insoluble fraction), were separated from the supernatants, containing the protein fraction non-associated with the cytoskeleton (soluble fraction). Both fractions were aliquoted and conserved at −20°C.

The nuclear and non-nuclear protein fractions were obtained as described in ref. (24). Briefly, cells were lysed in hypotonic buffer (20 mM N-2-Hydroxyethylpiperazine-N'-2-Ethane Sulfonic acid, pH 7, 10 mM KCl, 2 mM $MgCl_2$, 0.5% NP-40 and 1 mM sodium orthovanadate) containing a cocktail of protease inhibitors. Lysates were homogenized gently (30 strikes in a syringe with a 19 gauge needle and 30 strikes with a 25 gauge needle) and centrifuged at 1500*g* for 5 min at 4°C. The supernatants, containing the non-nuclear fraction, were cleared by centrifugation at 12 000*g* for 5 min and conserved at −70°C. The pellets, containing the nuclear fraction, were washed three times in hypotonic buffer. After homogenization in hypertonic buffer (hypotonic buffer containing 0.5 M NaCl and a cocktail of protease inhibitors), they were centrifuged at 12 000*g* for 10 min, and the supernatants were conserved at −70°C.

Aliquots of cell lysates containing the same amount of protein were resolved by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to Immobilon P membranes (Millipore, Billerica, MA). Membranes were incubated with the primary antibodies specific for β-catenin (BD Transduction Laboratories, Palo Alto, CA), α-tubulin (Sigma-Aldrich), β-actin (Sigma–Aldrich), Proliferating cell nuclear antigen (Signet Laboratories, Dedham, MA), P-Smad2,3 (Upstate) and Smad2,3 (Upstate) and with the appropriate peroxidase-conjugated secondary antibodies. Western blots were visualized using the enhanced chemoluminescent system (Amersham, Little Chalfont, Buckinghamshire, UK) to expose X-ray films.

RNA analysis

Total RNA was isolated from the cell lines by the guanidinium thiocyanate procedure. Reverse transcription was performed at 37°C for 2 h using the moroney murine leukemia virus enzyme (Promega), 1 μg RNA as template and oligo-dT as primer.

Quantitative polymerase chain reaction was performed using iTaq SYBR Green Supermix (Bio-Rad, Hercules, CA) and specific primers (SuperArray, Frederick, MD) in an IQ5 workstation (Bio-Rad).

Tumorigenicity assay

Suspensions of 3 × 10^6 cells were injected intradermically in the flanks of *scid* mice (Charles River Laboratories, Wilmington, MA). The animals were examined for the presence of tumors, which were measured periodically. The samples obtained after euthanizing the animals were analyzed by the Experimental Pathology Service of the Instituto de Investigaciones Biomedicas, Madrid, Spain.

Results

Functional blockade of Smad4 in PANC-1 cells abrogates the TGF-β1/Smad2,3 response

PANC-1 cells were co-transfected with a pcDNA3 vector encoding a neomycin resistance cassette and with a flag-tagged Smad4 construct lacking the 39 C-terminal amino acids, which exerts a dominant-negative effect because the truncated protein lacks the domain responsible for Smad heteromerization (20). After 2 weeks of G418 treatment, several individual clones were selected and isolated. PANC-1 cells were also transfected with the pcDNA3 empty vector only (Pneo), as a control.

Following TGF-β_1 activation, Smad2,3 associate with Smad4 and translocate to the nucleus, where the complex regulates the transcription of multiple genes (1). To analyze whether the expression of dominant-negative Smad4 led to a blockade of the Smad-signaling pathway in PANC-1 cells, we characterized the kinetics of Smad2,3 nuclear translocation in the parental cell line and in Pneo cells. Stimulation with 2 ng/ml TGF-β_1 resulted in enhanced nuclear localization of Smad2,3, that reached a maximum at 1 h of treatment, and decreased back to the basal level 3 h after the stimulation (data not shown). We selected two clones derived from the transfection with dominant-negative Smad4, dn5 and dn7, in which TGF-β1-induced Smad2,3 nuclear translocation was blocked (Figure 1A). To confirm that the Smad pathway was not functional in these clones, we assayed the activity of 3TPlux, a reporter gene that contains three Smad-binding elements from the PAI-1 promoter, one of the targets of Smad2,3. TGF-β_1 treatment led to a 3-fold increase of 3TPlux basal activity in Pneo cells, but had no effect in dn5 and dn7 clones (Figure 1B).

