TUMORIGENESIS AND NEOPLASTIC PROGRESSION

Smad4 Decreases the Population of Pancreatic Cancer—Initiating Cells through Transcriptional Repression of *ALDH1A1*

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Cancer progression involves a rare population of undifferentiated cancer-initiating cells that have stem cell-like properties for self-renewal capacity and high tumorigenicity. We investigated how maintenance of pancreatic cancer—initiating cells is influenced by Smad4, which is frequently deleted or mutated in pancreatic cancer cells. Smad4 silencing up-regulated the expression of aldehyde dehydrogenase 1A1 (ALDH1A1) mRNA, whereas forced expression of Smad4 in pancreatic cancer cells down-regulated it. Smad4 and ALDH1 expression inversely correlated in some human clinical pancreatic adenocarcinoma tissues, suggesting that ALDH1 in pancreatic cancer cells was associated with decreased Smad4 expression. We then examined whether ALDH1 served as a marker of pancreatic cancer—initiating cells. Pancreatic cancer cells contained ALDH1^{hi} cells in 3% to 10% of total cells, with high tumorigenic potential. Because Smad4 is a major mediator of transforming growth factor (TGF)- β family signaling, we investigated the regulatory mechanism of ALDH activity by TGF- β and bone morphogenetic proteins. Treatment with TGF- β attenuated ALDH1^{hi} cells in several pancreatic cancer cells, whereas bone morphogenetic protein-4 was not as potent. Biochemical experiments revealed that TGF- β regulated ALDH1A1 mRNA transcription through binding of Smad4 to its regulatory sequence. It appears that TGF- β negatively regulates ALDH1 expression in pancreatic cancer cells in a Smad-dependent manner and in turn impairs the activity of pancreatic cancer—initiating cells. (*Am J Pathol* 2015; 185: 1457–1470; <http://dx.doi.org/10.1016/j.ajpath.2015.01.011>)

Among cancer patients, the major causes of mortality are local recurrences and distant metastases. Recent evidence indicates that these events occur because of a specific population of the so-called cancer-initiating cells (CICs), also known as cancer stem cells, which exist within various types of tumors. Therefore, understanding and targeting CICs are essential for improving cancer treatment. CICs are capable of both self-renewal and forming non-CICs to maintain the heterogeneous lineages of cancer cells that comprise the tumor.¹ This hierarchy produces the diversity and heterogeneity of cancer cells found in tumor tissue. CICs can be identified by expression of cell surface markers, such as CD133 and CD44, by expression of the aldehyde dehydrogenase 1 (ALDH1) enzyme, or as a side population using flow cytometry. ALDH1 oxidizes retinal to retinoic acid and is responsible for resistance against

anticancer drugs, such as cyclophosphamide.² ALDH1 expression has been detected in normal stem cells and progenitor cells,^{3–5} as well as in several types of cancers, including breast, lung, colon, diffuse-type gastric, and

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pancreatic cancers.^{6–10} High ALDH1 expression correlates with poor prognosis in several types of cancers.^{8,11}

Transforming growth factor (TGF)- β is the prototypic member of a family of secreted proteins that includes TGF- β s, activins, and bone morphogenetic proteins (BMPs). At the cell surface, TGF- β binds to two different serine-threonine kinase receptors, type I (T β RI) and type II (T β RII), thus exerting many biological functions in both normal and cancer cells.¹² On ligand binding, two T β RIIs and two T β RIIs form a heteromeric complex, which transduces intracellular signals by phosphorylating receptor-regulated Smads (R-Smads): Smad2 and Smad3 for TGF- β signaling and Smad1 and Smad5 for BMP signaling. These phosphorylated R-Smads form heteromeric Smad complexes with the common-partner Smad Smad4. R-Smad and common-partner Smad complexes associate with various transcription factors and transcriptional co-activators or co-repressors in the nucleus, thereby regulating transcription of a wide spectrum of target genes. TGF- β also activates non-Smad pathways, including various mitogen-activated protein kinase pathways. TGF- β inhibits epithelial cell proliferation through the induction of p15^{INK4B} and p21^{CIP1/WAF1} or through the reduction of c-Myc; therefore, TGF- β signaling pathways have tumor-suppressive function during cancer progression. Accordingly, perturbation of TGF- β signaling is thought to be involved in many types of cancers. Alterations of genes encoding T β RII, T β RI, Smad4, and Smad2 have been reported to be responsible for progression of various types of cancers.¹³

Pancreatic cancer is one of the most aggressive cancers and is most commonly diagnosed when it is already at an advanced stage of either metastatic or locally advanced cancer. For the past 40 years, the 5-year survival rate of pancreatic cancer patients has remained only 6%. It appears that lesions occurring in the pancreatic ducts, pancreatic intraepithelial neoplasia (PanIN), are a precursor of pancreatic ductal adenocarcinoma. Numerous studies of PanIN and associated adenocarcinomas have identified common mutation patterns, particularly in *KRAS* and *CDKN2A* (encoding p16^{INK4A}). Coincident lesions also reportedly show similar patterns of loss of heterozygosity at chromosomes 9q, 17p, and 18q, which harbor *CDKN2A*, *TP53*, and *SMAD4*, respectively, with studies consistently showing an increasing number of gene alterations in higher grade PanINs.¹⁴ Blackford et al¹⁵ suggested that inactivation of the *SMAD4* gene correlates with metastasis and poor prognosis in patients with surgically resected pancreatic ductal adenocarcinoma.

We present evidence that TGF- β negatively regulates ALDH1 expression in pancreatic cancer cells in a Smad-dependent manner and that TGF- β in turn impairs the activity of pancreatic CICs. Our model predicts that this regulatory mechanism might be disrupted by mutations and deletions that occur in *SMAD4* in human pancreatic cancer cells.

