SMAD7 ABROGATES TGF-β1 MEDIATED GROWTH INHIBITION IN COLO-357 CELLS THROUGH FUNCTIONAL INACTIVATION OF THE RETINOBLASTOMA PROTEIN

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Running Title: Suppression of RB Function by Smad7
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Smad7 is overexpressed in 50% of human pancreatic cancers. COLO-357 pancreatic cancer cells engineered to overexpress Smad7 are resistant to the actions of transforming growth factor-beta-1 (TGF-β1) with respect to growth inhibition and cisplatin-induced apoptosis, but not with respect to modulation of gene expression. In order to delineate the mechanisms underlying these divergent consequences of Smad7 overexpression, we studied the effects of Smad7 on TGF-β1-dependent signaling pathways and cell cycle regulating proteins. TGF-β1 induced the phosphorylation of MAPK, p38-MAPK, and AKT2 irrespective of the levels of Smad7, and inhibitors of these pathways did not alter TGF-β1 actions on cell growth. By contrast, Smad7 overexpression interfered with TGF-β1-mediated attenuation of cyclin A and B levels, inhibition of cdc2 dephosphorylation and CDK2 inactivation, upregulation of p27, and the maintenance of the retinoblastoma protein (RB) in a hypophosphorylated state. Smad7 also suppressed TGF-β1-mediated inhibition of E2F activity but did not alter TGF-β1-mediated phosphorylation of Smad2, the nuclear translocation of Smad2/3/4, or DNA binding of the Smad2/3/4 complex. Although Smad7 did not associate with the type I TGF-β receptor (TßRI), SB-431542, an inhibitor of the kinase activity of this receptor, blocked TGF-β1-mediated effects on Smad-2 phosphorylation. These findings point toward a novel paradigm whereby Smad7 acts to functionally inactivate RB and de-repress E2F without blocking the activation of TßRI and the nuclear translocation of Smad2/3, thereby allowing for TGF-β1 to exert effects in a cancer cell that is resistant to TGF-β1-mediated growth inhibition.

The transforming growth factor-β (TGF-β) superfamily is a group of multifunctional cytokines that play an important role in regulating cell growth and differentiation, cell death (apoptosis), and morphogenesis in a variety of biological systems (1,2). TGF-βs regulate cell growth by enhancing the proliferation of cells that are of mesenchymal origin while inhibiting the proliferation of epithelial cell types. TGF-β1 action is mediated via a heteromeric complex of TGF-β type II (TßRII) and type I (TßRI) serine/threonine kinase receptors (TßRI and TßRII) that forms upon ligand binding to TßRII (3). The ensuing phosphorylation of serine and threonine...
residues within the GS domain of TßRI activates its kinase activity, thereby leading to the phosphorylation of the receptor-regulated Smads (R-Smads) Smad2 and Smad3. Activated Smad2/3 form a complex with Smad4 (a Co-Smad), and translocate to the nucleus to act as transcriptional modulators of TGF-ß1 regulated genes (4). Smad7, an inhibitory Smad, inhibits TGF-ß1 signaling by binding to the TßRI receptor and preventing phosphorylation of Smad2 and Smad3 (5). However, additional non-Smad signaling pathways can be activated including the mitogen-activated protein kinases (ERK1/2, p38 MAPK), (6) the stress activated protein kinases (JNK1/2) (7), and the phosphatidylinositol-3 kinase (PI3K) pathways (8) (9).

TGF-ß1 inhibits the growth of cells of epithelial origin by down-regulating components of the cell cycle and up-regulating cell cycle inhibitors. In most epithelial cell types TGF-ß1 acts in late G1 phase and prevents further progression to the G1/S phase transition (10). Cyclin-dependent kinases (CDK’s) and cyclin complexes phosphorylate specific target molecules, such as the retinoblastoma proteins pRB, p107, and p130 (11). The cyclin dependent kinase inhibitors (CDKI’s) mediate cell cycle arrest at different points of G1 (12). TGF-ß1 inhibitory actions in late G1 phase is mediated, in part, by the inhibition of cyclin D1 and cyclin E expression which prevents CDK kinase activity resulting in RB hypophosphorylation (13,14), and/or by the upregulation the expression of various CDKI’s such as p21, and p27 (15,16). The transcription factor E2F regulates the expression of S and G2 phase cyclins such as cyclin E, cyclin A, and cyclin B (17). Hypophosphorylated RB binds to and represses E2F by recruiting histone deacetylases (HDAC) and forming a repressor complex at E2F responsive promoters to block transcription of cyclins necessary for cell cycle progression (18,19).

In addition to inhibiting TGF-ß1 signaling, Smad7 may function as a TGF-ß1-independent transcriptional modulator. When Smad7 is complexed to the DNA binding domain of GAL4, there is increased transcription of a minimal retinoid acid receptor-ß2 GAL4-TGTA luciferase construct (20). Furthermore, Smad7 also associates with the transcriptional co-activator p300 (21), the transcriptional co-activator Yes-Associated Protein (YAP65) (22), and the cell cycle arrest and apoptotic associated protein Gadd34 (23). Thus, Smad7 may exert a distinct TGF-ß1-independent function by acting as a co-activator/co-repressor, which is dependent upon the cellular context of which it is expressed.