TGF-β_1 is a potent inhibitor of cell proliferation in many cell types, notably in epithelial cells (3). In PANC-1 cells, it has been described that TGF-β_1 plays a suppresor role (25). As this effect might be mediated by Smad4, we measured cell proliferation at different times in the presence of different concentrations of TGF-β_1. At 48 h of treatment with 5–10 ng/ml TGF-β_1, cell proliferation was inhibited in both the parental and Pneo cell lines for 50–80%. However, TGF-β_1, at the same concentrations, only inhibited 30% of cell proliferation in dn5 and dn7 cell clones (Figure 1C). This result led us to conclude that blockade of Smad4 suppressed the TGF-β_1 antiproliferative response in PANC-1 cells.

Blockade of Smad4 in PANC-1 cells leads to decreased cytoplasmic and nuclear β-catenin levels

PANC-1 cells display a rounded morphology and do not establish stable cell–cell contacts between adjacent cells due to the absence of E-cadherin (26). We neither observe any significant change in the morphological appearance of dn5 and dn7 with respect to PANC-1 or Pneo cells nor detect expression of E-cadherin in the transfected clones (data not shown).

In contrast, we observed reduced expression (~50%) of β-catenin in clones transfected with dominant-negative Smad4 when compared with the other clones (Figure 2A). As the function of β-catenin in signal transduction depends on its cytoplasmic/nuclear localization, we analyzed the distribution of the protein after the cells were lysed in 1% Triton X-100/1% NP-40. This allowed us to separate an insoluble fraction, containing proteins tightly associated with the cytoskeleton, from a soluble fraction, containing the rest of cellular proteins. We detected a 50% reduction in the levels of soluble β-catenin in dn5 and dn7 clones when compared with PANC-1 and Pneo cells, whereas no changes were observed in the levels of cytoskeleton-associated β-catenin among the different clones (Figure 2B). Cytoplasmic (free)