Materials and Methods

Cell Culture and Reagents

Human pancreatic adenocarcinoma Panc-1 and BxPC-3 cells were cultured as described previously.^{16,17} Human pancreatic adenocarcinoma SUIT-2 cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 U/mL of penicillin, and 100 μ g/mL of streptomycin. Cells were grown in a 5% CO₂ atmosphere at 37°C. All experiments were performed on heterogenous populations of shRNA-transfected or adenovirus-infected cells. Among three TGF- β isoforms, TGF- β 3 (R&D Systems, Minneapolis, MN) was used in this study. Recombinant BMP-4 was purchased from R&D Systems.

shRNA

Lentivirus vectors carrying shRNA were generated as described previously.¹⁸ pENTR4-H1 was used to insert shRNA specific for human *SMAD4* and *ALDH1A1* into the lentivirus vectors CSII-RfA-CG and CS-RfA, respectively. Vectors were also generated using control shRNA. The sequences of shRNAs are listed in Table 1. Lentiviruses were concentrated using Lenti-X Concentrator (Clontech, Mountain View, CA).

Transfection

Transient transfection of Smad4 was performed using FuGENE 6 (Roche Diagnostics, Basel, Switzerland) following the manufacturer's instructions. In a 6-well plate, BxPC-3 cells were transfected with 5 μ g of control vector or pcDNA3-Smad4 in the presence of 6 μ L of FuGENE 6 per well. Knockdown of Smad4 using siRNA was performed as described previously.¹⁹ The siRNA sequence targeting human *SMAD4* was 5'-UUACAUCCAACUGCACAC-CUUUGC-3'. Panc-1 cells were transfected with 100 nmol/L of either siRNA or control siRNA in the presence of 5 μ L of Lipofectamine 2000 (Invitrogen, Carlsbad, CA) per well of a 6-well plate.

RNA Isolation and RT-PCR

Total RNA was extracted using Isogen reagent (Nippon Gene, Toyama, Japan). Same cDNA was prepared from each cell, subjected to quantitative real-time RT-PCR analysis.²⁰ All samples were run in duplicate. Values were normalized by hypoxanthine guanine phosphoribosyl transferase 1 mRNA. The primers for quantitative real-time RT-PCR are listed in Table 1.

Immunoblotting

Immunoblotting was performed as described previously.²⁰ Anti-phospho-retinoblastoma protein (Ser 807/811) antibody

Table 1 Sequences of Oligonucleotides Used for Generation of shRNAs and Primers in RT-PCR

Gene	Oligonucleotides
Oligonucleotides used for generation of shRNAs	
<i>SMAD4</i>	Forward: 5'-GATCCCCAAGCAATGGAACACCAATACTCAGGGTGTGCTGTCCCTGAGTATTGGTGTTCATGTGCTT-TTTTGGAAAT-3' Reverse: 5'-CTAGATTTCCAAAAAAGCAATGGAACACCAATACTCAGGGGACAGCACACCCTGAGTATTGGTGTTC-CATGTGCTTGGG-3'
<i>ALDH1A1</i>	Forward: 5'-GATCCCCGTAGCCTTCACAGGATCAAACGTGTGCTGTCCGTTTGATCCTGTGAAGGCTACTTTTGGAAAT-3' Reverse: 5'-CTAATTTCCAAAAAGTAGCCTTCACAGGATCAAACGGACAGCACACGTTTGATCCTGTGAAGGCTACG-GG-3'
Control	Forward: 5'-GATCCCCGCGCGCTTTGTAGGATTCGGTGTGCTGTCCGAATCCTACAAAGCGCGCTTTTGGAAAT-3' Reverse: 5'-CTAGATTTCCAAAAAGCGCGCTTTGTAGGATTCGGGACAGCACACCGAATCCTACAAAGCGCGCGGG-3'
Primers used for quantitative real-time PCR in chromatin immunoprecipitation analysis	
<i>ALDH1A1</i>	Forward: 5'-ACCCAGGAGTCACTCAAGG-3' Reverse: 5'-ACTGTGGGCTGGACAAAGTAG-3'
<i>ALDH1A3</i>	Forward: 5'-TCTCGACAAGCCCTGAAGT-3' Reverse: 5'-TATTCGGCCAAAGCGTATTC-3'
<i>CDKN1A</i>	Forward: 5'-AGTGGACAGCGAGCAGCTGA-3' Reverse: 5'-CGAAGTTCCATCGCTCACGG-3'
<i>CDKN1B</i>	Forward: 5'-CGGTGGACCACGAAGAGTTAA-3' Reverse: 5'-GGCTCGCCTCTTCCATGTC-3'
<i>CDKN2B</i>	Forward: 5'-CCGCCACAACGACTTTATT-3' Reverse: 5'-CAGCCTTCATCGAATTAGGTG-3'
<i>CDC25A</i>	Forward: 5'-GCCTGTACCAACCTGAC-3' Reverse: 5'-CCAGGAGAATCTAGACAGAAACC-3'
<i>MYC</i>	Forward: 5'-CCACACATCAGCACAACACG-3' Reverse: 5'-CGGTTGTTGCTGATCTGTCTCA-3'
<i>CCNE1</i>	Forward: 5'-GCACTTTCTTGAGCAACACCT-3' Reverse: 5'-GTGTCGCCATATACCGGTCAA-3'
<i>SMAD4</i>	Forward: 5'-AAAACGGCCATCTTCAGCAC-3' Reverse: 5'-AGGCCAGTAATGTCCGGGA-3'
<i>NANOG</i>	Forward: 5'-ATTGAGGACAGCCCTGATCTTTC-3' Reverse: 5'-TTTTTGCACACTCTTCTCTGC-3'
<i>POU5F1</i>	Forward: 5'-GTGGAGAGCAACTCCGATG-3' Reverse: 5'-TGCTCCAGCTTCTCCTTCTC-3'
<i>SOX2</i>	Forward: 5'-CGAGTGGAACTTTTGTCCGA-3' Reverse: 5'-TGTGCAGCGCTCGCAG-3'
<i>PROM1</i>	Forward: 5'-TGGATGCAGAACTTGACAACGT-3' Reverse: 5'-ATACCTGCTACGACAGTCGTGGT-3'
<i>CXCR4</i>	Forward: 5'-CCTGCCCTCCTGCTGACTATT-3' Reverse: 5'-TCATCTGCCCTCACTGACGTTG-3'
<i>EPCAM</i>	Forward: 5'-CGGCGACGCGGACTTTTG-3' Reverse: 5'-GAGCCATTCAATTTCTGCCTTCATC-3'
<i>CD44</i>	Forward: 5'-TCAGAGGAGTCGGAGAGAGGAAAC-3' Reverse: 5'-GAAAAGTCAAAGTAACAATAACGTGG-3'
<i>CD24</i>	Forward: 5'-TGTTCTCTTGGGAAGTGAATC-3' Reverse: 5'-ACCTAAAAGTCAAATGCAATG-3'
<i>HPRT1</i>	Forward: 5'-TTTGCTTTCTTGGTCAGGC-3' Reverse: 5'-GCTTGCGACCTTGACCATCT-3'
Primers used for quantitative real-time PCR in chromatin immunoprecipitation analysis	
<i>HPRT1</i>	Forward: 5'-TGTTTGGGCTATTTACTAGTTG-3' Reverse: 5'-ATAAAATGACTTAAGCCCAGAG-3'
<i>ALDH1A1</i>	Forward: 5'-TGCAACAGGCATACTCCTT-3' Reverse: 5'-CAGGGCAGAAGAATCACAGA-3'
+25,060 to +27,715 bp	Forward: 5'-ACTGTGGTGCAAACAGCAACACC-3' Reverse: 5'-TTGGTGTGGTGGTACCCATAAGAGC-3'
Promoter	Forward: 5'-CCAAGCAGCTATTAGGTCTGGGACA-3' Reverse: 5'-CACCCCACTGAGGGTCTTGGGA-3'
Intron11	Forward: 5'-CCATCTGGTGAAACATGCTG-3' Reverse: 5'-TACTGCGAGGCTTCTGTGA-3'
-124,970 to -123,530 bp	

