In vivo disruption of TGF-β signaling by Smad7 leads to premalignant ductal lesions in the pancreas

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Pancreatic cancer represents the fifth leading cause of cancer death in the United States, with a median survival of 4–6 months. According to a latest estimate, there were 31,860 new cases of pancreatic cancer and 31,270 deaths from this disease in 2004 (1). Pancreatic cancer has the worst prognosis of all gastrointestinal cancers, with 5-yr survival rates <5% (2), largely due to a lack of specificity in clinical presentations in the early stage of cancer development and the resistance to conventional cancer therapy. Therefore, it is of paramount importance to develop model systems with early lesions of pancreatic cancers to facilitate the diagnosis and therapy for this deadly disease.

Various genetic changes have contributed to the development of pancreatic cancers. Activation of the Kras proto-oncogene has been found in >90% of pancreatic cancers (3–5). On the other hand, inactivation of various tumor-suppressor genes such as p16INK4a and p53 has been identified in most invasive pancreatic cancers (6). In addition, >50% of pancreatic cancers bear homozygous deletions or inactivating mutations of Sma- and Mad-related protein (Smad) 2 (7). Because Smad4 plays a central role in the tumorigenesis of pancreatic cancers (6), TGF-β signaling plays a critical role in the tumorigenesis of pancreatic cancers (6).

TGF-β superfamily members control cellular growth, differentiation, and apoptosis, as well as embryonic development (8, 9). TGF-β family members regulate gene expression via serine/threonine kinase receptors at the cell surface (10) and a group of intracellular transducers called Smad proteins (11). According to their functional and structural features, Smads are classified as receptor-specific Smads (R-Smads), a common-Smad (Co-Smad or Smad4), and inhibitory Smads (I-Smads) (11–13). TGF-β signaling starts by binding of the ligand with the type II receptor, followed by recruitment of the type I receptor. The activated type I receptor phosphorylates the R-Smads, including Smad2 and Smad3, which then form a heteromeric complex with the Co-Smad, Smad4. The R-Smad/Smad4 complex is translocated into the nucleus where it regulates the transcription of target genes. Smad7 is a member of the I-Smads that is able to antagonize TGF-β signaling by direct interaction with the type I receptor (14).

To provide in vivo evidence that TGF-β signaling is implicated in the development of pancreatic cancer, we generated and analyzed a transgenic mouse model with specific disruption of TGF-β signaling. This study showed that this myc-tag did not affect the inhibitory activity of Smad7 on TGF-β and activin signaling (17).

The plasmid construct was linearized and used in microinjection to generate the transgenic mice that were identified by both Southern blot analysis and PCR genotyping (Fig. 1 B and C). From a total of 78 offspring in the C57 background given by the pseudopregnant foster mothers, 5 founders were identified to carry the transgenes. The mice positive for the transgene were crossed with mice of DBA2 strain to generate transgenic mice used in this study. All mice positive for the transgene showed no signs of health problems up to 10 months of age as compared with the WT littermates.

Smad7 Transgene Was Expressed only in the Pancreas. Of the five founders that carried the transgene, only one of them was found to have relatively high expression of the myc-tagged Smad7 by Western blotting analysis with the pancreas isolated from the offspring of the founders (data not shown). All of the phenotypic and histological analyses were based on the offspring from this founder. At 6 months of age, pancreata were isolated from the transgenic pancreata of the offspring.

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Abbreviations: PanIN, pancreatic intraepithelial neoplasia; Smad, Sma- and Mad-related protein; PCNA, proliferating cell nuclear antigen.

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mice as well as their WT littermates. At that age, we found that the pancreas from most transgenic mice was significantly enlarged in comparison with the WT animals (25 of 29 transgenic mice as compared with 7 WT animals). As shown in Fig. 2A, the pancreas from the WT mouse was ~1.5 cm in length. However, the pancreas from the transgenic animal was ~2.5 cm in length. This observation was in contrast to those gathered at 2 months of age, when the size of the pancreas from the transgenic mice was indistinguishable from that of the WT (data not shown). To confirm that the expression of the Smad7 gene driven by the elastase I promoter/enhancer is limited to the pancreas, we performed Western Blotting analysis using an anti-myc antibody with tissues isolated from one representative transgenic animal at 6 weeks of age. As shown in Fig. 2B, the myc-tagged Smad7 was found only in the pancreas, but not in other tissues including the liver, lung, spleen, kidney, and many other tissues. We next used immunofluorescent staining with an anti-myc antibody to analyze the localization of the exogenously expressed Smad7. As shown in Fig. 2C, no fluorescence signal could be detected in the pancreas from the WT animal. However, Smad7 expression could be easily detected in pancreatic ducts (Fig. 2B), some acinar cells (Fig. 2C), and a few islets (data not shown) in the pancreas from the transgenic mouse. These data, therefore, indicate that Smad7 had a specific expression in the pancreas under the control of the elastase I promoter/enhancer.

