Suppression of Tumorigenesis and Induction of p15\textsuperscript{ink4b} by Smad4/DPC4 in Human Pancreatic Cancer Cells


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ABSTRACT

Purpose: The tumor suppressor gene Smad4/DPC4, a key transcription factor in transforming growth factor β (TGF-β) signaling cascades, is inactivated in 50% of pancreatic adenocarcinomas. We seek to determine the role of Smad4/DPC4 in the suppression of tumor cell growth and in the regulation of TGF-β-mediated expression of cell-cycle regulatory genes p15\textsuperscript{ink4b} and p21\textsuperscript{waf1}.

Experimental Design: Smad4/DPC4 is overexpressed by adenoviral infection in CFPac-1 pancreatic cancer cells, in which Smad4/DPC4 is homozgyously deleted, and in Capan-1 pancreatic cancer cells, in which Smad4/DPC4 is not expressed. Expression of the TGF-β downstream target gene p21\textsuperscript{waf1}, regulation of the p15\textsuperscript{ink4b} promoter, anchorage-independent growth, and tumorigenesis were examined.

Results: We demonstrate that expression of Smad4/DPC4 in Capan-1 cells reduced anchorage-independent growth by more than 50%, and inhibited xenograft tumor growth. However, overexpression of Smad4/DPC4 did not inhibit CFPac-1 cell growth. Interestingly, Smad4/DPC4 induced expression of p15\textsuperscript{ink4b}, p21\textsuperscript{waf1}, and TGF-β-responsive reporter gene in Capan-1 but not in CFPac-1 cells. Furthermore, we found a previously unidentified Smad4 binding element (SBE) located in the region between −356 and −329 bp of the p15\textsuperscript{ink4b} promoter. The p15\textsuperscript{ink4b} promoter reporter gene assays revealed that Smad4-dependent transcriptional activation is mediated by this SBE, which indicates that p15\textsuperscript{ink4b} is one of the downstream target genes regulated by Smad/DPC4.

Conclusion: These results explain the role of Smad4/DPC4 in TGF-β-mediated inhibition of cell proliferation in vitro and in vivo. Moreover, these results suggest that Smad4/DPC4-mediated tumor suppression and induction of TGF-β-regulated cell-cycle-inhibitory genes may depend on additional factors that are absent in CFPac-1 cells.

INTRODUCTION

Pancreatic adenocarcinoma is the fifth leading cause of cancer death in adults in the United States (1). The epidemiology of pancreatic cancer provides few clues as to its etiology and pathogenesis, and there are no methods for its early detection. Only about 20% of patients with pancreatic cancer have resectable tumors. Because chemotherapy and irradiation are largely ineffective (1, 2), the chance of cure is small, and therapeutic options in patients with advanced disease are few. Thus, only 1–3% of all patients diagnosed with pancreatic cancer can expect to survive 5 years; clearly, new treatments based on a better understanding of the biology of this cancer must be established.

Deletions and point mutations of the tumor suppressor gene Smad4/DPC4 have been identified in about 50% of pancreatic adenocarcinomas but only in about 10% or less of other cancers, which suggests that Smad4/DPC4 may have a specific role in pancreatic tumorigenesis (3–5). A functional role for Smad4/DPC4 protein was first suggested by its peptide sequence (6), which is similar to those of the Drosophila Mad homologues, Drosophila melanogaster Mad protein and the Caenorhabditis elegans Mad homologues sma-2, sma-3, and sma-4, which regulate cell differentiation (7). Several Smad proteins are phosphorylated by the cognate type I serine/threonine kinase receptor in response to ligand binding. Once phosphorylated, Smad proteins translocate to the nucleus in which they function as transactivators to regulate expression of downstream target genes (8–11). The Drosophila Mad protein binds to a specific DNA sequence (12), whereas other Smad proteins form complexes with DNA-binding partners, such as FAST1, to function as transcriptional regulators (13, 14). Furthermore, both human Smad3 and Smad4/DPC4 proteins recognize SBE(3), an 8-bp palindromic sequence (5′-GTCTA-3′) of the SBE, Smad-binding element; TGF-β, transforming growth factor β; pfu, plaque-forming unit(s); CMV, cytomegalovirus; β-gal, β-galactosidase; MOI, multiplicity/multiplicities of infection; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FBS, fetal bovine serum.

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GAC-3’), and act as sequence-specific transcriptional activators (15).