and anti-poly (ADP-ribose) polymerase antibody were purchased from Cell Signaling Technology (Beverly, MA). Anti-ALDH1 antibody was purchased from BD Biosciences (San Jose, CA), and anti-Smad4 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti- α -tubulin antibody was purchased from Sigma-Aldrich (St Louis, MO).

Cell Proliferation Assay

Cells from each cell stock were seeded in triplicate in 6-well plates (1×10^4 cells per well). At the indicated days after preparation, cells were counted using a hemocytometer as described previously.²⁰

Colony Formation Assay

The colony formation assay was performed as described previously.²¹ Cells were seeded in 0.3% agar at 1×10^4 cells per well and cultured for 2 weeks. Diethylaminobenzaldehyde (DEAB) (100 mmol/L; Sigma-Aldrich) was diluted in ethanol and added to the medium of each well at a concentration of 100 μ mol/L every 3 days. The colony diameters were measured using Cellsens Standard (Olympus, Tokyo, Japan).

ChIP Analysis

Chromatin immunoprecipitation (ChIP) analysis was performed as described previously.²² Anti-Smad4 antibody

(R&D Systems) and anti-Smad2/3 antibody (BD Biosciences) were used for immunoprecipitation.²³ Genomic DNA eluted in 100 μ L of Tris-EDTA buffer was used for quantitative real-time RT-PCR. The primers for quantitative real-time RT-PCR in ChIP analysis are listed in Table 1.

Reporter Assay

The upstream region (−25,060 to −27,715 bp) and downstream region (+124,970 to +123,530 bp) of human *ALDH1A1* were subcloned into the promoter-reporter construct pGL4.10 luc2 (Promega, Madison, WI). Panc-1 cells were seeded in a 24-well plate (5×10^4 cells per well) and transfected with promoter-reporter constructs using FuGENE 6, following the manufacturer's instructions. Twenty-four hours later, cells were treated with TGF- β 3 for an additional 24 hours and then lysed. Luciferase activity was measured with the Dual Luciferase Reporter Assay System (Promega) using Mithras LB 940 (Berthold Technologies, Oak Ridge, TN). Luciferase activity was normalized to that of pGL4.10-TK-hRLuc, which was co-transfected.

Aldefluor Assay

Aldefluor assay was performed using the ALDEFLUOR Kit (StemCell Technologies, Vancouver, BC, Canada) as described previously.¹⁰ MoFlo Astrios (Beckman Coulter, Pasadena, CA) was used for cell sorting, and an Epics-XL flow cytometer (Beckman Coulter) was used for analysis.

Subcutaneous Xenograft Models

Male BALB/c *nu/nu* mice (4 weeks of age) were purchased from the Oriental Yeast Company (Tokyo, Japan). The same stocks of cells were prepared, a total volume of 100 μ L of cells in Matrigel (BD Biosciences) was subcutaneously injected into the left flank of each mouse, and tumors were measured as described previously.²⁴ All animal experiments were performed in accordance with the policies of the Animal Ethics Committee at The University of Tokyo.

Immunohistochemistry

Formalin-fixed, paraffin-embedded pancreatic tissues were obtained from patients with pancreatic adenocarcinoma at The University of Tokyo Hospital. All patients gave their informed consent. Hematoxylin and eosin staining of tissues, antigen retrieval, and immunodetection were performed as described previously.²⁵ Anti-Smad4 antibody (Santa Cruz Biotechnology) and anti-ALDH1 antibody (BD Biosciences) were the primary antibodies used for section immunostaining. All studies were conducted using protocols approved by

Figure 1 Smad4 is essential for the *in vivo* pancreatic tumor growth. **A:** SMAD4 mRNA expression in pancreatic cancer cells was determined by quantitative real-time RT-PCR. **B:** Cell lysates were immunoblotted with the indicated antibodies. **C:** Human pancreatic adenocarcinoma Panc-1 cells were infected with lentiviruses carrying control shRNA (Panc-1-shNTC) or shRNA targeting *SMAD4* (Panc-1-shSMAD4). SMAD4 mRNA expression in these cells was determined by quantitative real-time RT-PCR. **D:** Cell lysates were immunoblotted with the indicated antibodies. **E:** BALB/c *nu/nu* mice were subcutaneously injected with Panc-1-shNTC ($n = 4$) and Panc-1-shSMAD4 cells ($n = 5$), and tumor volume was measured. **F:** The numbers of Panc-1-shNTC and Panc-1-shSMAD4 cells were counted at 1, 2, and 3 days after seeding. Data are presented as means $\pm$ SD. * $P < 0.05$. ND, not detected.

Research Ethics Committee at The University of Tokyo, Graduate School of Medicine.