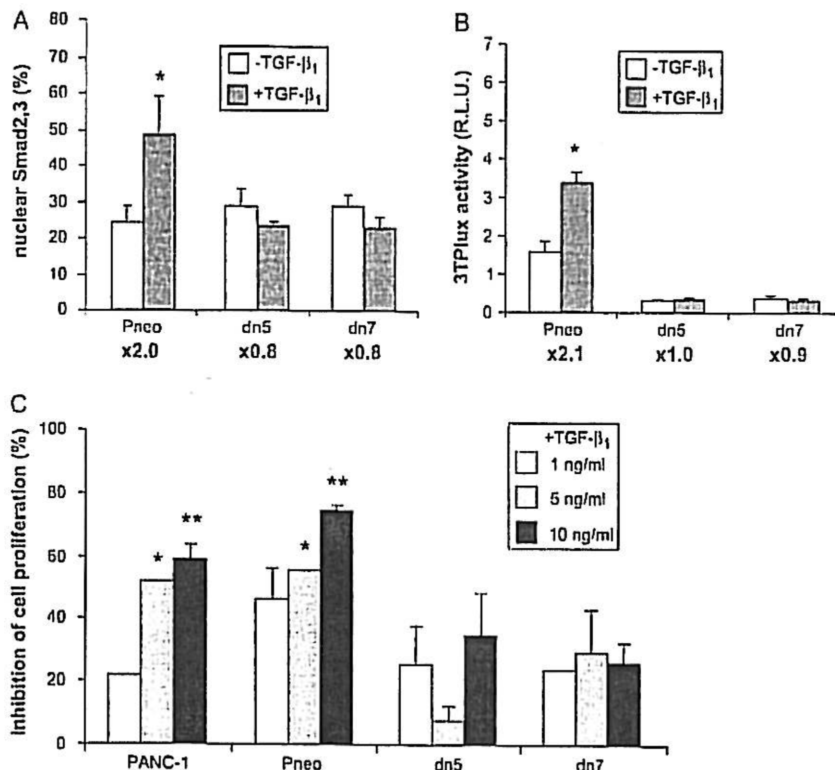

Fig. 1. Blockade of the TGF-β/Smad response in dominant-negative Smad4 PANC-1 transfectants. (A) Nuclear translocation of Smad2,3 after treatment with 2 ng/ml TGF-β_1 during 1 h. Smad2, 3 were visualized by immunofluorescence staining with an antibody directed against both proteins. The percentage of cells showing nuclear staining was determined in 10 fields per experiment at least and the average and standard deviation were calculated. (B) Measurement of 3TPlux reporter activity. Cells were transiently transfected with the 3TPlux and pRL-TK plasmids and grown for 24 h in the presence or absence of 5 ng/ml TGF-β_1. Renilla activity was used to normalize luciferase activity, and the result was expressed in R.L.U. (relative light units). The average and standard deviation of triplicate determinations of two independent experiments were plotted. (C) Cell proliferation assay in PANC-1 transfectants. Cells were plated, allowed to settle overnight and treated with TGF-β_1 for 48 h. For each treatment, the inhibition of cell proliferation was calculated as described in Materials and Methods in triplicate determinations of two independent experiments, and the average and the standard deviation were plotted. $^*P < 0.05$ and $^{**}P < 0.01$, Student's t-test.

β-catenin can translocate to the nucleus, where it regulates gene transcription. We analyzed the presence of β-catenin in the purified nuclear fraction as well as in nuclei-free extracts. The expression of the protein was significantly decreased in the nuclear fractions of clones dn5 and dn7, in which it was barely detectable (Figure 2C).

To investigate the possibility that the reduction in β-catenin levels observed in dn5 and dn7 cells was a consequence of inhibiting TGF-β1 signaling, we treated PANC-1 cells with SB431542, a selective inhibitor of the TGF-β type I receptors activin receptor-like kinase (ALK)4, ALK5 and ALK7 (27). The treatment with this agent did not affect β-catenin levels in PANC-1 cells (Figure 3A), but it blocked Smad2,3 transcriptional activity, as it was determined using the 3TPlux reporter (Figure 3B). If β-catenin levels were directly related to TGF-β1 signaling in PANC-1 cells, the treatment with the factor would have induced an increase in β-catenin levels as it has been observed in desmoid tumors (28). However, neither a short-time (Figure 3C) nor a long-time treatment of PANC-1 cells with TGF-β1 (data not shown) affected β-catenin levels. In these conditions, Smad2,3 were phosphorylated, indicating a normal cellular response to TGF-β (Figure 3C). Altogether, these observations suggest a requirement of Smad4, rather than activation of the TGF-β1 pathway, is involved in the regulation of β-catenin protein levels in PANC-1 cells.

Blockade of Smad4 leads to accelerated proteasomal degradation of β-catenin in PANC-1 cells

To determine the mechanism involved in the regulation of β-catenin in PANC-1 clones, we analyzed the expression of β-catenin transcripts by quantitative reverse transcription–polymerase chain reaction. We did not detect any significant difference in β-catenin messenger RNA expression among the different clones (Figure 4A). This result led us to conclude that the differences in β-catenin levels in dn5 and dn7 clones with respect to parental and control cells are not due to a transcriptional mechanism.

The cytoplasmic pool of β-catenin is very unstable in the absence of Wnt signals and the protein is rapidly phosphorylated and degraded. To compare the half-life of β-catenin in the different cell lines, we treated Pneo and dn5 with cycloheximide, an inhibitor of protein synthesis, at different times (Figure 4B). β-Catenin half-life is shorter in dn5, in which β-catenin levels decreased shortly after treatment with cycloheximide. For instance, after a treatment of 2 h, the amounts remaining of β-catenin in dn5 and Pneo clones were ~30 and 65%, respectively.