Human pancreatic cancer cells are generally resistant to the growth inhibitory effects of TGF-ß1. This resistance is the consequence of a number of genetic alterations, including decreased expression of the receptors (TßRI and TßRII) (24), mutations and homozygous deletions of Smad4/DPC4 (25), and overexpression of the TGF-ß1 inhibitors Smad6 and Smad7 (26,27). COLO-357 pancreatic cancer cells engineered to overexpress Smad7 are resistant to TGF-ß1-mediated growth inhibition but exhibit increased PAI-1 expression in response to TGF-ß1 (27) and elevated levels of thioredoxin (28). In order to elucidate the mechanisms whereby TGF-ß1-mediated growth inhibition is abolished by Smad7 in the face of its persistent ability to modulate gene expression, in the present study we examined the effects of Smad7 overexpression on the cell cycle machinery and on the nuclear translocation of Smad2/3. We now report that Smad7 attenuates TGF-ß1 inhibitory actions on cyclin A and cyclin B levels, cdc2 phosphorylation at Thr161, and
RB hypophosphorylation without interfering with TGF-ß1-mediated nuclear translocation of Smad2/3. These findings point to a novel mechanism for the functional inactivation of RB and for attenuating the growth inhibitory actions of TGF-ß1 without blocking Smad2/3 activation or modulation of gene expression by TGF-ß1.

Materials and Methods

Cell Culture - COLO-357 human pancreatic cancer cells (29) were grown in Dulbecco’s Modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin (Irvine Scientific, Irvine, CA) in 5% CO2. Sham and myc epitope tagged Smad7 transfected cells were grown in the presence of 400 µg/ml G418 (Sigma Aldrich, St. Louis, MO). For FACS analysis and western blotting, cells (70% confluent) were incubated in serum free medium (0.1% BSA, 5 µg/ml transferrin, 5 ng/ml sodium selenite and antibiotics) for 24 hours before TGF-ß1 (Genentech, Inc., South San Francisco, CA) addition. COS-7 cells were grown in Dulbecco’s Modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin (Irvine Scientific, Irvine, CA) in 5% CO2. Sham and myc epitope tagged Smad7 transfected cells were grown in the presence of 600 µg/ml G418 (Sigma Aldrich, St. Louis, MO).

Immunoblotting - Cells were washed and lysed in complete lysis buffer (1% NP-40, 10% glycerol 20mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaVO4, and 10 mM NaPO4) in the presence of a protease inhibitor solution consisting of 1mM phenylmethyl sulfonylfluoride (PMSF), 1µg/ml aprotonin, 1µg/ml leupeptin, and 1µg/ml pepstatin (PALP). Lysates were then subjected to SDS-PAGE, transferred to nitrocellulose membranes (25 µg/lane), and incubated with the indicated antibodies. Anti-Cyclin A, B1, and E, phospho-Smad2, and myc tag antibodies were from Upstate Biotechnologies (Waltham MA). Anti-Cyclin D1, D2, and D3 were from Lab Vision-Neomarkers (Fremont, CA). Anti-Smad2/3 and Grb2 were from BD Transduction Laboratories (San Jose, CA). Anti-Smad4, p27, p21, Cdk2, Cdk4, and Erk2 were from Santa Cruz Biotechnologies (Santa Cruz, CA).

CDK2 Kinase Assay - Sham and Smad7 overexpressing cells untreated or treated with TGF-ß1 for 24 hours were lysed in kinase lysis buffer (50 mM Tris-Cl, pH 7.5, 250 mM NaCl, 0.1% NP-40, 5 mM EDTA, 0.1% NaVO4, 10 % glycerol, and PALP protease inhibitors). The lysates were immunoprecipitated with an anti-CDK2 antibody (Santa Cruz Biotechnologies, Santa Cruz, CA) and protein A/G agarose added for 1 hour. The complex was washed extensively with kinase lysis buffer and twice with kinase assay buffer (50mM Tris-Cl, pH 7.5, 10mM MgCl2, 10 mM B-glycerophosphate, 0.1mM NaVO4, 1mM DTT). The reaction mixture (50 ul) contained kinase assay buffer, 1 µg purified RB protein (Santa Cruz Biotechnologies, Santa Cruz, CA), and 10 µCi γ-32P-ATP. The reactions were carried out at 37°C for 30 minutes and stopped by adding SDS-
PAGE loading buffer. The samples were boiled for 5 minutes and resolved on a 10% SDS-PAGE gel. The gel was dried and phosphorylated RB detected by radiography.

**Cell Fractionation** - Cytosolic and nuclear fractions were isolated according to the protocol of Wong et al. (30) with minor modifications. Cells (150 mm culture dishes) were scraped in phosphate buffered saline (PBS) containing 10% glycerol, 1 mM NaVO₃, and a protease inhibitors (PALP), washed twice with PBS containing PALP, and resuspended in hypotonic lysis buffer (20 mM HEPES, pH 7.6, 20% glycerol, 10 mM NaCl, 1.5 mM MgCl, 0.2 mM EDTA, 25 mM NaF, 25 mM β-glycerophosphate, 1mM sodium phosphate, 0.5 mM DTT, 0.1% Triton-X 100, and PALP). Following lysis at 4°C and removal of the supernatants (cytosolic component), the nuclear pellets were washed in hypotonic lysis buffer and incubated for 30 minutes at 4°C in the same buffer containing 500 mM NaCl to extract all nuclear proteins. The nuclear lysates were cleared by centrifugation, and cell fractions were then subjected to western blot analysis.