Statistical Analysis

Two-sided Student's *t*-test was used for examining differences in gene expression levels, tumorigenic potentials, and *in vitro* proliferative ability in multiple groups. Repeated-measures analysis of variance was used for examining

Figure 2 Expressions of pluripotent stem cell markers are increased in pancreatic cancer cells on knockdown of SMAD4. **A:** Expression levels of pluripotent stem cell markers in Panc-1-shNTC and Panc-1-shSMAD4 cells were determined by quantitative real-time RT-PCR. **B:** BxPC-3 cells were transfected with control vector (–) or SMAD4. Expression levels of SMAD4 mRNA and of pluripotent stem cell markers in these cells were determined by quantitative real-time RT-PCR. Data are presented as means $\pm$ SD. * $P < 0.05$.

differences in the size of tumors. $P < 0.05$ was considered significant.

Results

Knockdown of *SMAD4* in Pancreatic Cancer Cells Leads to Enrichment of CICs

SMAD4 is frequently mutated in pancreatic cancer cells. We examined Smad4 expression in three human pancreatic cancer cells: Panc-1, BxPC-3, and SUIT-2. Consistent with a previous report,²⁶ quantitative real-time RT-PCR analysis and immunoblotting revealed that *SMAD4* mRNA and Smad4 protein were expressed in Panc-1 and SUIT-2 cells but remained undetected in BxPC-3 cells (Figure 1, A and B). On the basis of these findings, Panc-1 and SUIT-2 cells were used as Smad4-expressing pancreatic cancer cells in this study.

To determine the role of Smad4 expression in tumorigenesis of pancreatic cancer cells *in vivo*, we established Panc-1 cells with knockdown of Smad4 using shRNA. *SMAD4* mRNA and its protein were successfully silenced in cells introduced with shRNA targeting *SMAD4* (Panc-1-shSMAD4 cells) but not in control cells (Panc-1-shNTC cells) (Figure 1, C and D). These cells were then xenografted into nude mice, and tumorigenic potential was monitored (Figure 1E). Tumors generated by Panc-1-shSMAD4 cells were larger than those by Panc-1-shNTC cells, suggesting that Smad4 plays a tumor suppressive role in the formation of pancreatic cancer *in vivo*.

It is assumed that rapid cell growth is critical for the tumor-forming ability of cancer cells; thus, we assessed the proliferation of these cancer cells *in vitro* (Figure 1F). Panc-1-shSMAD4 and Panc-1-shNTC cells exhibited similar *in vitro* proliferation rates. Smad4 knockdown did not alter the expression levels of cyclin-dependent kinase (CDK) regulators (*CDKN1A*, *CDKN1B*, *CDKN2B*, *CCNE1*, *MYC*, and *CDC25A*) or phosphorylation of

retinoblastoma proteins, suggesting that Smad4 did not influence cell cycle progression of pancreatic cancer cells (Supplemental Figure S1, A and B). Moreover, poly (ADP-ribose) polymerase cleavage, which is an indicator of apoptotic cell death, was not enhanced in Panc-1-shSMAD4 cells (Supplemental Figure S1C).

These results suggested that Panc-1-shSMAD4 cells have high tumorigenic activity despite the fact that proliferative activity does not differ between Panc-1-shSMAD4 cells and Panc-1-shNTC cells. In xenograft models, many factors have been implicated in tumor growth, including anchorage-independent growth, escape from various apoptotic stimuli, and interactions between cancer cells and tumor microenvironment. In addition to these mechanisms, we postulated that heterogeneity of pancreatic cancer cells might be altered by the expression of Smad4. Thus, we examined whether certain population of cancer cells (eg, CICs) were enriched in pancreatic cancer cells on knockdown of SMAD4. It is thought that the specific expressions of transcription factors Nanog and Oct4 are important for the characteristics of pancreatic CICs.²⁷ Smad4 silencing in Panc-1 cells elevated the expressions of these pluripotent stem cell markers, including *NANOG*, *POU5F1* (encoding Oct4 protein), and *SOX2* (Figure 2A). On the other hand, forced expression of Smad4 in BxPC-3 cells uniformly decreased the expression levels of these markers (Figure 2B). These results support the hypothesis that Smad4 expression may negatively regulate the number of CICs in pancreatic cancer cells.

ALDH1 Expression Inversely Correlates with Smad4 Expression in Human Pancreatic Cancer

To isolate the CIC population in pancreatic cancer cells, we investigated the expression levels of markers that were highly expressed in pancreatic cancer cells with knockdown

Figure 3 Knockdown of SMAD4 in pancreatic cancer cells leads to increased expression of ALDH1. **A:** Expressions of cancer-initiating cell markers in Panc-1-shNTC cells and Panc-1-shSMAD4 cells was determined by quantitative real-time RT-PCR. **B:** Cell lysates were immunoblotted with the indicated antibodies. Data are presented as means $\pm$ SD. * $P < 0.05$. ND, not detected.

of SMAD4. CD133 (alias PROM1), CD44, CD24, epithelial specific antigen (alias epithelial cell adhesion molecule), and CXCR4 are reportedly useful for isolation of pancreatic CICs.^{28–30} ALDH1 activity is also reported to be able to identify CICs in pancreatic cancers.³¹ Quantitative real-time RT-PCR analysis revealed much higher expression of ALDH1A1 mRNA in Panc-1-shSMAD4 cells than in control Panc-1-shNTC cells (Figure 3A). The amounts of ALDH1 protein were also remarkably up-regulated in Panc-1-shSMAD4 cells (Figure 3B). Because other ALDH1

isoforms are reportedly expressed in certain types of CICs,³² their expressions in Panc-1 cells were also examined (Figure 3A). ALDH1A2 mRNA was not detected in these cells. Expression of ALDH1A3 mRNA was suppressed in Panc-1-shSMAD4 cells, implying that this gene was not important for pancreatic CIC identification. Together, these findings suggested that ALDH1A1-expressing pancreatic cancer cells were enriched in cancer cells with SMAD4 knockdown.