The current model for Smad function suggests that Smad4/DPC4 is the key transactivator that regulates TGF-β-inducible gene expression. Signal-dependent phosphorylation and nuclear translocation of Smad proteins are the key regulatory steps in TGF-β signaling (16, 17), TGF-β was first identified as one of the factors promoting the transformed phenotype in fibroblasts (18); however, it was quickly discovered to have antiproliferative activity in most of the mammalian epithelial cell lines tested (19). TGF-β may regulate the growth of cultured epithelial cells in part by up-regulating cell-cycle inhibitors such as p15ink4b and p21 waf1, which induce G1 cell-cycle arrest (20, 21). Many cancer cells have been shown to lose responsiveness to TGF-β-induced growth inhibition (7, 22), which suggests that a defect in the TGF-β signaling cascades may eliminate a critical negative control for cell proliferation.

We previously demonstrated that deletion or mutational inactivation of the Smad4/DPC4 gene correlates with a loss of responsiveness to TGF-β-induced growth inhibition and TGF-β-inducible p21 waf1 expression and that Smad4/DPC4 can reestablish TGF-β-inducible reporter gene activity in a Smad4/DPC4-null human pancreatic adenocarcinoma cell line (23). Zhou et al. (24) have shown that Smad4/DPC4 is required for the induction of TGF-β- and activin-responsive reporter gene activity in a Smad4-null colorectal cancer cell line generated by a gene-targeting technique for somatic cells. Our recent work has demonstrated that the presence of Smad4/DPC4 is necessary for the growth-inhibitory effects of TGF-β signaling and p21 waf1 induction in human carcinoma cells in vitro (25). Experiments using chromosome transfer have provided evidence for tumor suppression by Smad4/DPC4 (26). Deletion of either Smad4/DPC4 and APC genes or deletion of Smad4/DPC4 alone in mice resulted in intestinal tumorigenesis (27–29). However, it remained unclear whether expression of Smad4/DPC4 inhibits or decreases tumorigenicity in Smad4/DPC4-null pancreatic cancer cells in vivo. To test the hypothesis that overexpression of Smad4/DPC4 can inhibit tumorigenic growth of human pancreatic adenocarcinoma cells and that Smad4/DPC4-mediated growth inhibition and tumor suppression depend on the induction of Smad4/DPC4 downstream target genes such as TGF-β-regulated cell-cycle inhibitory genes, we sought to determine the in vitro and in vivo growth-inhibitory effects of Smad4/DPC4 overexpression in Smad4/DPC4-null pancreatic cancer cells.

MATERIALS AND METHODS

Cells Lines and Cell Culture. CF Pac-1, Panc-1, and Capan-1 human pancreatic cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 µg/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml ampicillin (Life Technologies, Inc., Grand Island, NY). All of the cell lines were incubated at 37°C (5% CO2).

Recombinant Adenovirus. We constructed a replication-defective adenovirus expressing a 1.6-kb Smad4/DPC4 cDNA (adenovirus Ad5/Smad4/DPC4). This adenovirus was prepared as previously described and purified by two rounds of cesium chloride ultracentrifugation (25, 30). The purified virus was dialyzed twice against buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM MgCl2, and 10% glycerol for 6 h at 4°C, divided into aliquots, and stored at −80°C until used. Viral titer was determined by UV-spectrophotometric analysis (viral particles/ml) and by plaque assay (pfu/ml). Final viral concentrations for in vitro and in vivo infections were made by diluting the stock virus with PBS. Ad5/Smad4/DPC4 was shown to express 1.6-kb Smad4/DPC4 mRNA and Smad4/DPC4 protein after transduction of both normal cells and tumor cells.

Analysis of Gene Transduction and Expression. Twenty-four h after infection with the Ad5/CMV/β-gal virus, cells were washed with PBS at 40°C, fixed with ice-cold 1.25% gluteraldehyde, and stained with X-gal (5-bromo-4-chloro-3-indolyl-b-galactoside; Life Technologies, Inc.) as described previously (25). Transduction efficiencies were defined by the percentage of cells that stained positive (blue) for β-gal activity in a cell count of 1000 cells/well (in triplicate). After infection of each cell line with Ad5/Smad4/DPC4 at increasing MOI (0, 50, 100, and 250 pfu/ml), Smad4/DPC4 gene expression was determined by Northern blot analysis.

Northern Blot Analysis. To determine the presence or absence of Smad4/DPC4 expression in the three cell lines, RNA was isolated as described by Chomczynski and Sacchi (31), and Northern blot analysis was performed as described previously (25). Briefly, 10 µg of total RNA was subjected to electrophoresis through a 1% agarose gel containing formaldehyde, transferred to a Magna Charge nylon transfer membrane (Micron Separations Inc. Westboro, MA), UV cross-linked, and hybridized with a 32P-labeled 2.8-kb Smad4/DPC4 or GAPDH probe prepared by random primer labeling method. All of the blots were exposed to Kodak Biomax MR film (Kodak) at −80°C, and bands were quantified with a Phosphor Imager (Molecular Dynamics, Sunnyvale, CA) or with a Bios personal densitometer (Molecular Dynamics).