**Immunocytofluorescence** - Cells were plated onto glass cover slips and allowed to adhere overnight. Cells were serum starved for 24 hours before treatment with 500 pM TGF-β1 for 1 hour. Slides were then fixed for 30 minutes in 4% formaldehyde, washed extensively with PBS, permeabilized in 0.2% Triton-X 100, and blocked for 1 hour in blocking buffer (PBS, 0.1% Triton-X 100, 2% goat serum). Slides were then incubated overnight at 4°C with rabbit anti-Smad2/Smad3 (Zymed, San Francisco, CA) or anti-myc tag (Upstate Biotechnologies, Waltham, MA) antibodies. After washing with PBS, slides were incubated for 1 hour with Cy3 or AlexaFluor secondary antibodies (Molecular Probes, Eugene, Oregon), and the nuclei were counterstained with 1ug/ml Hoechst 3358 dye for 10 minutes at room temperature. Immunocytofluorescence was detected using a Nikon Eclipse T200 microscope and the MetaFluor program.

**Luciferase Assay** - Cells were plated in 12 well plates at a density of 150,000 cells/well and allowed to adhere overnight. Transfections with the plasminogen activator inhibitor (PAI-1) luciferase (Dan Rifkin, New York University), or the pE2F-TA-Luciferase (Clontech, Palo Alto, CA) constructs (1.0 μg), along with the CMV-beta galactosidase plasmid (0.25 μg) were performed at 25°C (30 minutes) using 0.75 μg of Nupherin Neuron (Bio Mol, Plymouth Meeting, PA). The resulting complexes were incubated with Lipofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Cells were then transfected for 12 hours and allowed to recover overnight in complete medium before treatments. Relative luciferase activity was calculated by dividing the luciferase values by the beta galactosidase values.

**FACS Analysis** - Cells were plated in 6 well plates at a density of 100,000 cells/well and allowed to adhere overnight. Cells were serum starved for 24 hours before treatment with 500 pM TGF-β1 for an additional 24 hours. Cells were then trypsinized and fixed in 3.7% formaldehyde, stained with propidium iodide in PBS, and subjected to FACS analysis.

**Growth Assay** - The 3-(4,5-methylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) growth assay (31), which in pancreatic cancer cells correlates well with results obtained by cell counting with a hemocytometer and by monitoring [3H] thymidine incorporation (32,33), was used to monitor cell growth. Cells were plated at a density of 8,000 cells/well, using 96 well microtiter plates (Costar), and allowed to adhere overnight prior to TGF-β1 addition.
for the indicated times. Colorimetric changes were measured using a Microtiter plate reader with a 570 nm filter. The MEK inhibitor U0126, the p38 MAPK inhibitor SB203580, and the PI3K inhibitor LY294002 were purchased from Alexis Pharmaceuticals (San Diego, CA).

EMSA - Cells were scraped in PBS containing protease inhibitors (PALP) and 1 mM NaVO₃ and, pelleted, and resuspended in hypotonic buffer A (10 mM HEPES, pH 7.5, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 0.5 mM DTT, and protease inhibitors) and allowed to swell for 10 min on ice. NP-40 was added to a final concentration of 0.5% and cells were incubated on ice for 10 min before centrifugation for 2 min at 10,000 rpm. The supernatants were removed and nuclei resuspended in high salt buffer C (20 mM HEPES, pH 7.5, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, and PALP) and incubated with gentle shaking for 15 min on ice before centrifugation at 10,000 rpm for 2 min (34). EMSA reactions were performed with 5 μg nuclear extracts and 50,000 cpm an SBE consensus oligonucleotide (Santa Cruz Biotechnology). Binding reactions were carried out at 23°C for 30 min. Samples were analyzed for protein/DNA complex formation on a 5% non-denaturing polyacrylamide, and gels were exposed to Kodak Biomax film.

Smad7 Immunoprecipitation - Smad7 overexpressing COLO-357 cells were washed in PBS and lysed in complete lysis buffer containing 0.5% NP-40 buffer. Cell lysates (0.5 mg) were incubated with 2 μg of an anti-myc tag and the resulting complexes were bound with protein AG-Agarose beads, washed, and subjected to SDS-PAGE and immunoblotting with an anti-TβRII antibody (Santa Cruz Biotechnology, Santa Cruz CA). Bound antibody was visualized using enhanced chemiluminescence from Pierce (Rockford, IL).

RESULTS

COLO-357 cells express wild type Smad4 and are known to be growth inhibited by TGF-β1 (27). In the present study, TGF-β1 inhibited the growth of Sham transfected COLO-357 cells in a dose-dependent manner, maximal inhibition occurring at a concentration of 1.0 nM (Fig. 1A). This inhibition was completely abolished in Smad7 overexpressing cells (Fig. 1A), and occurred in conjunction with an increased percentage of cells in G1 and a decreased percentage of cells in G2 (Fig. 1B).