Next, we used Aldefluor assay to detect the cell population that possessed high ALDH1 enzymatic activity among these pancreatic cancer cells (Figure 4A). This assay specifically distinguished cells with high enzymatic activity from those with low activity by using DEAB, which inhibits the function of all members of the ALDH family. Cells with high ALDH activity were regarded as having high ALDH1A1 activity (ALDH1^{hi} cells) in this study. These ALDH1^{hi} cells comprised 3% to 10% of the pancreatic cancer cells (Figure 4A). Next, we examined the correlation between Smad4 expression and ALDH1 activity in Panc1 cells. siRNA targeting *SMAD4* was introduced into Panc-1 cells, and Aldefluor assay was conducted (Figure 4, B and C). The introduction of siRNA targeting *SMAD4* increased both ALDH1A1 expression and ALDH1 enzymatic activity in these cells.

We then profiled the expression patterns of the Smad4 and ALDH1 proteins in clinical samples from patients with pancreatic cancer. In 16 tissue samples of moderately differentiated adenocarcinoma, immunohistochemical analysis using anti-Smad4 antibody revealed two groups: tissues containing Smad4-expressing cancer cells at a low frequency and tissues exhibiting a high frequency of these cells (Figure 5). In both tissue types, intense ALDH1 staining was detected mainly in cells expressing low levels of Smad4, and Smad4 was also mainly observed in cells that did not show intense ALDH1 staining, with few exceptions. Overall, Smad4 expression was inversely correlated with that of ALDH1 in cancer cells within some human pancreatic cancer tissues (Table 2). These results support the possibility that ALDH1 expression in pancreatic cancer cells was suppressed by Smad4-mediated signal transduction.

ALDH1 Expression Is Essential for the Tumorigenic Ability of Pancreatic Cancer Cells

To investigate whether ALDH1 served as a marker for pancreatic CICs, ALDH1^{hi} cells and ALDH1[−] cells were sorted from pancreatic cancer cells and subjected to further analyses. To assess the differentiation potency of each population, these sorted cells were cultured *in vitro* for a week and then reanalyzed by flow cytometry (Figure 6A). Among both SUI-2 cells and Panc-1 cells, only ALDH1[−] cells were detected in the cultured ALDH1[−] cells. In contrast, cultured ALDH1^{hi} cells produced both ALDH1^{hi} cells and ALDH1[−] cells, suggesting that ALDH1^{hi} cells possessed multiple differentiation potency. In accordance with these findings, pluripotent stem cell markers, including

Figure 4 ALDH1^{hi} cells are enriched in pancreatic cancer cells on knockdown of SMAD4. **A:** Pancreatic cancer cells were treated with BODIPY-aminoacetaldehyde (BAAA) in the presence or absence of diethylaminobenzaldehyde (DEAB) for 45 minutes. Then the fraction of ALDH1^{hi} cells was analyzed by flow cytometry. The percentage of ALDH1^{hi} cells within the total population of viable cells is indicated in the histogram and graph. **B:** Panc-1 cells were transfected with control siRNA (siNTC) or siRNA targeting *SMAD4* (siSMAD4). Expression levels of *SMAD4* and *ALDH1A1* mRNA were determined by quantitative real-time RT-PCR. **C:** The Panc-1 cells in **B** were treated with BAAA in the presence or absence of DEAB for 45 minutes. Then the fraction of ALDH1^{hi} cells was analyzed by flow cytometry. The numbers in the histograms show the percentage of ALDH1^{hi} cells within the total population of viable cells. Data are presented as means $\pm$ SD (**B**). * $P < 0.05$.

NANOG, *POU5F1*, and *SOX2*, were highly expressed in ALDH1^{hi} pancreatic cancer cells (Supplemental Figure S2), whereas expression of other CIC markers was not elevated (Supplemental Figure S3).

We next investigated the tumor-forming ability of each fraction *in vitro* and *in vivo*. The colony formation assay revealed that the ALDH1^{hi} cells formed larger colonies than ALDH1⁻ cells, which was the case among cells sorted from both the SUIT-2 cells and Panc-1 cells (Figure 6B). Similarly, when equal amounts of ALDH1^{hi} cells and ALDH1⁻ cells sorted from SUIT-2 cells were xenografted into nude mice, large tumors were observed in mice bearing ALDH1^{hi} cells (Figure 6C). To directly compare the tumor-forming abilities of ALDH1^{hi} and ALDH1⁻ cells, various amounts of each population were subcutaneously xenografted (Table 3). Tumor formation from ALDH1^{hi} cells required injection of 100 cells, whereas injection of 100 ALDH1⁻ cells failed to form tumors in all mice examined. These observations suggested that the ALDH1^{hi} cells within pancreatic cancer cells included a greater proportion of CICs than the ALDH1⁻ cells.

We then examined whether ALDH1 expression was essential for the tumorigenic properties of CICs. Panc-1

cells stably containing shRNA targeting *ALDH1A1* (Panc-1-shALDH1A1) were established. The shRNA specifically knocked down the expression of *ALDH1A1* mRNA without affecting that of *ALDH1A3* mRNA (Figure 7A). Aldefluor assay revealed that ALDH1 activity was completely abolished in Panc-1-shALDH1A1 cells (Figure 7B). Cell proliferation did not differ between Panc-1-shALDH1A1 cells and control cells (Panc-1-shNTC) (Figure 7C); however, Panc-1-shALDH1A1 cells exhibited decreased colony-forming ability compared with Panc-1-shNTC cells, which could mimic the inhibitory effect of TGF- β on tumor formation (Figure 7D). Moreover, pharmacologic inhibition of ALDH1 activity by DEAB potentially reduced the colony-forming ability of parental Panc-1 cells (Figure 7D). Thus, we postulated that ALDH1 serves as a marker for pancreatic CICs, and that enzymatic ALDH1 activity is crucial for the tumorigenic potential of pancreatic CICs.

Smad4 Negatively Regulates ALDH1A1 Transcription in Pancreatic Cancer Cells in a TGF- β -Dependent Manner

Smad4 forms hetero-oligomeric complexes with the R-Smads (ie, Smad2 and Smad3 for TGF- β signaling and

Figure 5 ALDH1 is expressed in cells expressing low levels of Smad4 in human pancreatic cancer. Moderately differentiated adenocarcinoma tissues, specifically from the invasive front ($n = 8$) and center region ($n = 8$), from pancreatic tumor patients were stained with hematoxylin and eosin (H&E), anti-Smad4 antibody, and anti-ALDH1 antibody. Representative images of immunohistochemical analyses and H&E staining of tissues are shown. Smad4-expressing cancer cells and ALDH1-expressing cancer cells are also indicated (inner frame). Lesion sites were classified based on whether they contained a low or high density of Smad4-expressing cancer cells. **Red arrows** indicate high Smad4-expressing cancer cells, and **blue arrows** indicate low Smad4-expressing cancer cells. The **boxed regions** correspond to the **insets** at higher magnification ($\times 400$). Scale bar = 30 μ m.