A major mechanism for protein degradation in mammalian cells is mediated by the proteasome. This pathway seems to be the main mediator of β-catenin degradation (10). To analyze the implication of the proteasome in β-catenin stability in the clones, we treated the cell lines with ALLN and MG132, two different inhibitors of proteasomal activity (Figure 4C). Both agents induced accumulation of the protein equalizing the levels of β-catenin in the cell lines. Since ALLN is also an inhibitor of calpain (29), we used calpeptin, a specific inhibitor of calpain. As shown in Figure 4C, calpeptin had no effect in β-catenin expression. These results suggest that the expression of dominant-negative Smad4 accelerates β-catenin degradation through the proteasome in PANC-1 cells.

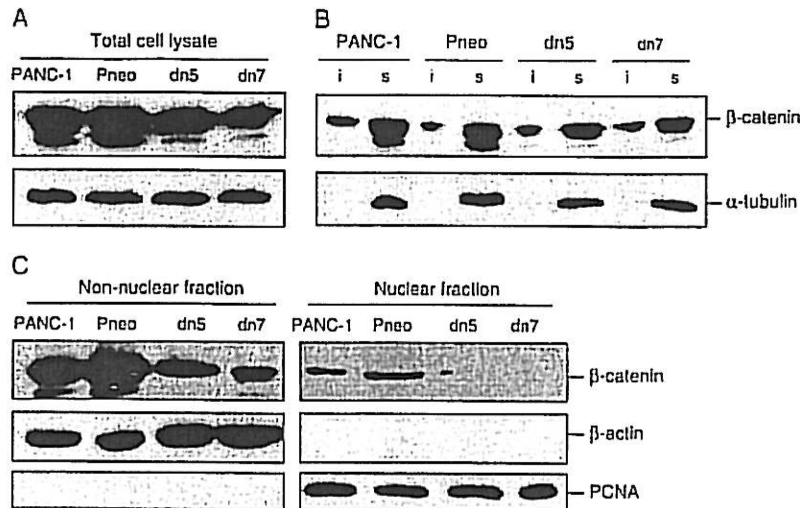

Fig. 2. Reduced β-catenin protein expression in dominant-negative Smad4 clones. (A) Western blot analysis in total cell lysates showed that β-catenin expression decreases in dn5 and dn7 clones. The membranes were reincubated with an anti-α-tubulin antibody as a control for protein loading. (B) Analysis of the distribution of β-catenin in the 1% Triton X-100 and 1% NP-40 insoluble (i) and soluble (s) fractions. Western blot for α-tubulin was performed as a control for protein loading. (C) Comparison between β-catenin expression in the non-nuclear and nuclear protein fractions from PANC-1 clones. The membranes were reincubated with an anti-β-actin antibody, as a control of a non-nuclear protein, and with an anti-proliferating cell nuclear antigen antibody, as a control of a nuclear protein.