Previous studies have shown Smad-independent pathways, including the MAPK, p38 MAPK, and PI3K are activated by TGF-β1 in certain cell types (6-8,35). In order to determine whether TGF-β1 activated these alternate pathways in COLO-357 cells, Sham and Smad7 overexpressing cells were incubated in the absence or presence of 500 pM TGF-β1 for up to one hour and blotted with anti-phospho-specific p42/44, p38 MAPK, and ATK2. Phosphorylation of ATK2, a downstream target of PI3K, was used as a marker for PI3K activity (36). In both Sham and Smad7 overexpressing COLO-357 cells, p38 MAPK, MAPK, and AKT2 phosphorylation occurred within 15 minutes of TGF-β1 addition, and then slowly declined after one hour (Fig. 2A), indicating that all three pathways are activated by TGF-β1 in COLO-357 cells, even in the presence of high levels of Smad7. Moreover, the MEK inhibitor U0126, the p38 MAPK inhibitor SB203580, and the PI3K inhibitor LY294002 did not alter TGF-β1-mediated growth inhibition in Sham cells, and did not attenuate the ability of Smad7 to prevent TGF-β1 mediated growth inhibition (Fig. 2B). Thus, these pathways do not contribute to the growth suppressive ability of TGF-β1.
in COLO-357 cells and do not modulate the actions of Smad7 on cell growth.

TGF-β1 acts in late G1 phase and prevents further progression to the G1/S phase transition through inhibiting the expression of S/G2 cyclins (10). Therefore the levels of cyclin A, cyclin B, and the D-type cyclins in response to TGF-β1 were examined next. In sham cells, TGF-β1 (500 nM) markedly decreased cyclin A and B levels, and slightly decreased cyclin D3 levels, whereas in Smad7 overexpressing cells TGF-β1 did not alter the levels of these cyclins (Fig. 2A). In both cell types, TGF-β1 increased cyclin D1 expression, as previously reported (37), without altering cyclin D2, cyclin E, Cdk2, or Cdk4 levels (Fig. 3A). Thus, in the presence of high levels of Smad7, TGF-β1 is incapable of suppressing cyclin A and B levels.

TGF-β1 induces cell cycle arrest through the cooperative actions of the cell cycle inhibitors p27 and p21 (15,16), therefore we next sought to determine the effects of Smad7 on p21 and p27 protein levels. In the absence of TGF-β1, p21 protein levels were decreased in Smad7 overexpressing cells by comparison with Sham, whereas p27 levels were similar in the two groups of cells (Fig. 3B). By contrast, TGF-β1 (500 pM) increased p21 levels in both Sham and Smad7 overexpressing cells, and increased p27 levels in Sham, but not in Smad7 overexpressing cells. Thus, Smad7 does not block TGF-β1-mediated growth inhibition in COLO-357 cells by altering p21 levels, but may act, in part, by preventing the up-regulation of p27. Although TGF-β1 can induce the ubiquitination and degradation of Smad7 in certain cells (38), monitoring the expression of myc-tagged Smad7 by western blotting revealed that Smad7 expression persisted in transfected cells for at least 24 hours following TGF-β1 addition (Fig. 3D).

Thus, TGF-β1 does not induce Smad7 degradation in COLO-357 cells.

The activity of CDK2 is necessary for progression through the G1 phase of the cell cycle. In both Sham and Smad7 overexpressing cells the protein levels of cyclin E and CDK2 remained elevated in the presence of TGF-β1. However, the levels of the CDK2 inhibitor p27 were increased by TGF-β1 in the Sham cells, suggesting that in the presence of high levels of cyclin E and CDK2, the kinase activity of CDK2 is decreased due to association with p27. Therefore the activity of CDK2 was analyzed next in an in-vitro kinase assay. CDK2 activity decreased in Sham cells treated with TGF-β1 for 24 hours but remained elevated in the Smad7 overexpressing cells (Fig. 3C). Thus, the elevated level of p27 in the Sham cells was sufficient to inhibit CDK2 activity in the presence of cyclin E.

The activity of cdc2 is crucial for the entry of cells into mitosis, and phosphorylation of cdc2 on Thr\textsuperscript{161} is required for this activation in order for cdc2 to associate with cyclins A and B (39). The phosphorylation of cdc2 was analyzed next using an antibody that recognizes cdc2 phosphorylated on Thr\textsuperscript{161}. After 24 hours, 500 pM TGF-β1 caused a decrease in the levels of phosphorylated cdc2 in Sham but not in Smad7 overexpressing cells (Fig. 3C). Thus, Smad7 prevents the TGF-β1 mediated decrease in cdc2 phosphorylation on Thr\textsuperscript{161}, thereby impeding its inactivation and possibly its dissociation from cyclin A and cyclin B.

RB is phosphorylated in G1 by the Cdk4/cyclin-D and Cdk2/cyclin-E complexes (11,40). Hyper-phosphorylation of RB abolishes its inhibitory activity, thereby releasing the transcription factor E2F and allowing for induction of S and G2 phase genes such as cyclin A and cyclin B.
Because cdc2 inactivation was attenuated in Smad7 overexpressing cells and cyclin A and cyclin B levels remained elevated, the phosphorylation state of RB was analyzed next using anti-RB antibodies recognizing either Ser\textsuperscript{795} or Ser\textsuperscript{807/811}. After 24 hours, 500 pM TGF-β\textsubscript{1} caused a marked decrease in the levels of phosphorylated RB in Sham cells. By contrast, phosphorylated RB levels remained elevated in the Smad7 overexpressing cells (Fig. 3C). RB protein levels remained constant over the 24 time hour period in both cell types, indicating that the increased phosphorylation observed in the Smad7 overexpressing cells was not due to increased RB protein levels in these cells.