Western Blot Analysis. Cells were lysed at 4°C in a lysis buffer containing 150 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, MO), and 25 mM Tris (pH 7.5). The lysates were cleared by centrifugation and boiled for 5 min at 100°C in Laemmli’s SDS-PAGE sample buffer containing 100 mM DTT. Proteins were resolved at 100 V on 10% polyacrylamide gels and transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA), UV cross-linked, and hybridized with a 32P-labeled double-stranded SBE oligonucleotides from Santa Cruz Biotechnology Inc., Santa Cruz, CA) or with a β-actin monoclonal antibody to (Sigma Chemical Co.). An enhanced chemiluminescence kit (Amersham, Arlington Heights, IL) was used for detection.

Nuclear Extract Preparation and Electrophoretic Mobility Shift Assay. Panc-1 Capan-1, and CF Pac-1 cells were treated with the buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM MgCl2, and 10% glycerol (i.e., mock-infected) or were infected with Ad5/Smad4/DPC4 for 48 h. Nuclear extracts were prepared according to the method of Liu et al. (10). DNA binding assays for Smad4 proteins were performed with 10 µg of nuclear extract, as described by Zawel et al. (15). Ten ng of 32P-labeled double-stranded SBE oligonucleotides from the p21 waf1 gene promoter region (5’-AGACAGACAAT-GTCTAGTCTATTGAAATGCCTGA-3’) was used as a...
probe. Competition assays were performed with a 50-fold excess of unlabeled wild-type or mutant (5’-AGACAGACAAT-GTTTATCTATTTGAAATGCGCTGA-3’) SBE oligonucleotides. The reactions were analyzed on 4% polyacrylamide gels containing 0.5 × Tris-borate-EDTA buffer.

In Vitro Cell Growth-inhibition Assay. The standard cell proliferation assay was performed as described previously (23–25). Briefly, the cell growth inhibition mediated by Ad5/Smad4/DPC4 was determined with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Pancreatic cancer cells were seeded into 96-well plates (1 × 10⁴ cells/well) in 100 μl of medium and incubated for 24 h at 37°C in 5% CO₂. The next day, the medium was aspirated from the wells, and the cells were either mock-infected (i.e., media control) or infected with Ad5/luciferase (control virus) or Ad5/Smad4/DPC4 in a 50-μl volume at a MOI of 100 pfu/ml. After 2 h, 150 μl of medium were added to each well. Forty μl of 2 mg/ml thiazolyl blue (Sigma Chemical Co.) were added to each well at completion of the treatment, and plates were incubated at 37°C for 4 h. Wells were then aspirated, and 100 μl of DMSO (Fisher Scientific Co., Pittsburgh, PA) were added to each well. Plates were read at 570 nm in a spectrophotometer (Emax Precision Microplate Reader; Molecular Devices Corp., Sunnyvale, CA). P was calculated using repeated measure ANOVA.

Anchorage-independent Growth Assay. Each of the cell lines was mock-infected or infected with Ad5/luciferase or Ad5/Smad4/DPC4 at a MOI of 100 pfu/ml (25). Aliquots of 5 × 10⁵ cells were mixed with Sea Plaque agarose (Hoefer Scientific Instruments, San Francisco, CA) and DMEM containing 10% FBS and were overlaid on a 0.8% agarose layer containing DMEM and 10% FBS in a 35-mm plate. The plates were incubated for 2–3 weeks, and colonies (>50 μm) were counted with a colony counter. All of the assays were performed in triplicate. P was calculated using repeated measure ANOVA.

Tumorigenicity Assay in Nude Mice. Female nu/nu mice, 6–8 weeks old, were maintained in a specific-pathogen-free animal care facility according to institutional guidelines. Xenografts of tumors were established by injecting 5 × 10⁶ cells (suspended in PBS) from each cell line into the s.c. tissues of the flank of each mouse. After the tumors reached an approximate volume of 100 mm³, 5 × 10⁶ cells were either mock-infected (i.e., media control) or infected with Ad5/luciferase (control virus) or Ad5/Smad4/DPC4 at a MOI of 100 pfu/ml (25). Aliquots of 5 × 10⁵ cells were mixed with Sea Plaque agarose (Hoefer Scientific Instruments, San Francisco, CA) and DMEM containing 10% FBS and were overlaid on a 0.8% agarose layer containing DMEM and 10% FBS in a 35-mm plate. The plates were incubated for 2–3 weeks, and colonies (>50 μm) were counted with a colony counter. All of the assays were performed in triplicate. P was calculated using repeated measure ANOVA.