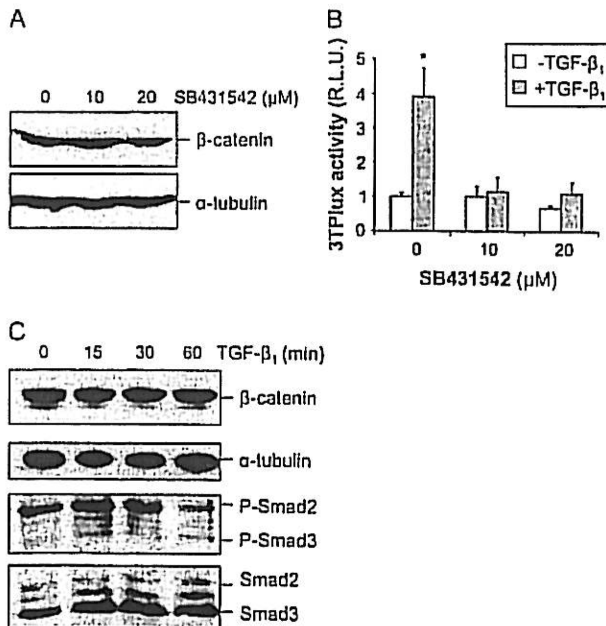

Fig. 3. Effect of TGF-β1 signaling on Smad4 expression in PANC-1 cells. (A) Effect of the TβRI kinase inhibitor SB431542 in β-catenin protein expression in PANC-1 cells. The inhibitor was added at the indicated concentrations during 24 h in the presence of 10% serum. Western blot for α-tubulin was performed as a control for protein loading. (B) The TβRI kinase inhibitor SB431542 blocks Smad2,3-dependent signaling in PANC-1 cells. 3TPlux activity was measured as described for Figure 1B. *$P < 0.05$, Student's t-test. (C) Effect of TGF-β_1 treatment in β-catenin protein expression in PANC-1 cells. PANC-1 cells were treated with 5 ng/ml TGF-β_1 for the indicated times in the presence of 10% serum. Western blot for α-tubulin was performed as a control for protein loading. Smad2 and Smad3 were phosphorylated in PANC-1 cells in response to TGF-β_1 treatment. The same samples were blotted for P-Smad2,3 and for total Smad2,3.

β-Catenin signaling is decreased in Smad4 dn clones

The presence of low nuclear levels of β-catenin suggested that β-catenin signaling was impaired in clones dn5 and dn7 with respect to Pneo and PANC-1 cells. This was shown to be the case by using the TOP-FLASH reporter construct, in which three consensus sites for TCF/Lef factors are fused to the luciferase gene (22). These consensus sites are mutated in the FOP-FLASH promoter, which was used as a control (Figure 5A). The ratio between TOP- and FOP-FLASH activities was 3 in PANC-1 and Pneo, indicating that β-catenin-dependent gene transcription is active in these cells. In contrast, the ratio between TOP- and FOP-FLASH activities in dn5 and dn7 falls to 1, meaning that this response is blocked in these clones. However, it was recovered in cells expressing increasing mutant β-catenin S33Y, which cannot be phosphorylated by the degradation complex (23), confirming that impaired β-catenin/TCF transcriptional activity in dn5 and dn7 cells is due to the rapid degradation of β-catenin. Similarly, overexpression of wild-type Smad4 in dn5 and dn7 cells increased both β-catenin/TCF and 3TPlux transcriptional responses (Figure 5C and D), indicating that the block in β-catenin signaling observed in these cells was a consequence of the loss of Smad4. Finally, we found that the expression of peroxisome proliferator activated receptorδ and CD44, two β-catenin/TCF target genes (8), was reduced in dn5 and dn7 clones with respect to Pneo and PANC-1 cells (Figure 5E and F).

Blockade of Smad4 attenuates the tumorigenic potential of PANC-1 cells

To determine the consequences of the block of Smad and β-catenin/TCF-signaling pathways in the tumorigenicity of PANC-1 cells, we injected the different clones intradermically in both flanks of *scid* mice. Tumor incidence after injection of dn5 and dn7 clones was lower than that of control cells, 67 versus 100%, with slightly longer latency periods in tumors induced by the dominant-negative Smad4-expressing clones (Figure 6). The difference in the onset of tumors induced by dn5 and dn7 versus Pneo cells was statistically significant ($P < 0.001$, Student's t-test). All tumors were histologically typed as undifferentiated carcinomas (data not shown). Taken together, these data suggest that dn5 and dn7 cell lines are less tumorigenic than control or PANC-1 cells.