**Overexpression Smad7 in the Pancreas Disrupts TGF-β Signaling in Vivo.** Smad7 is an inhibitory Smad protein that blocks TGF-β signaling through interaction with the TGF-β type I receptor. One of the major signaling events after TGF-β receptor activation is Smad2 phosphorylation (18, 19). We hypothesized that the overexpression of Smad7 in the transgenic animal would attenuate TGF-β signaling through a reduced Smad2 phosphorylation. To address this issue, we performed Western blotting analysis using protein lysates extracted from either transgenic or WT mice. An antibody specific for the phosphorylated Smad2 was used to determine the phosphorylation status of Smad2, and an anti-Smad2 antibody was used to detect the total Smad2 proteins. As shown in Fig. 3A, we found that Smad2 phosphorylation was reduced in the pancreas isolated from the transgenic mice in comparison with the pancreas from the WT littermate. However, the total amount of Smad2 proteins was not changed in both samples. Furthermore, we used immunofluorescent staining to detect phosphorylation of Smad2 with pancreas sections (Fig. 3B). Phosphorylation of Smad2 was clearly observed in the pancreas of the WT mice. However, the fluorescent signal for phospho-Smad2 was almost absent in the pancreas section from the transgenic mice. In addition, we analyzed the expression of Smad4 in the pancreas of the mice by immunostaining. As shown in Fig. 3C, Smad4 was expressed in the pancreas of both the transgenic and WT animals at a similar level, indicating that the Smad7 transgene didn’t affect the expression of Smad4. Taken together, these data demonstrated that overexpression of Smad7 was able to inactivate TGF-β signaling in the pancreas.

**In Vivo Disruption of TGF-β Signaling in the Pancreas Induces Premalignant Ductal Lesions.** We next analyzed the histological changes of the pancreas associated with the exogenous expression of Smad7. At 2 months of age, the size and histology of the pancreata from the transgenic mice were indistinguishable from those of the WT animals. However, the pancreas from the transgenic mice at 6 months of age had an obvious change with the characteristics of PanIN lesion that is believed to be a precursor to invasive pancreatic cancers (6, 20).

In the pancreas from WT animals, our histological analyses revealed an abundant distribution of acinar tissue with scattered
protruded into the lumen. However, we could rarely observe typical
the ductal epithelial cells were hyperproliferated so that they
creatic ducts in the transgenic mice (Fig. 4A). A spectrum of pseudostratified epithelium, cell tufting, micropap-
[52x65]PanIN lesions in a 6-month-old transgenic mouse.
[52x85]antibody together with Hoechst 33342 staining. The arrowheads indicate
fluorescent staining in pancreas sections from WT or transgenic mice. The
used in Western blotting with an anti-phosphorylated Smad2 antibody. The
Proteins were isolated from pancreata of the WT or transgenic mouse and
pseudostratified architecture. The PanIN-1B lesions were fre-
quently observed in our transgenic mice with exogenous expression
pseudostratified phenotype, characteristics of the PanIN-1A lesions.
The PanIN-2 lesion is characterized by moderate cytological
[52x105]signaling by association with the TGF-
[52x115]/H9252
[52x125]/H20862
[52x157]inhibition of TGF-β signaling by Smad7 in the transgenic mouse. (Fig. 4A).
PanIN lesions are premalignant lesions classified into PanIN-1A, PanIN-1B, PanIN-2, and PanIN-3 based on the
degree of cytological and architectural atypia of pancreatic sections
PanIN-1A and -1B lesions are represented by a transition from
the normal cuboidal epithelium to a columnar
transition from the normal cuboidal epithelium to a columnar
phenotype, characteristics of the PanIN-1A lesions.
The PanIN-2 lesion is characterized by moderate cytological
atypia in the ductal epithelium. We occasionally observed PanIN-2
lesions in the pancreas from the transgenic animals (Fig. 4G). In these
lesions, the hyperproliferative ductal cells budded into the
lumen with moderate atypia. Accompanying the PanIN lesions, an
increased fibrosis could be observed in the regions surrounding the
ductal lesion (Fig. 4B–H). In a few cases, we also observed
lymph-mononuclear cell infiltration adjacent to the PanIN lesion
(Fig. 4H), indicating a local inflammatory reaction induced by the
ductal alteration. Taken together, these observations demonstrated
that inactivation of TGF-β in the pancreas is able to lead to
hyperproliferation of the ductal epithelium, with accelerated fibro-
sis around the ductal lesion.