Immunostaining. Formalin-fixed, paraffin-embedded tissues were prepared from the tumor-bearing animals treated with Ad5/Smad4/DPC4 or control virus Ad5/luciferase. The specimens were cut into 4-μm-thick sections, mounted on slides, and stained with H&E. Immunohistochemical staining was performed with monoclonal anti-Smad4 antibody (Santa Cruz Biotechnology, Inc.) as described previously (4).

Transduction Efficiency and Overexpression of Smad4/DPC4 in Pancreatic Cancer Cell Lines. Because our previous report showed that the TGF-β signaling pathway is functional in Panc-1 cells (25), the activity of Ad5/Smad4/DPC4 virus was confirmed using Panc-1 cells. As shown in Fig. 1A, overexpressed Smad4/DPC4 protein was detected using Western blot analysis in Panc-1 cells infected with Ad5/Smad4/DPC4 but not in the cells infected with the control virus (Ad5/luciferase). Expression of p21waf1 was induced by over-
expression of Smad4/DPC4 (Fig. 1A). Smad4/DPC4 DNA-binding activities were markedly induced in the Ad5/Smad4/DPC4-infected cells but not in Ad5/luciferase-infected and the mock-infected cells (Fig. 1B). These results show the activity of Ad5/Smad4/DPC4 virus, which is consistent with the activity that we described previously in detail (25).

Smad4/DPC4 is wild type in Panc-1 cells but is homozygously deleted in CFPac-1 cells and has a point mutation and an undetectable level of mRNA in Capan-1 cells (5, 6, 25). The transduction efficiencies of Capan-1 and CFPac-1 pancreatic cancer cell lines were determined using the Ad5/CMV/H9252-gal reporter gene construct with the Panc-1 cell line as a positive control. The cell lines varied in terms of transduction efficiency: at a MOI of 100 pfu/ml, H9252-gal was expressed in 75, 45, and 30% of Panc-1, Capan-1, and CFPac-1 cells, respectively (data not shown). Overexpressed Smad4/DPC4 mRNA was detected in Capan-1 and CFPac-1 cells infected with Ad5/Smad4/DPC4 but not in those infected with Ad5/luciferase (Fig. 2, A and B). At increasing MOI, Smad4/DPC4 mRNA levels increased dose-dependently in these cell lines (Fig. 2, A and B). To examine the DNA-binding activity of virally expressed Smad4/DPC4 protein, nuclear extracts were isolated from Capan-1 and CFPac-1 cells infected with Ad5/Smad4/DPC4 and Ad5/luciferase, and electrophoretic mobility assays were performed. Smad4/DPC4-specific DNA-binding activities were markedly induced in the Ad5/Smad4/DPC4-infected cells but not in those infected by Ad5/luciferase (Fig. 2C, Lane 1, 2, 5, and 6). The competition showed that the Smad4-DNA binding activity was specific (Fig. 2C, Lane 3, 4, 7, and 8), and supershift assays showed that the DNA binding consisted of Smad4 because the Smad4 antibody interfered with Smad4 DNA-binding activity (Fig. 2C, Lanes 9 and 10). Taken together, these data show that re-expression of Smad4/DPC4 can be achieved by infecting cells with Ad5/Smad4/DPC4 adenovirus; that overexpressed Smad4/DPC4 protein binds to a SBE probe and induces its downstream target gene, p21waf1. These findings indicate that this approach may be useful in analyzing the effect of Smad4/DPC4 on the inhibition of transformation in vitro and on the suppression of tumorigenesis in vivo.

Effect of Wild-Type Smad4/DPC4 on in Vitro Cell Proliferation Is Analyzed. We have shown that p21waf1 expression induced by overexpression of Smad4/DPC4 is independent of TGF-β receptor activation (25). To determine whether the growth of pancreatic cancer cells can be inhibited by overexpression of Smad4/DPC4, the three pancreatic cancer cell lines were either mock-infected or infected with the Ad5/Smad4/DPC4 adenovirus or the Ad5/luciferase control virus. The assay for anchorage-independent growth was performed. As shown in Fig. 3, Capan-1 and Panc-1 cells that were infected with Ad5/Smad4/DPC4 at a MOI of 100 pfu/ml demonstrated far fewer colonies (defined as >50 μm) in soft agarose at 21 days than did mock-infected or control virus-infected cells. The difference at 21 days was statistically significant for both Ad5/Smad4/DPC4-infected Capan-1 (P = 0.017) and Panc-1 (P = 0.037) cells. Again, growth of CFPac-1 cells was not affected by Ad5/
Smad4/DPC4 infection. No significant apoptosis was observed (data not shown).