E2F-1 transcriptional activity is primarily repressed by binding to hypophosphorylated RB and RB (41). Since RB remained highly phosphorylated in the smad7 overexpressing cells in the presence of TGF-β\textsubscript{1}, the effects of Smad7 on E2F activity were examined next. As determined in an E2F-Luciferase reporter assay, TGF-β\textsubscript{1} markedly decreased E2F activity in Sham cells, but not in Smad7 overexpressing cells (Fig. 4). Thus, the ability of Smad7 to interfere with TGF-β\textsubscript{1}-mediated RB hypophosphorylation is functionally important, inasmuch as it prevents the inhibition of E2F-1 transcriptional activity.

Immunofluorescent studies in the absence of TGF-β\textsubscript{1} revealed that Smad2/3 exhibited nuclear and cytoplasmic staining in Sham and Smad7 overexpressing cells. Following TGF-β\textsubscript{1} addition, there was a marked translocation of cytoplasmic Smad2/3 into the nucleus, irrespective of the Smad7 levels (Fig. 5A). To confirm that the myc-tagged Smad7 expression construct was fully functional, the same experiments were performed in COS-7 cells, in which Smad7 is known to block Smad2/3 translocation (5). In contrast to the findings with COLO-357 cells, Smad2/3 was predominantly cytoplasmic in both sham transfected and Smad7 overexpressing COS-7 cells, and TGF-β\textsubscript{1} induced the nuclear translocation of Smad2/3 in the sham-transfected but not in the Smad7 overexpressing COS-7 cells (Fig. 5B). Inasmuch as Smad7 protein levels were similar in transfected COS-7 and COLO-357 clones (data not shown), these observations indicate that the actions of Smad7 with respect to the nuclear translocation of receptor activated Smads is cell type specific. In support of this conclusion, TGF-β\textsubscript{1} increased the activity of a PAI-1-luciferase construct to the same extent in Sham and Smad7 overexpressing cells (data not shown).

It is generally accepted that Smad7 binds TβRI in response to TGF-β\textsubscript{1} treatment thereby blocking the phosphorylation and nuclear translocation of Smad2 and Smad3, and suppressing TGF-β\textsubscript{1}-mediated growth inhibition (5). Therefore, we next sought to determine whether Smad7 overexpression altered TGF-β\textsubscript{1} actions on Smad2 phosphorylation. Surprisingly, TGF-β\textsubscript{1} increased Smad2 phosphorylation to the same extent in Sham and Smad7 overexpressing cells (Fig. 6A), the phosphorylation of Smad2 remaining elevated for up to 12 hours in both groups of cells (Fig. 6B). Further, Western blotting of nuclear and cytoplasmic fractions revealed that a large portion of Smad2/3 was already present in the nucleus in the absence of exogenously added TGF-β\textsubscript{1}, and that the cytoplasmic portion of Smad2/3 translocated to the nucleus upon TGF-β\textsubscript{1} addition, irrespective of the levels of Smad7 (Fig. 6C). Smad4 was also predominantly localized to the nucleus in the absence of exogenously added TGF-β\textsubscript{1}. Moreover, the basal distribution of all three Smad proteins was not altered by TGF-β neutralizing antibodies (data not shown).
In most cell types Smad7 associates with TβRI, thereby inhibiting Smad2/3 phosphorylation (5). Inasmuch as Smad phosphorylation was not blocked in Smad7 overexpressing COLO-357 cells, the ability of Smad7 to associate with TβRI was analyzed next. Cells were treated with TGFβ for 1 to 12 hours and co-immunoprecipitation of TβRI with Smad7 was examined by western blotting. Association of Smad7 with TβRI could not be detected at any time point (Fig. 7A). By contrast, under similar incubation conditions, Smad7 was found to associate with TβRI. These observations suggest that the inability of Smad7 to block the phosphorylation and activation of the Smads is due to the failure of Smad7 to bind TβRI in COLO-357 cells. Alternatively, TGFβ may act in COLO-357 cells through a pathway that is independent of TβRI. To exclude this possibility, the effects of SB-431542, a highly specific TβRI kinase inhibitor, were examined next. There was a dose-dependent inhibition of TGF-β1-mediated phosphorylation of Smad2 in both Sham and Smad7 overexpressing clones, maximal inhibition occurring at 0.5 μM SB-431542. Therefore, activation of the Smad pathway by TGF-β1 is dependent upon TβRI kinase activity in COLO-357 cells, irrespective of the absence or presence of Smad7.

Smad6, an inhibitor of BMP signaling, binds the BMPRII receptor to inhibit the phosphorylation of Smad1/5. In addition, Smad6 can bind Smad4 and inhibit Smad1/5/4 complex formation and DNA binding. Since Smad7 failed to block the phosphorylation and nuclear translocation of Smad2/3, it was possible that Smad7 may function at a nuclear level to inhibit the DNA binding of the Smad complex in COLO-357 cells. Therefore, we next examined DNA binding of the Smads in Sham and Smad7 overexpressing cells. Smad7 did not prevent the DNA binding of the Smad2/3/4 complex in COLO-357 cells, as evidenced by EMSA using an SBE probe (Fig. 8). Taken together, these results confirm that the mechanism of abrogation by Smad7 of TGF-β1 mediated growth inhibition in COLO-357 is Smad2/3/4 independent.