In addition to PanIN lesions, ductal stasis and dilation were
observed in most pancreas sections (Fig. 4B and E), suggesting
that pancreatic ducts were either functionally impaired or par-
tially blocked. Patchy hyperplasia and degeneration could be
observed among the acinar cells (Fig. 4I), consistent with the
scattered expression pattern of the Smad7 transgene in the
acinar cells (Fig. 2C).

Epithelial Features of the PanIN Lesions in the Transgenic Mouse. To
define the PanIN lesions in the transgenic mice, we applied
Alcian blue staining and immunohistochemistry analysis. Alcian
blue is one of the most widely used cationic dyes to detect
glycosaminoglycans that are abundantly synthesized by the pancre-
atic ductal epithelium. We observed that Alcian blue stain was
positive in the ductal epithelium overlying the pancreatic ducts in
both WT and transgenic mice (Fig. 5A and B). An intense Alcian
blue staining was observed in PanIN lesions in the transgenic mice,
indicating the epithelial nature of PanINs in the transgenic mice. To
further confirm this finding, we performed immunohistochemistry
studies to detect expression of cytokeratin-19 (CK-19), a specific
epithelial cell marker for pancreatic ducts. As expected, intense
CK-19 signals were detected in normal pancreatic ducts as well as in
PanIN lesions (Fig. 5C and D). Taken together, these data
[52x309]Fig. 3. Disruption of TGF-β signaling by Smad7 in the transgenic mouse. (A) Proteins were isolated from pancreata of the WT or transgenic mouse and
used in Western blotting with an anti-phosphorylated Smad2 antibody. The
same blot was also analyzed by an anti-total-Smad2 antibody. Note the
reduction of Smad2 phosphorylation in the pancreas from the transgenic
animal. (B) The anti-phosphorylated Smad2 antibody was used in immuno-
fluorescent staining in pancreas sections from WT or transgenic mice. The
cells were stained with Hoechst 33342. (C) Immunostaining with Smad4
antibody together with Hoechst 33342 staining. The arrowheads indicate
PanIN lesions in a 6-month-old transgenic mouse.

Discussion

In this report, we demonstrated that disruption of TGF-β
signaling is thought to be a major tumor
suppressing pathway due to its anti-proliferative activity (8, 9). We
hypothesized that inactivation of TGF-β signaling by Smad7 in the
pancreas would promote cell proliferation. We assessed this issue by
detecting proliferating cell nuclear antigen (PCNA) expression. As
shown in Fig. 5E, the basal level of PCNA expression was low in the
pancreas of WT animals, and the strong PCNA-positive ductal cells
were seldom observed. A few of the PCNA-positive acinar cell were
scattered through the exocrine portion of the pancreas (Fig. 5E). In
the transgenic mice, the ductal cells with PanIN lesions were found
to express high levels of PCNA (Fig. 5F). The PCNA expression
level in acinar cells in the transgenic mice was elevated as well.
Therefore, it seemed that overexpression of Smad7 in the pancreas
can induce cellular escape from TGF-β-mediated antiproliferative
activities.

Disruption of TGF-β Signaling Promotes Proliferation of Ductal and
Acinar Cells. TGF-β signaling is known to be a major tumor
suppressing pathway due to its anti-proliferative activity (8, 9). We
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Therefore, it seemed that overexpression of Smad7 in the pancreas
can induce cellular escape from TGF-β-mediated antiproliferative
activities.