These results suggest that the inhibition of anchorage-independent growth observed in Capan-1 and Panc-1 cells occurred as a result of overexpression of Smad4/DPC4 and that Smad4/DPC4-dependent growth inhibition may in part rely on the induction of Smad4/DPC4-inducible genes.

Overexpression of Smad4/DPC4-inhibited Tumor Growth. To determine the effect of Smad4/DPC4 on the suppression of tumor cell growth in vivo, a xenograft tumorigenicity assay was carried out in nude mice, and the measurements of tumor volumes were analyzed statistically. Our analyses showed that growth was significantly suppressed in tumors derived from Capan-1 cells after treatment with Ad5/Smad4/DPC4 (P = 0.004), but not after Ad5/luciferase treatment, which led to an overall reduction in tumor size at the end of the experiment (Fig. 4A). In contrast, tumors derived from CFPac-1 pancreatic cancer cells, infected with Ad5/Smad4/DPC4 or Ad5/luciferase virus, grew equally well (Fig. 4B). Consistent with the results of the in vitro experiments (Fig. 3, A and B), the growth of tumors derived from Ad5/Smad4/DPC4-infected Capan-1 cells was significantly suppressed by overexpression of Ad5/Smad4/DPC4, whereas the growth of tumors derived from Ad5/Smad4/DPC4-infected CFPac-1 pancreatic cancer cells was resistant to the growth-inhibitory effects of Ad5/Smad4/DPC4.

To confirm that Smad4/DPC4 is expressed in both cell lines after infection with Ad5/Smad4/DPC4, immunohistochemical analysis was performed on Capan-1 and CFPac-1 tumor tissues 2 weeks after the infection with Ad5/Smad4/DPC4 and Ad5/luciferase virus. As shown in Fig. 4C, immunohistochemical staining demonstrated that Smad4/DPC4 was expressed in both tumors after infection with Ad5/Smad4/DPC4 virus. In summary, both the in vivo and the in vitro experiments indicated that overexpression of Smad4/DPC4 suppressed tumorigenesis in Capan-1 cells but not in CFPac-1 cells.

**Smad4/DPC4-induced p15ink4b Expression.** To understand the differential response to Smad4/DPC4 overexpression in Capan-1 and CFPac-1 cell lines, we first determine whether overexpression of Smad4/DPC4 could induce p15ink4b expression. Western blot analysis was performed using Capan-1 and CFPac-1 cell lines that were mock-infected or that were infected with Ad5/Smad4/DPC4 or Ad5/luciferase at various MOIs. The expression of p15ink4b gene and Smad4/DPC4 and the level of β-actin control were analyzed 48 h after infection. As shown in Fig. 5, overexpression of Smad4/DPC4 was detected in the Ad5/Smad4/DPC4-infected Capan-1 and CFPac-1 cell lines, and the level of expression was proportional to the MOI. It was interesting that, in Capan-1 cells, the expression of p15ink4b was induced (Fig. 5A). However, no expression of p15ink4b was detected in the CFPac-1 cell line (Fig. 5B). Subsequently, Southern blot analysis revealed the presence of an intact p15ink4b gene in Capan-1, Panc-1, and CFPac-1 cells (data not shown), which suggested that the induction of p15ink4b expression by Smad4/DPC4 is blocked by additional defects present in this cell line. Taken together, our data indicate that p15ink4b is one of the downstream target genes regulated by Smad4/DPC4.

**p15ink4b Gene Promoter Has Smad4 Binding Element and Is Inducible with Smad4.** To determine whether the p15ink4b promoter contains any functional SBE, the genomic fragments of p15ink4b were isolated from a human-placenta genomic DNA library by using the Apal/BamHI fragment of the p15ink4b cDNA as a probe. One of the genomic clones contains 3.4 kb 5’ to the ATG translation start site of the p15ink4b gene;
the sequence in this clone is identical to that in human chromosome 9p21 cosmide clone 86, which contains the p15ink4b gene. This 3.4-kb genomic fragment was cloned into the pGL-luciferase vector. A schematic diagram of the p15ink4b promoter region is shown in Fig. 6A.

To identify the region of the p15ink4b promoter that is responsive to Smad4/DPC4, 3.4-kb, 2.88-kb, 1.23-kb, 0.73-kb, 0.48-kb, and 0.19-kb p15ink4b promoter deletions were constructed by using XbaI/NdeI, XbaI/PstI, XbaI/Tth1111, XbaI/SpeI, XbaI/AflII, and XbaI/SmaI, respectively, and a 0.29-kb deletion was constructed by deleting 0.19 kb from the SmaI site of the 0.48-kb p15ink4b promoter. Luciferase reporter gene assays indicated that the Smad4-responsive region was located in this 0.29-kb region between −403 and −113 bp of the p15ink4b gene promoter (Fig. 6B). DNA sequencing analysis revealed the existence of three SBEs (GTCT) in the region between −403 and −113 bp of the p15ink4b gene promoter.