Smad1 and Smad5 for BMP signaling) and transduces specific signals. To identify which signals were involved in regulating ALDH1 expression in pancreatic cancer cells, SUIT-2 cells and Panc-1 cells were exogenously stimulated with TGF- β and BMP-4 (Figure 8, A and B). TGF- β inhibited the transcription of ALDH1A1 mRNA in both SUIT-2 cells and Panc-1 cells immediately, which in turn reduced ALDH1 protein translation. In contrast, BMP-4 reduced ALDH1 mRNA only in SUIT-2 cells but not in Panc-1 cells. BMP-4 reduced ALDH1 protein in neither SUIT-2 nor Panc-1 cells. In both SUIT-2 cells and Panc-1 cells, ALDH1 activity was inhibited by TGF- β and less potently by BMP-4 (Figure 8C). These results suggested that ALDH1 regulation by Smad4 in pancreatic cancer cells was dependent mainly on TGF- β signaling. These findings also predict that TGF- β may attenuate the tumorigenic potentials of pancreatic cancer cells through the reduction of ALDH1 expression. Accordingly, TGF- β strongly inhibited colony formation of both SUIT-2 cells and Panc-1 cells, and BMP-4 was less potent than TGF- β (Figure 8D).

Finally, we performed biochemical experiments aiming to uncover the molecular mechanism of transcriptional

regulation of ALDH1A1 mRNA by Smad-dependent TGF- β signaling. Because TGF- β down-regulated ALDH1A1 mRNA expression even after 24 hours stimulation, it was expected that *ALDH1A1* was a direct target of the Smad pathway (Figure 8A). Our previous ChIP and ChIP-sequencing analysis of human diffuse-type gastric cancer cells, OCUM-2MLN,²¹ predicted four loci around the *ALDH1A1* locus to be candidate Smad-binding sites: upstream region (–25,060 to –27,715 bp), promoter, intron 11, and downstream region (+124,970 to +123,530 bp) (unpublished data). We performed a new ChIP analysis to ascertain whether direct Smad binding to the *ALDH1A1* genome was involved in pancreatic cancer cells. ChIP analysis using anti-Smad2/3 antibody revealed Smad2/3 binding in some loci, but the binding profiles differed between SUIT-2 cells and Panc-1 cells (Figure 9A). In contrast, ChIP analysis using anti-Smad4 antibody revealed common binding of Smad4 to the upstream region and the downstream region in both types of cancer cells (Figure 9B). Moreover, these bindings of Smad4 were enhanced by treatment with TGF- β , suggesting that these sites were important for TGF- β –Smad-mediated regulation of ALDH1A1 mRNA. Constructs containing these Smad4-binding candidate sites were introduced into Panc-1 cells, and reporter activities were examined in the presence or absence of TGF- β (Figure 9C). The reporter activity of the construct that contained the +124,970 to +123,530 bp region was modestly suppressed by TGF- β . Together, these results suggested that TGF- β might negatively regulate *ALDH1A1* transcription in pancreatic cancer cells through the binding of Smad4 to this region.

Discussion

There is increasing evidence that small fractions of CICs in tumor tissues may be responsible for recurrence in many cancers. However, little is known about the regulatory mechanisms involved in their maintenance. The present results document the importance of Smad-mediated TGF- β signals in the maintenance of ALDH1^{hi} cells within pancreatic cancer.

Table 2 Immunohistochemical Analyses of Moderately Differentiated Adenocarcinoma Tissues Using Anti-Smad4 Antibody and Anti-ALDH1 Antibody

Patient no.	Invasive front		Center	
	Smad4	ALDH1	Smad4	ALDH1
1	Low	High	Low	High
2	High	High	Low	High
3	High	High	High	High
4	High	Low	Low	High
5	Low	Low	Low	High
6	High	Low	High	Low
7	Low	High	High	Low
8	High	High	High	Low

Figure 6 ALDH1 serves as a cancer-initiating cell marker of pancreatic cancer cells. **A:** ALDH1⁻ cells and ALDH1^{hi} cells were sorted from human pancreatic adenocarcinoma SUI-2 and Panc-1 cells using Aldefluor assay. Seven days after fractionation, cells were reanalyzed by flow cytometry. Numbers beside the graphs indicate the percentages of ALDH1^{hi} cells within the total population of viable cells. **B:** ALDH1⁻ cells and ALDH1^{hi} cells from SUI-2 or Panc-1 cells were cultured in soft agar for 2 weeks. Colony diameters and representative images are shown. **C:** BALB/c *nu/nu* mice were subcutaneously injected with 1×10^4 of ALDH1⁻ cells ($n = 6$) and ALDH1^{hi} cells ($n = 4$) from SUI-2 cells, and tumor volume was measured. Data are presented as means $\pm$ SD (**B** and **C**). * $P < 0.05$. Scale bar = 100 μ m.

The Role of Smad4 during Pancreatic Cancer Progression

During cancer progression, mutations and deletions occur in many types of genes. Among them, *SMAD4*, a candidate tumor suppressor gene on chromosome 18q21, has high frequencies of allelic losses in many types of tumors.^{33–35} *SMAD4* mutations have been found in 17% to 56% of patients with juvenile polyposis syndrome and in 22% to 46% of patients with advanced colorectal cancers.^{33,34} Takaku et al³⁶ reported polyps phenotypically similar to human juvenile polyps in the stomach and duodenum of *Smad4* heterozygous knockout mice. They also observed that intestinal polyps in *Smad4*(+/-) *Apc* ^{Δ 716}(+/-) mice developed into more malignant polyps than those in the simple *Apc* ^{Δ 716}(+/-) heterozygotes.³⁷ These results indicate that Smad4 plays a tumor suppressive role in the malignant progression of these tumors. Bardeesy et al³¹ addressed the role of Smad4 deficiency in pancreatic cancer in experiments using genetically engineered mice. Although the activated *Kras*^{G12D} allele alone elicited PanIN, the combination of *Kras* activation and *Smad4*

deficiency resulted in the rapid development of tumors that resembled intraductal papillary mucinous neoplasia, a human pancreatic ductal adenocarcinoma precursor. Another *in vivo* experiment revealed that the absence of *Pten* initiated widespread premalignant lesions in the pancreas, which were significantly accelerated by *Smad4* deficiency.³⁸ These findings indicate that *SMAD4* mutations or deletions accelerate the malignant progression of cancer in combination with other gene alterations.