DISCUSSION

Smad7 was first identified in human umbilical vein endothelial cells (HUVEC) as a gene that was upregulated in response to shear stress (42). Further experiments revealed that Smad7 is an inhibitor of TGF-β1 signaling in bovine aortic endothelial cells where in response to TGF-β1 treatment Smad7 bound directly to the type I TGF-β receptor (TβRI) and prevented the association, phosphorylation, and activation of Smad2 (5). Smad7 also blocks TGF-β1 mediated growth inhibition in a variety of cell lines such as Mv1-Lu, HepG2 liver cells, keratinocytes, mammary epithelial cells, and COLO-357 pancreatic cancer cells (27,43-46), and TGF-β1 up-regulates Smad7 expression in Mv1Lu cells and other cell types (47). This up-regulation has been suggested to constitute a negative feedback loop that may act to dampen TβRI-dependent signaling.

RB was originally identified as a gene that was either mutated or deleted in retinoblastoma (48), and was subsequently shown to be frequently lost or mutated in osteosarcomas and small cell lung cancers, and to a lesser extent in other human cancers (49). In addition, functional inactivation of RB by DNA tumor viruses has been documented in many certain malignancies. For example, human papilloma virus type E7 binds to RB, which has been implicated in cervical cancer, and suppresses its function (50). Aberrant phosphorylation of RB can also lead to its functional inactivation. This may occur as a
consequence of a number of alterations, such as cyclin D1 overexpression, or mutation/silencing of the p16 gene, all of which can lead to excessive activation of cdk4/6 and loss of RB-mediated growth suppression (51).

In the present study, we determined that high levels of Smad7 interfered with the ability of TGF-β1 to maintain RB in a hypophosphorylated state. Three distinct lines of evidence suggest that the hyperphosphorylating effect of Smad7 on RB was not due to a non-specific effect on the cell cycle. First, Smad7 did not alter TGF-β-mediated up-regulation of p21, indicating that it did not interfere with this Cdk inhibitor. Similarly, increased p21 induction in response to TGF-β1 is not blocked by Smad7 expression in hepatic stellate cells, but is blocked in Mv1Lu cells, keratinocytes, and in HepG2 cells, (52,53), pointing to cell type dependent differential regulation of p21 induction by Smad7. Second, Smad7 attenuated TGF-β-mediated inhibition of cdc2 phosphorylation on Thr161. Inasmuch as cyclin B and cdc2 interact to promote the G2/M transition during the cell cycle, this observation implies that high levels of Smad7 can interfere with the ability of TGF-β1 to suppress cell cycle progression through the G2/M phase of the cell cycle, as evidenced by our cell sorting experiments (FACS analysis). TGF-β1 inhibits cdc2 phosphorylation at Thr161 by inhibiting CAK activity (54). It is possible therefore that Smad7 may interfere with CAK inhibition. In support of this hypothesis, the inhibitory effect of TGF-β1 on CDK2 activation, which is also CAK dependent (54), was completely blocked by Smad7 overexpression. Third, Smad7 did not alter TGF-β-mediated up-regulation of cyclin D1, but interfered with TGF-β1-mediated attenuation of cyclin A and B levels. This up-regulatory effect by TGF-β-1 on cyclin D1 has been previously reported to occur in COLO-357 cells (37), as well as in cells of mesenchymal origin such as 3T3-F442A and skeletal myoblasts (55,56). By contrast, our findings with respect to cyclin A, cyclin B, and cdc2 phosphorylation imply a novel function for Smad7 in cell cycle regulation.

RB hyperphosphorylation is a multi-step process during which mitogenic signaling activated by many tyrosine kinase receptors lead to the up-regulation of cyclinD-CDK4/6 activity and initial phosphorylation of RB, followed by up-regulation of cyclin E-CDK2 activity and the additional phosphorylation of RB, and subsequent release of E2F. In Sham cells, TGF-β1 maintained RB in a hypophosphorylated state and upregulated p27 levels while suppressing cyclin A and B levels and inhibiting CDK2 and Cdc2. By contrast, in Smad7 overexpressing cells, TGF-β1 mediated up-regulation p27 and down regulation of cyclins A and B were blocked. Moreover, the high levels of Smad7 interfered with TGF-β1 mediated inhibition of CDK2 and cdc2, as well as with TGF-β1-mediated inhibition of E2F activity. Together these observations suggest that impedance of RB dephosphorylation may represent a novel mechanism for E2F de-repression.

High levels of Smad7 did not interfere with TGF-β1-mediated nuclear translocation of Smad2/3, as determined by cell fractionation and immunofluorescent localization studies. Smad7 also failed to associate with TβRI and did not interfere with Smad2 phosphorylation in COLO-357 cells. Nonetheless, the actions of TGFβ1 on Smad2 phosphorylation were completely blocked by SB-431542, a specific inhibitor of the kinase activity of TβRI. By contrast, TGF-β-mediated growth inhibition was not altered by inhibition of MEK, p38 MAPK, or PI3K. Together, these observations point to a novel paradigm whereby Smad7 does
not directly interact with TβRI and does not interfere with Smad2/3 activation, but acts, instead on TGF-β1 mediated hypophosphorylation of RB by blocking TGF-β1-mediated growth inhibition. This mechanism explains why Smad7 does not attenuate TGF-β1-mediated induction of PAI-1 (27), cyclin D1 (37), p21 (57), or thioredoxin (28). In theory, Smad7 may modulate gene expression through several different mechanisms. First, by maintaining RB in a hyperphosphorylated state, Smad7 may allow for the release of E2F from RB-dependent inhibition, thereby indirectly promoting gene transcription. Second, the Smad7 MH2 domain has the potential to act as a transcriptional activator, albeit less efficiently than the MH2 domains of Smad4 and Smad1 (58) and it is possible, therefore, that high levels of Smad7 may directly modulate gene transcription. Together these observations explain the divergence between the suppressive actions of Smad7 on TGF-β1-mediated inhibition of cell proliferation and its permissive effects with respect to TGF-β1-mediated modulation of gene expression.