To determine whether these two SBEs interact with proteins, electrophoretic mobility shift assays were performed with various lengths of DNA fragments (L1, L2, L3, and L4) generated by PCR from the AflII/SmaI fragment (−403 to 113 bp) of the p15ink4b gene promoter (Fig. 6A). Two SBEs (GTCT) were found in L1, one possible SBE in L2, and no SBEs in L3 or L4. These results showed that only L1, one possible SBEs in L2, and no SBEs in L3 or L4. These results showed that only L1, which contains the two SBEs, had binding activity (Fig. 6C). Using an oligonucleotide probe (p15-oligoA) corresponding to the SBE (GTCT) detected Smad DNA binding activity in p15ink4b gene promoter (Fig. 6D, Lane 1) but not in control AsPc-1/puro cells. This binding activity disappeared in competition with unlabeled p15-oligoA but remained unchanged when the mutant p15-oligoA was used.
suggest that the loss of Smad4/DPC4-inducible expression of failed to induce p21waf1 expression in CFPac-1 cells. However, Smad4/DPC4 overexpression induced. As shown in Fig. 7B, 3TP-luciferase reporter gene activity was induced by overexpression of Smad4/DPC4, and it was further stimulated by TGF-β in Capan-1 cells. However, 3TP-luciferase reporter gene activity was not induced by TGF-β in Smad4/DPC4-transfected CFPac-1 cells (Fig. 7B). These findings are consistent with the differential expression of p15ink4b and p21waf1 genes in these two cell lines (Figs. 5, A and B, and 7A). The levels of expression of Smad2 and Smad3 are very similar in Panc-1, Capan-1, and CFPac-1 cell lines (data not shown). Therefore, these data suggest that additional unidentified defects in the TGF-β signaling pathways may result in the loss of TGF-β- and Smad4/DPC4-regulated gene expression in CFPac-1 cells.

**DISCUSSION**

This report shows that overexpression of Smad4/DPC4 inhibited the growth of Panc-1 and Capan-1 human pancreatic adenocarcinoma cell lines in vivo and that Smad4/DPC4 overexpression suppressed tumor growth in Capan-1 cells but not in CFPac-1 cells in vitro. These results suggest that Smad4/DPC4-mediated growth inhibition and tumor suppression is in part dependent on the induction of TGF-β-regulated genes, including cell-cycle-inhibitory genes. Our results also show that p15ink4b is one of the target genes regulated by Smad4/DPC4 and that Smad4/DPC4-induced p15ink4b gene transcription is mediated by a previously unidentified SBE located in the region between −356 and −329 bp of the p15ink4b promoter.

To determine whether the 15ink4b promoter contains any functional SBE, we isolated several p15ink4b genomic clones from a human-placenta genomic DNA library and analyzed one of the genomic clones containing 3.4 kb 5' to the ATG translation start site of p15ink4b gene (Fig. 6). Smad4/DPC4-induced p15ink4b gene transcription is mediated by a previously unidentified SBE located in the region between −356 and −329 bp of the p15ink4b promoter. Our results show that the SBE in the region between −356 and −329 bp of the p15ink4b promoter is much more active than the SBEs between positions −95 and −46 region of the p15ink4b promoter identified from the 113-bp promoter of the p15ink4b gene (Fig. 6B; Ref. 33).