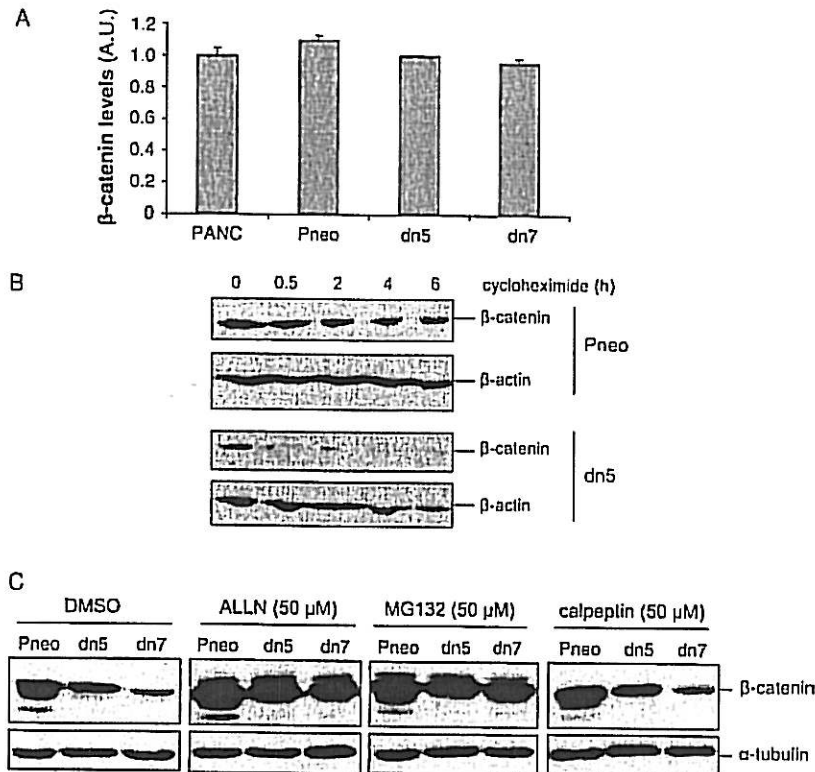

Fig. 4. Blockade of Smad4 accelerates the turnover of β-catenin in PANC-1 clones. (A) Analysis of β-catenin RNA expression by quantitative polymerase chain reaction. The values were normalized for glyceraldehydes 3-phosphate dehydrogenase expression and expressed in arbitrary units (A.U.). (B) The half-life of β-catenin is shorter in dn5 clone. The Pneo and dn5 clones were treated at different times with 10 μg/ml cycloheximide in the presence of 10% serum. β-Catenin levels were detected by western blot. β-Actin was used as a control for protein loading. (C) Effect of treatment with the proteasome inhibitors ALLN and MG132 and the calpain inhibitor calpeptin in β-catenin protein expression. The agents were added at the indicated concentrations during 9 h in the presence of 10% serum. Control treatments were performed in 0.5% dimethyl sulfoxide (DMSO). The pictures shown are from membranes equally exposed. An anti-α-tubulin antibody was used as a control for protein loading.

Discussion

The alteration of the cellular responses to TGF-β1 is a hallmark of many carcinogenic processes. In cells that have undergone malignant progression, the cytokine no longer exerts antiproliferative or proapoptotic actions but, instead, it promotes invasion and metastasis (2,3). Although the molecular mechanisms responsible for this switch still remain to be fully determined, many authors have proposed that the integration of different signaling pathways plays an important role in the response of transformed cells to TGF-β1. The most paradigmatic situation is the cross talk between Ras- and TGF-β1-signaling pathways. TGF-β1 can activate Ras independently of Smads and this stimulation produce different effects on malignant progression (see refs 30,31 for review). One of the components of the TGF-β-signaling cascade that is subject to alteration during carcinogenesis is *Smad4*, a key mediator of the Smad-signaling pathway and a tumor suppressor (32). In a previous study, we demonstrated the collaboration between loss of Smad4 function and hyperactivation of Ras in transformed keratinocytes. Thus, in cells containing a *Ras* oncogene, loss-of-function of *Smad4* led to increased Ras basal activity associated with the transition from a squamous to more aggressive spindle tumor phenotype (33). Moreover, we have observed that this mechanism also operates in human colon carcinoma cells (Diana Romero, Maite Iglesias and Miguel Quintanilla). However, cross talk between Ras and Smad signaling seems to be highly dependent on the cell status. Thus, in PANC-1 cells, we did not detect any change in Ras-signaling activity after the loss of Smad4 (data not shown), a fact probably related with the extremely high levels of active Ras that these cells contain at the basal state, that makes unlikely that it can be increased further.