To our knowledge, the present study provides the first evidence that *SMAD4* deletion is involved in pancreatic CIC maintenance. ALDH1A1 mRNA expression in

Table 3 Incidences of Tumor Formation of ALDH1⁻ Cells and ALDH1^{hi} Cells from SUI-2 Cells

Cell	Tumor incidence			
	10,000	3000	1000	100
ALDH1 ⁻	2/2	3/3	3/4	0/3
ALDH1 ^{hi}	2/2	6/6	5/5	4/4

Incidence of tumor formation of SUI-2 cells in Figure 6. Tumor formation was determined at 2 months after injection.

Figure 7 ALDH1 is important for the tumorigenic potential of pancreatic cancer cells. **A:** Expression levels of ALDH1A1 mRNA and ALDH1A3 mRNA in Panc-1-shNTC and Panc-1-shALDH1A1 cells were determined by quantitative real-time RT-PCR. **B:** Panc-1-shNTC and Panc-1-shALDH1A1 cells were treated with BODIPY-aminoacetaldehyde (BAAA) in the presence or absence of diethylaminobenzaldehyde (DEAB) for 45 minutes. Then the fraction of ALDH1^{hi} cells within the total population of viable cells. **C:** The numbers of Panc-1-shNTC and Panc-1-shALDH1A1 cells were counted at 1 and 3 days after seeding. **D:** Panc-1-shNTC and Panc-1-shALDH1A1 cells were cultured in soft agar for two weeks. Untreated Panc-1 cells and cells treated every 2 days with 3 μ L of ethanol (EtOH), 100 μ M/L DEAB, or 1 ng/mL of transforming growth factor (TGF)- β 3 were also prepared. Colony diameters and representative images are shown. Data are presented as means $\pm$ SD. * P < 0.05. Scale bar = 100 μ m.

pancreatic cancer cells was up-regulated by silencing of Smad4, which in turn increased the population of ALDH1^{hi} tumorigenic cancer cells (Figures 3, A and B, and Figure 4, B and C). This concept was further reinforced by our examination of clinical specimens. In human pancreatic cancer tissues, Smad4 protein expression levels were inversely correlated with those of ALDH1 protein (Figure 5 and Table 2). These observations suggested that disruption of Smad4-mediated TGF- β signaling is important for pancreatic CIC regulation.

Duda et al³⁹ reported that restoration of Smad4 into SMAD4 homozygous-deleted human pancreatic tumor cells reduced *in vivo* tumorigenesis. They also documented that expression of vascular endothelial growth factor was down-regulated in Smad4-restored pancreatic cancer cells. Because angiogenesis is thought to provide a favorable vascular niche for CICs, both CIC maintenance and formation of a suitable microenvironment for CICs are likely affected by Smad4 expression in pancreatic cancer cells.

The Role of ALDH1 Expression in Pancreatic CICs

Recent evidence indicates that specific cell populations of CICs exist in various cancers, which may be identified by specific markers, such as CD133 and CD44, and as a side population by flow cytometry.^{8,28–30} The present study revealed that ALDH1 may be useful for the isolation of

pancreatic CICs (Figure 6). We also found that TGF- β diminished ALDH1^{hi} populations of pancreatic cancer cells (Figure 8). CICs are thought to possess properties similar to those of normal stem cells, including the ability to self-renew while producing differentiated daughter cells. In this study, ALDH1^{hi} cells within pancreatic cancer cell populations exhibited strong expressions of NANOG, POU5F1 (OCT4A), and SOX2, indicating that ALDH1^{hi} pancreatic cancer cells possessed the same characteristics as pluripotent stem cells (Supplemental Figure S2). Expression levels of NANOG, POU5F1, and SOX2 were also increased in Panc-1-shSMAD4 cells (Figure 2A), suggesting that these markers were regulated by Smad4-mediated signaling. However, silencing of ALDH1 in pancreatic cancer cells did not alter the expression levels of these markers (data not shown); thus, NANOG, POU5F1, and SOX2 may be regulated by TGF- β in an ALDH1-independent manner.^{40,41}

It is now believed that CICs are resistant to conventional treatments, including chemotherapy and irradiation. For example, the ATP-binding cassette (ABC) drug transporters protect CICs from many chemotherapeutic agents. Directly targeting CICs may be crucial for preventing cancer recurrences and metastases. To investigate this issue, Panc-1-shALDH1 cells were established and characterized for their tumorigenic potentials. We found that tumor-forming ability was disturbed by ALDH1 silencing (Figure 7). Many previous reports have documented the importance of ALDH1 for the proliferation, differentiation, and metabolism of several

Figure 8 The ALDH1^{hi} population within pancreatic cancer cells is diminished by transforming growth factor (TGF)-β and less potently by bone morphogenetic protein (BMP)-4. **A:** Human pancreatic adenocarcinoma SUIT-2 and Panc-1 cells were treated with 1 ng/mL of TGF-β3 or 30 ng/mL of BMP-4 for the indicated number of hours. ALDH1A1 mRNA expression levels were determined by quantitative real-time RT-PCR. **B:** SUIT-2 and Panc-1 cells were treated with 1 ng/mL of TGF-β3 or 30 ng/mL of BMP-4 for 48 hours. Cell lysates were immunoblotted with the indicated antibodies. **C:** SUIT-2 and Panc-1 cells were treated with BODIPY-aminoacetaldehyde (BAAA) in the presence or absence of diethylaminobenzaldehyde for 45 minutes. Then the fraction of the ALDH1^{hi} cells was analyzed by flow cytometry. The percentages of ALDH1^{hi} cells within the total population of viable cells are indicated in the histograms and graph. **D:** SUIT-2 and Panc-1 cells were treated with 1 ng/mL of TGF-β3 or 30 ng/mL of BMP-4 every 2 days and cultured in soft agar for 2 weeks. Colony diameters and representative images are shown. Data are presented as means ± SD fold change under TGF-β3 or BMP-4 stimulation (**A**) and means ± SD (**D**). **P* < 0.05. Scale bar = 200 μm.

agents in cells.^{42–45} However, the present results provide the first evidence that ALDH1 expression may directly affect the tumor-forming ability of pancreatic cancer cells. As shown in Figure 7D, pharmacologic inhibition of ALDH1 enzymatic activity using DEAB or related compounds could eradicate the CICs in pancreatic cancer tissue and thus may have a therapeutic benefit in pancreatic cancer treatment.