Adenoviral infection is an effective delivery system that can be used to introduce and overexpress specific genes of interest. Variability in gene delivery is dependent on the transduction efficiency of the cells studied; within this constraint, however, increasing the MOI results in a dose-dependent increase in gene expression within the cells. Our results, which showed that infection with Ad5/Smad4/DPC4 adenovirus achieved overexpression of the Smad4/DPC4 gene, are similar to the findings in other pancreatic cancer cells infected with various adenoviruses (32, 34, 35). Infection with the Ad5/Smad4/DPC4 adenovirus inhibited foci formation and anchorage-independent growth in culture and suppressed tumor growth in nude mice. Our previous report (25) suggested that overexpression of Smad4 induced p21waf1 expression and SBE4-luc promoter reporter activity in MDA-MB–468 cells, in both p15ink4b and p21waf1 might have been caused by additional defects in the TGF-β signaling pathways in CFPac-1 cells. To confirm this, 3TP-luciferase reporter gene assays were carried out to determine whether this reporter gene activity can be induced by TGF-β stimulation in Capan-1 and CFPac-1 cells transfected with Smad4/DPC4. As shown in Fig. 7B, 3TP-luciferase reporter gene activity was induced by overexpression of Smad4/DPC4, and it was further stimulated by TGF-β in Capan-1 cells. However, 3TP-luciferase reporter gene activity was not induced by TGF-β in Smad4/DPC4-transfected CFPac-1 cells (Fig. 7B). These findings are consistent with the differential expression of p15ink4b and p21waf1 genes in these two cell lines (Figs. 5, A and B, and 7A). The levels of expression of Smad2 and Smad3 are very similar in Panc-1, Capan-1, and CFPac-1 cell lines (data not shown). Therefore, these data suggest that additional unidentified defects in the TGF-β signaling pathways may result in the loss of TGF-β- and Smad4/DPC4-regulated gene expression in CFPac-1 cells.
which the Smad4/DPC4 gene is known to be homozygously deleted; in MiaCaPa-2 cells, which have lost expression of the type II TGF-β receptor; and in TGF-β-responsive Panc-1 cells with or without TGF-β stimulation. This would indicate that overexpressed Smad4/DPC4 can bypass the TGF-β receptor activation and translocate into the nucleus to provide an essential transcriptional activation function for TGF-β signaling. One possibility is that Smad4 with Smad1 or Smad2 forms constitutive heteromeric complexes that might be regulated by the high basal level of Smad1 or Smad2 phosphorylation in the tumor cells used in our study. This is supported by the report by Wisotzkey et al. (36) that Medea (Drosophila Smad4) and MAD are physically associated and that this interaction is induced by the high basal phosphorylation of MAD in COS cells but is not augmented by cotransfection with activated DPP receptors. The introduction of p53 into pancreatic cancer cells has been associated with apoptotic changes (32, 34, 35, 37), whereas the introduction of Smad4/DPC4 resulted in growth inhibition without indication of apoptotic changes.5 This finding is consistent with the growth-inhibitory effect of TGF-β on epithelial cells and the effect of the up-regulation of the TGF-β-inducible cell-cycle inhibitor p21\textsuperscript{ras1} by Smad4/DPC4 (23, 37). It is also consistent with the role of Smad4/DPC4 in the activation of downstream cell-cycle-inhibitory proteins that induce growth arrest rather than cell death (25, 32, 34, 35, 37). TGF-β-induced growth inhibition that is independent of Samd4 has also been reported. For example, TGF-β-mediated growth inhibition in DPC4−/− MEF cells and in a Smad4-null pancreatic cancer cell line, BxPc-3, does not require a functional Smad4/DPC4 gene (38, 39). TGF-β-induced and phosphatase-mediated inhibition of extracellular signal-regulated protein kinase (ERK) activation or the inhibition of p70 S6 kinase by protein phosphatase 2A was reported to involve Smad4/PDC4-independent signaling pathways (40, 41). These results suggest a novel TGF-β signaling pathway leading to the inhibition of cell growth. Our earlier report showed that the TGF-β-mediated growth inhibition in Capan-1 and CFPac-1 cells requires a functional Smad4/DPC4 gene, which implies that the novel TGF-β signaling pathway that is independent of Smad4/PDC4 is defective in Capan-1 and CFPac-1 cells (23).

Homozygous deletion of the Smad4/DPC4 gene and the absence of genetic alterations in p15\textsuperscript{ink4b} in the CFPac-1 cell line have been reported previously (3–5). Both p15\textsuperscript{ink4b} and p16\textsuperscript{ink4a} are located in the 9p21 genomic region, and deletions of this region frequently occur in pancreatic carcinomas (42, 43). Codeletions of p15\textsuperscript{ink4b} and p16\textsuperscript{ink4a} have been reported in pancreatic cancer cell lines; it has been suggested that p16\textsuperscript{ink4a} is more likely than p15\textsuperscript{ink4b} to be the target gene of this region (44). However, it has been suggested that p15\textsuperscript{ink4b} aberrations may occur independently (45). Because no deletion or other gross genetic alteration was detected in CFPac-1 cells (data not shown) and no p21\textsuperscript{ras1} mutations have been reported in pancreatic cancer, other mutations, in addition to the Smad4/DPC4 homozygous deletion, may cause defective TGF-β signaling pathways in CFPac-1 cells.