Surprisingly, in PANC-1 cells, functional loss of Smad4 led to decreased cytoplasmic and nuclear β-catenin levels. These findings bring back a new element to the complex network of TGF-β signaling in carcinoma cells. Our data provide evidence for enhanced β-catenin degradation through the proteasome in the absence of Smad4, a phenomenon that can be rescued by proteasomal inhibitors. Moreover, β-catenin-dependent transcriptional activity was abrogated as a consequence of the rapid turnover of β-catenin. These changes correlate with reduced tumorigenicity in *scid* mice. The molecular mechanisms responsible for β-catenin stabilization in the presence of Smad4 remain to be determined. We show that increased β-catenin degradation in dominant-negative Smad4 expressing is dependent on Smad4 but independent of TGF-β signaling. It could be a direct interaction between Smad4 and β-catenin within the cytoplasm that would protect β-catenin from phosphorylation and subsequent degradation. Although our attempts to coimmunoprecipitate both proteins from PANC-1 cell lysates were unsuccessful, this interaction has been described in *X.laevis* by Nishita *et al.* (15). These authors have detected a weak, and probably indirect, interaction between Smad4 and β-catenin.

It has been well established that alterations of Wnt signaling components play a central role in colon carcinogenesis. The best-known example is in hereditary colorectal cancer, in which *APC* mutation is the initiation event (17). In contrast, few data are available about the implication of Wnt signaling in pancreatic carcinoma. It has been