Biochemical Analysis of the Regulatory Mechanism of ALDH1 Expression by Smad4

Previous studies have identified several molecular mechanisms that regulate ALDH1 expression. Moreb et al⁴⁶ found that co-stimulation with interleukin-1 and tumor necrosis factor-α increased the expression of ALDH1 mRNA and its protein in human bone marrow cells. Treatment of human mammary epithelial cells with miR-146a inhibitor also

decreases the percentage of ALDH1-positive cells.⁴⁷ Elizondo et al⁴⁸ proposed a model in which retinoic acid receptor-α and CCAAT/enhancer binding protein-β activate the *ALDH1* gene promoter through the retinoic acid response element and the CCAAT/enhancer binding protein-β response element in mouse hepatoma Hepa-1 cells. Furthermore, ALDH1 enzyme expression and activity are reduced by inhibition of the Notch signaling pathway in certain types of mouse osteosarcoma cells.⁴⁹ However, the molecular mechanism behind the transcription of *ALDH1A1* mRNA in cancer cells remains unknown. We performed biochemical analyses to examine the regulatory mechanism of TGF-β-mediated expression of *ALDH1A1* mRNA in pancreatic cancer cells (Figure 9C). Through these studies, we identified several Smad4-binding sites that might be important for the transcriptional regulation of *ALDH1A1* (Figure 9). Our results suggested that TGF-β negatively regulates the

Figure 9 Transcription of *ALDH1A1* mRNA is regulated by Smad4 in a transforming growth factor (TGF)- β -dependent manner. **A** and **B**: Human pancreatic adenocarcinoma SUIT-2 and Panc-1 cells were stimulated with 1 ng/mL of TGF- β 3 for 1.5 hours, and cells were fixed and harvested. Genomic DNA was immunoprecipitated by anti-Smad2/3 (**A**) or anti-Smad4 antibody (**B**). Smad2/3- or Smad4-bound DNA was determined by quantitative real-time PCR using the primers designed around the *ALDH1A1* locus. Numbers indicate the genomic positions relative to the transcriptional start site of *ALDH1A1*. The *HPRT1* intronic region was used as a negative control for chromatin immunoprecipitation (ChIP). **C**: Panc-1 cells were transfected with *ALDH1A1* reporter-promoter constructs, and cells were treated with 1 ng/mL of TGF- β 3 for 24 hours. Relative luciferase activities were determined. Data are presented as means $\pm$ SD.

transcription of *ALDH1A1* mRNA through the binding of Smad4 (Figure 9).

We previously performed ChIP-sequencing analysis using anti-Smad4 antibody and found that Smad4 binding was concentrated in specific genomic sequences, including an AP1 site and a GC-rich motif.⁵⁰ This finding suggested the involvement of other transcription factors in the binding of Smad4. Zeb2 (also known as SIP1) forms a complex with Smads and regulates transcription of target genes.⁵¹ In the present study, AP1 sites were present in the Smad binding sites both upstream and downstream of the *ALDH1A1* locus, and Zeb2/SIP1 binding motifs were observed downstream of this locus (data not shown). These transcription factors may be required for the Smad4-dependent regulation of *ALDH1A1* transcription.

Smad4 affected the percentage of *ALDH1*^{hi} cells but not that of *CD133*⁺ cells (Figure 4, data not shown). Lonardo et al⁵² reported that nodal/activin signaling is important for the characteristics of *CD133*⁺ pancreatic CICs. However, we found that the *ALDH1*^{hi} cell population was diminished by TGF- β but not by activin (data not shown). These data suggest that the TGF- β -*ALDH1* pathway and nodal/activin-*CD133* pathway may function independently in the stemness of pancreatic cancer cells.

Similar to the present findings, previous reports have indicated that TGF- β from the cancer microenvironment leads to decreased side population cells and *ALDH1*^{hi} cells in diffuse-type gastric carcinoma.^{10,21} Tang et al⁵³ reported that TGF- β down-regulates the expression of inhibitor of DNA-binding 1 in breast cancer cells, which leads to the differentiation and decrease of CICs. TGF- β also restricts the self-renewal activity of $\alpha 6\beta 1$ ^{hi}*CD34*^{hi} cells in cutaneous squamous cell carcinomas,⁵⁴ suggesting that TGF- β plays a tumor suppressive role in this type of tumor. In the present study, tumor-forming ability of pancreatic cancer cells was significantly attenuated by TGF- β (Figure 8D). We postulated that TGF- β negatively regulates the transcription of *ALDH1A1* mRNA through Smad-dependent signaling pathway (Figure 9). However, tumor-forming ability of BxPC-3 cells was inhibited by TGF- β 3 (data not shown). Because BxPC-3 cells do not express Smad4 (Figure 1A), Smad-independent signaling pathway might be also involved in the TGF- β -mediated tumor suppressive role. Numerous reports have also found that the TGF- β family plays an opposite role in cancer progression. In several types of cancer, induction of epithelial-mesenchymal transition generates cells with stem cell properties.^{55–57} In glioma cells, TGF- β maintains the self-renewal

activity and tumorigenicity of glioma-initiating cells through the leukemia inhibitory factor–induced JAK/STAT pathway and SOX4-SOX2 pathway.^{58,59} Overall, it appears that the roles of TGF- β family members are context dependent. It is possible that the bidirectional functions of TGF- β family members are determined by many factors,⁶⁰ including types of cancer, types of molecular markers, and the types of isoforms of TGF- β family used in each study.

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Supplemental Data

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