5 J. B. Fleming and P. J. Chiao, unpublished observations.
Tumor cells are heterogeneous. The specific growth-inhibition patterns induced by overexpression of Smad4/DPC4 may reflect the individual genetic makeup of each cell line. Although homozygous deletions and inactivating intragenic mutations in the Smad4/DPC4 have been reported in more than 50% of pancreatic cancer cell lines and xenograft tumors (3–5), there may be additional genetic alterations that affect TGF-β signaling. We have demonstrated that the tumor cell growth in vitro and in vivo was inhibited by overexpression of Smad4/DPC4 in Capan-1 cells, not in CFPac-1 cells, and p21 expression was induced by overexpression of Smad4/DPC4 in Capan-1 cells, not in CFPac-1 cells. These results are consistent with the 3TP luciferase reporter gene activity (Fig. 7). We noticed that there were small increases or decreases (less than 3-fold) in luciferase report gene activity in Capan-1 and CFPac-1 cells with or without TGF-β stimulation, respectively. These may also be attributable to the small variations commonly seen and may be the expected variation for this type of reporter gene assay. Recently, it was shown that re-expression of Smad4/DPC4 in the Smad4/DPC4-null cell line Hs667n did not induce growth inhibition with TGF-β stimulation (46). It is interesting that re-expression of Smad4/DPC4 inhibited vascular endothelial growth factor and up-regulated thrombospondin-1 expression in Hs667n cells in the absence of TGF-β stimulation (46), thereby suppressing tumorigenesis through the inhibition of angiogenesis. It will be important to determine how Smad4/DPC4 regulates the transcription of VEGF and thrombospondin-1.

Two of the three cells lines in our study did not express wild-type Smad4/DPC4, but all three of the cell lines overexpressed Smad4/DPC4 after adenoviral infection. The lower transduction efficiency of the CFPac-1 cell line relative to that of the other two cell lines may have contributed to its decreased response to adenoviral treatment but does not explain the absence of a growth-inhibitory effect at a MOI of 100 pfu/ml. The TGF-β and Smad4/DPC4-mediated growth inhibition of CFPac-1 cells correlated with the loss of Smad4/DPC4-regulated p15ink4b and p21waf1 expression and the lack of TGF-β, and Smad4/DPC4-inducible 3TP-luciferase reporter gene activity. Our findings suggest that these defects in TGF-β and Smad4/DPC4 signaling pathways in CFPac-1 cells may be caused by the inactivation of additional coactivators for Smad4/DPC4-mediated transcription of downstream target genes in CFPac-1 cells. Several approaches for the identification of this coactivator are being used in the investigations in progress. Inactivation of p15ink4b and Smad4/DPC4 has been found in experimental models of metastases, which suggests that the two proteins play important roles in the growth regulation of human pancreatic cancer cells (43, 44). The absence of growth-inhibitory effects in a pancreatic cancer cell line derived from metastatic pancreatic cancer cells has been found in experimental models of metastases, which suggests that the two proteins play important roles in the growth regulation of human pancreatic cancer cells. These findings suggest that the genes regulated by Smad4/DPC4 play a key role in Smad4/DPC4-mediated growth inhibition and tumor suppression.

Recent progress in vector-mediated gene delivery has made gene therapy a promising approach to cancer treatment. The adenoviral vector is a safe method of gene transfer that has a high rate of gene transduction independent of target-cell growth and to inhibit tumorigenesis through the inhibition of angiogenesis. It will be important to determine how Smad4/DPC4 regulates the transcription of VEGF and thrombospondin-1.

Recent progress in vector-mediated gene delivery has made gene therapy a promising approach to cancer treatment. The adenoviral vector is a safe method of gene transfer that has a high rate of gene transduction independent of target-cell growth activity. Our results suggest that an adenoviral vector can be used to restore functional Smad4/DPC4 to Smad4/DPC4-deficient pancreatic cancer cells in vitro and to inhibit tumorigenesis in vivo through the bypass of the TGF-β receptor activation. Ad5/Smad4/DPC4, a replication-defective adenoviral vector bearing the wild-type Smad4/DPC4, controlled by a constitutively active CMV promoter/enhancer, can, in dose-dependent fashion, induce an overexpression of Smad4/DPC4 in pancreatic cancer cells that lack normal growth control because of mutational inactivation and loss of the wild-type allele of Smad4/DPC4. By inducing the downstream target genes, including the cell cycle inhibitors p21waf1 and p15ink4b, Smad4/DPC4 inhibits the growth of Capan-1 cells. However, overexpression of Smad4/DPC4 cannot inhibit growth and is unable to activate the key downstream target genes such as cell cycle inhibitor p21waf1 and p15ink4b in CFPac-1 pancreatic cancer cells. Thus, the molecular heterogeneity of pancreatic cancer will challenge even the most sophisticated therapeutic strategies.

**Table 7**

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<th>Capan-1</th>
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Suppression of Tumorigenesis and Induction of p15\(^{ink4b}\) by Smad4/DPC4 in Human Pancreatic Cancer Cells


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