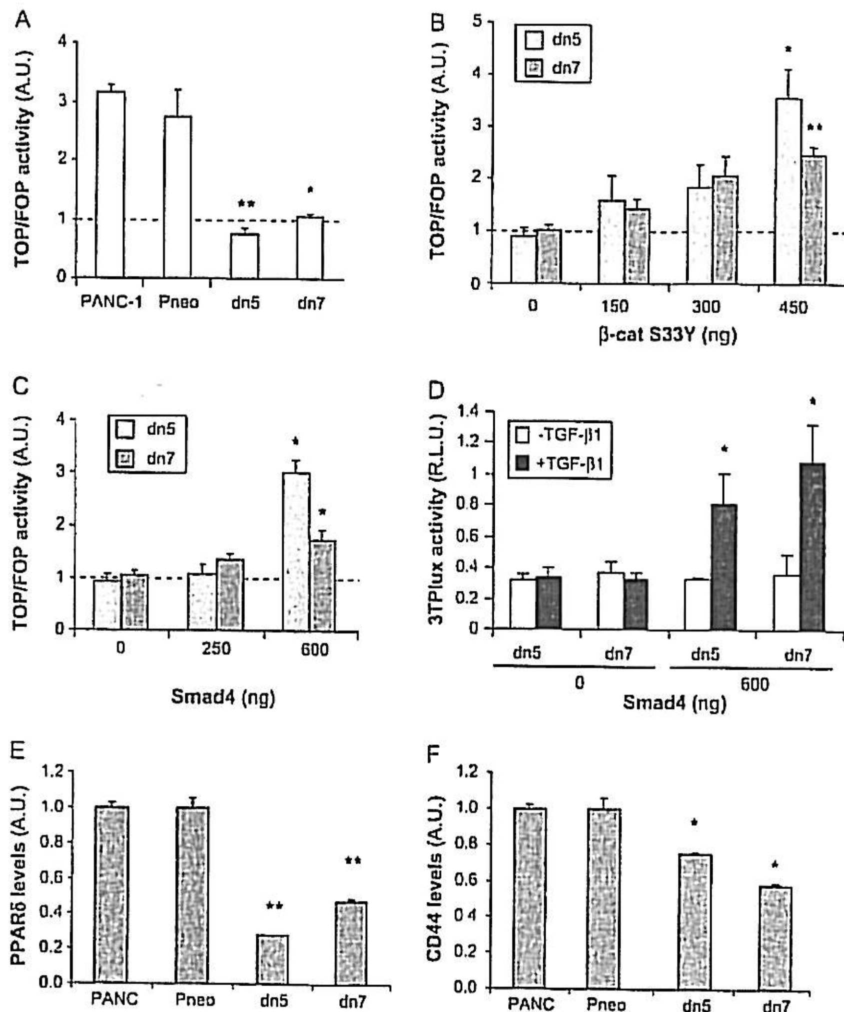

Fig. 5. β-Catenin transcriptional response is blocked in dominant-negative Smad4 PANC-1 clones. (A) Determination of β-catenin activity with the TOP/FOP reporter system. Cells were transiently transfected with the TOP-FLASH or the FOP-FLASH reporters and with the pRL-TK plasmid. The luciferase activity was measured 24 h later and corrected for renilla activity. The ratio between TOP-FLASH and FOP-FLASH activities was determined in triplicate determinations of two independent experiments, and the average and standard deviation of these values were expressed in A.U. (arbitrary units). (B) Expression of mutated β-catenin rescues TOP/FOP activity in dn5 and dn7 clones. Cells were co-transfected with the TOP-FLASH or FOP-FLASH and pRL-TK plasmids, and with different amounts of β-catenin bearing the S33Y mutation, that cannot be phosphorylated and degraded by the proteasome. (C) Overexpression of wild-type Smad4 in dn5 and dn7 clones restores β-catenin transcriptional response. The clones were co-transfected with the TOP-FLASH or the FOP-FLASH and the pRL-TK plasmids and with different amounts of pCMV5-full-length Smad4. (D) Overexpression of Smad4 restores TGF-β1 signaling through the Smad pathway in dn5 and dn7 clones. 3TPlux activity was measured as described for Figure 1B. (E and F) Analysis of the expression of target genes of β-catenin signaling in PANC-1 clones. peroxisome proliferator activated receptorδ (E) and CD44 (F) RNA expression was analyzed by quantitative polymerase chain reaction. The values were normalized for glyceraldehydes 3-phosphate dehydrogenase expression and expressed in arbitrary units (A.U.). *$P < 0.05$ and **$P < 0.01$, Student's t-test.

described that *APC* or *β-catenin* is frequently mutated in some rare malignant tumors of the pancreas, like adenomatous polyposis-associated pancreoblastoma and pancreatic acinar cell carcinoma (34,35). Pujal *et al.* (36) have recently analyzed Wnt-signaling activity in a panel of pancreatic carcinoma cell lines. This study concluded that only a small subset of cell lines displayed a high basal activity of the Wnt-signaling pathway. Although many groups have studied the integration of TGF-β and Wnt signals during embryonic development (15,16,37,38), the cross talk between these pathways in carcinogenesis remains largely unexplored. The few data available come from studies carried out in transgenic mice (39,40). The conclusions of these reports indicate that loss of both *Smad4* and *APC* cooperate in malignant progression. Since *APC* is unaltered in PANC-1 cells, our results suggest that, in the presence of normal *APC*, loss of *Smad4* results in reduced β-catenin protein levels and signaling activity that leads to decreased tumorigenicity.

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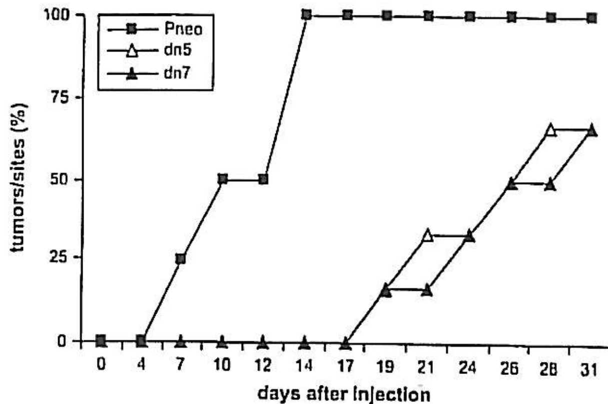

Fig. 6. Tumorigenicity of PANC-1 clones in *scid* mice. Cells were injected in both flanks of three mice per group. The animals were examined periodically for the appearance of tumors. A mouse from the Pneo group died shortly after injection and could not develop visible tumors.

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