Several lines of evidence suggest that Smad7 actions are cell type specific. First, Smad7 is induced by TGF-β in many cell types and then associates with TβRI, thereby preventing Smad2/3 activation (4). Moreover, Smad7 is expressed at high levels in fibroblasts from patients with scleroderma (59), and there is an increased association of Smad7 with TβRI in these fibroblasts (59). By contrast, in the present study, we determined that Smad7 does not associate with TβRI in COLO-357 cells. Second, in endothelial cells, Smad6 up-regulates PAI-1, whereas Smad7 blocks this effect (60), an action opposite to the effects observed in COLO-357 cells. Third, in prostate cancer cells, Smad7 up-regulates Bim and is required for methoxyestradiol-induced apoptosis (61). By contrast, Smad7 induces resistance to apoptosis in COLO-357 cells, and this effect is due to activation of NFκB (28). These divergent findings in different cell types serve to underscore the fact that Smad7 is more than a TGF-β-activated negative regulator of TβRI.

RB mutations are rare in pancreatic ductal adenocarcinoma (PDAC) (62,63). By contrast, p16 mutations (90%) and/or p16 silencing (remaining 10%) occur in virtually all PDAC samples studied to date (64,65). Furthermore, many of these cancers overexpress mitogenic growth factors and their cognate high affinity tyrosine kinase receptors, overexpress cyclin D1, and harbor mutated K-ras (90% of PDACs), thereby leading to enhanced mitogenic signaling (66). TGF-β1-mediated growth inhibition is abrogated in these cells as a result of the presence of Smad4 mutations, decreased expression of TβRI, and increased expression of Smad7 and Smad6. The present findings document a novel mechanism whereby increased levels of Smad7 functionally inactivate RB and de-repress E2F, thereby interfering with the ability of TGF-β1 to inhibit cell cycle progression while allowing for TGF-β1 to induce the nuclear translocation of Smad2/3 and to modulate gene expression (Fig. 9). Smad7 also interferes with TGF-β1-mediated upregulation of p27, thereby preventing CDK2 inactivation (Fig. 9). In view of the importance of RB inactivation in cancer progression, our findings suggest that Smad7-induced RB dysfunction may provide a distinct growth and survival advantage to pancreatic cancer cells, and may therefore represent an important therapeutic target in PDAC.
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**FIGURE LEGENDS**

**Fig. 1.** Smad7 Abrogates TGF-β1 Mediated Growth Inhibition. *A*, Sham and Smad7 overexpressing cells were plated at a density of 8,000 cells/well in 96 well microtiter plates, and allowed to adhere overnight. Cells were then incubated for 72 hours in the absence or presence of the indicated concentrations of TGF-β1, and MTT assays were performed as described in the Methods section. *B*, Sham and Smad7 overexpressing cells were serum starved for 24 hours and then treated with 500 pM TGF-β1 for 24 hours, trypsinized and stained with propidium iodide and subjected to FACS analysis.

**Fig. 2.** TGF-β1 Activates MAPK, p38 MAPK and AKT2 Pathways. *A*, Sham and Smad7 overexpressing cells were incubated in the absence of presence of 500 nM TGF-β1 for up to one hour. Protein lysates were subjected to western blot analysis using anti-phospho-specific p42/44 (MAPK-P), p38 MAPK, and AKT2. The membranes were then stripped and probed for ERK2 to confirm for equal loading. *B*, Sham and Smad7 overexpressing cells were plated at a density of 8,000 cells/well in 96 well microtiter plates and allowed to adhere overnight. Cells were incubated in the absence (open bars) or presence of 10 μM U0126 (closed bars), SB203580 (hatched bars), or LY294002 (stippled bars) for one hour and then incubated for 72 hours in the
absence or presence of 500 pM TGF-β1. MTT assays were performed as described in the
Methods section.

**Fig. 3.** Effects of Smad7 on Cell Cycle Regulating Proteins. Sham and Smad7 overexpressing
cells were serum starved for 24 hours and then treated with 500 pM TGF-β1 for 24 hours. *A*,
Protein lysates were subjected to western blot analysis using antibodies specific for cyclin A,
cyclin B, cyclins D1-D3, cyclin E, and CDK2/4. *B*, Membranes were immunoblotted with
antibodies specific for the CDK inhibitors p21 and p27. *C*, The levels of CDK2 activity, cdc2,
and RB phosphorylation were also analyzed by an in-vitro kinase assay, an antibody that
recognizes cdc2 phosphorylated on Thr\(^{161}\), and antibodies that recognize Ser\(^{795}\) and Ser\(^{807/811}\) of
hyper-phosphorylated RB. Membranes were then stripped and re-probed for total cdc2 and RB
protein. *D*, Membranes were immunoblotted with an anti-myc tag antibody to monitor Smad7-
myc tagged protein levels. All membranes were stripped and probed for ERK2 to assess protein
loading.

**Fig. 4.** Effects of Smad7 on E2F Activity. *A*, Sham and Smad7 cells were seeded in 12 well
plates and transiently transfected with an E2F-TALuc construct. Cells were serum starved for 12
hours and then incubated for 24 hours in the absence (open bars) or presence (closed bars) of 500
pM TGF-β1. Relative E2F-Luciferase activity was calculated by dividing the luciferase values
by the beta galactosidase values. *p<0.034 when treated with TGF-β1 compared to values
observed in untreated Sham.

**Fig. 5.** Effects of Smad7 on Smad2/3 Translocation in COLO-357 and COS-7 Cells. *A*, Sham
and Smad7 overexpressing COLO-357 cells were plated onto glass coverslips and allowed to
adhere overnight. After a 24 hour incubation in serum free media, the cells were treated with
500 pM TGF-β1 for 1 hour. The cells were fixed in 4% formaldehyde and stained for Smad2/3
with an anti-Smad2/3 antibody. The nuclei were counterstained with Hoechst 3358 to visualize
nuclei. *B*, Sham and Smad7 overexpressing COS-7 cells were plated onto glass coverslips and
allowed to adhere overnight, serum starved and then treated with 500 pM TGF-β1 for 1 hour.
The cells were fixed in 4% formaldehyde and stained for Smad2/3 with an anti-Smad2/3
antibody. Hoechst 3358 was used to visualize nuclei.

**Fig. 6.** Smad7 Does Not Block Smad2 Phosphorylation or Smad Nuclear Translocation. *A*,
Sham and Smad7 overexpressing cells were serum starved overnight prior to the addition of 500
pM TGF-β1 for 30 minutes. Protein extracts were subjected to western blot analysis with a
phospho-specific anti-Smad2 antibody. *B*, The levels of phospho-Smad2 were assessed after 2,
6, and 12 hours following TGF-β1 addition. The membranes were re-probed for ERK2 to assess
protein loading. The nuclear translocation of Smad 2/3, Smad4, and Smad7 were assessed after
treatment with 500 pM TGF-β1 for one hour. *C*, Nuclear and cytosolic fractions were subjected
to western blot analysis for Smad2/3, Smad4, and Smad7 cellular localization. The membranes
were then stripped and probed for cyclin A (predominantly nuclear) and Grb2 (predominantly
cytoplasmic) to confirm fractionation procedure.

**Fig. 7.** TβRI Kinase Activity is Required for Smad2 Phosphorylation. *A*, Absence of Receptor
Binding. Smad7 overexpressing cells were serum starved overnight and incubated for one to 12
hours with 500 pM TGF-β1. Cell lysates (1.0 mg) were incubated with 2 μg of an anti-myc tag
antibody and the resulting complexes were bound with protein AG-Agarose beads, washed, and
subjected to SDS-PAGE and immunoblotting with an anti-TβRI antibody to identify co-
immunoprecipitated Smad7. *B*, Sham and Smad7 overexpressing cells were serum starved
overnight and incubated for one hour with the indicated concentrations of SB-431542 prior to the
addition of 500 pM TGF-β1 for 30 minutes. Protein extracts were subjected to western blot
analysis with a phospho-specific anti-Smad2 antibody. The membranes were re-probed for ERK2 to assess protein loading.

**Fig. 8.** Smad7 Does Not Inhibit Smad2/3/4 DNA Binding. Sham and Smad7 cells were serum starved for 24 hours and treated with TGF-β1 for 1 hour. Nuclear extracts (5 μg) were incubated with 50,000 cpms of a ³²P labeled SBE oligonucleotide. Binding reactions were carried out at 23°C for 30 minutes. Samples were analyzed for protein/DNA complex formation on a 5% non-denaturing polyacrylamide. The gel was dried and exposed to Kodak Biomax film.

**Fig. 9.** Model Depicting Smad7 Actions. Mitogenic signaling and stimulation of CDK4/6 leads directly to RB hyperphosphorylation. Mitogenic signaling also up-regulates Smad7, and high levels of Smad7 also contribute to increased RB inactivation by hyperphosphorylation. Activation of growth inhibitory pathways by factors such as TGF-β1, leads to an increase in p21 and p27, a decrease in S/G2 phase cyclins, and hypophosphorylation of RB. However, in the presence of high levels of Smad7, p27 is not upregulated and the increase in p21 is not sufficient to inhibit cell cycle progression. Thus, high levels of Smad7 contribute to suppression of TGF-β-mediated growth inhibition and to E2F de-repression.
Figure 1

A

![Graph showing cell growth (%) of control over TGF-β1 concentrations.]

Cell Growth (% of Control)

- S7M1
- S7M2
- Sham

72 Hours TGF-β1

0 pM 100 pM 500 pM 1 nM

B

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B

Cell Growth (% of Control)

- - + + - - + + - - + +
Sham Smad7
Figure 4
Figure 6

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Figure 7

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Smad2-P

ERK2
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SBE

NS

Probe
Figure 9
SMAD7 abrogates TGF-β1 mediated growth inhibition in colo-357 cells through functional inactivation of the retinoblastoma protein
Nichole Boyer Arnold and Murray Korc

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