Discussion

In this report, we demonstrated that disruption of TGF-β
signaling is able to induce PanIN formation in the mouse.
Through a rat elastase I promoter/enhancer, we targeted pan-
creas-specific expression of Smad7, an inhibitory Smad that
antagonizes TGF-β signaling by association with the TGF-β type
receptor (14). Our immunoblotting analysis confirmed that the
exogenous Smad7 was expressed only in the pancreas, but not in
other tissues of the animal. The inhibition of TGF-β signaling
was confirmed by a reduction of Smad2 phosphorylation in the
found that the exogenous Smad7 was expressed in ductal epithelium. Therefore, overexpression of DNR under the MT1 promoter is mainly limited to acinar expression in the pancreas (23, 24). Therefore, overexpression of DNR under the MT1 promoter is mainly associated with lesions of acinar cells, but not of ductal epithelium. Our study used the −205/+8-bp region of rat elastase I promoter/enhancer. In our immunohistochemistry studies, we found that the exogenous Smad7 was expressed in ductal epithelium and acinar cells (Fig. 2C) as well as islet (data not shown). This finding is consistent with our observation that Smad7 overexpression in the transgenic mouse is associated with PanIN lesions in the ductal epithelium accompanied by an enhanced proliferation of the acinar cells. In addition, we observed premalignant lesions in the pancreas only in 6-month-old transgenic mice, but not in 2-month-old animals. Therefore, it is likely that other genetic alterations accumulated over time are needed for the formation of these premalignant changes.

The cellular origin of ductal pancreatic carcinoma has been a controversial issue (25, 26). Studies by Grippo et al. (27) using the elastase promoter driving Kras expression in the pancreas demonstrated acinar-to-ductal metaplasia in aged mice, but no ductal lesions in young animals. However, our studies with Smad7 transgene revealed clear PanIN lesions, without obvious acinar dysplasia surrounding the ductal changes, indicating that the premalignant changes of the ductal epithelium are not likely derived from acinar cells. However, it is possible that the premalignant ductal cells are originated from islet cells or multipotent precursor cells due to transdifferentiation. This possibility is supported by our observation that the PanIN lesions are frequently neighbored by islets (data not shown). In addition, this issue can be addressed in the future by analysis using lineage-specific markers such as Pdx-1.

It is noteworthy that Smad7 is also able to block activin signaling by interacting with the activin type I receptor (17). Activin ligands and receptors are expressed in the developing pancreas (28), and activin shares a similar antiproliferative effect as TGF-β in most cell types (29). Overexpression of Smad7 in the transgenic mouse, therefore, may also antagonize the activin function in the pancreas. Expression of dominant negative activin type II receptors in the pancreas was associated with islet hypoplasia and impaired differentiation of both endocrine and exocrine cells, but without PanIN lesions (30, 31). In our study, overexpression of Smad7 in the pancreas is able to induce characteristic PanIN lesions distinct from
The phenotypes of transgenic mice that express either dominant negative TGF-β receptors or dominant negative activin receptors. At present, we could not rule out the possibility that our observation with Smad7 overexpression might be caused by a combined antagonization of both TGF-β and activin signaling in the pancreas. To a certain extent, Smad7 overexpression in our model is equivalent to deletion of Smad4 in human pancreatic carcinomas in which the loss of Smad4 is expected to abrogate the signaling pathways of both TGF-β and activin. In addition, it is noteworthy that Smad7 may exert its functions independent of Smad2/Smad3 activation upon TGF-β signaling, such as activation of p38 mitogen-activated protein kinase (MAPK) via TGF-β-activated kinase 1 (TAK1) and MAPK kinase 3 (MKK3) (32). Interestingly, activin is also reported to activate p38 MAPK to stimulate expression of pro-endocrine gene neurogenin 3 in pancreatic endocrine cells (33). Therefore, it needs to be determined in the future whether deregulation of p38 MAPK by Smad7 overexpression is implicated in the formation of PanIN lesions in the pancreas.

In humans, PanIN has been considered a direct noninvasive neoplastic precursor to pancreatic cancers (6). A stepwise molecular progression model for the carcinogenesis in the pancreas has been postulated based on the stages of PanIN and the associated genetic mutations from human studies (6, 34). The early event in PanIN is always associated with mutations of Kras (3–5) and telomere shortening (35). The loss of p16INK4a commonly occurs at the intermediate stage. Mutations of p53 and Smad4 have been considered as late events that occur in PanINs with increasing severity (6, 34, 36). The involvement of these mutations in the development of pancreatic cancers has been extensively investigated in mouse models (25). Consistent with the molecular progression model of pancreatic cancer formation, only activating Kras mutation has been shown to be able to directly induce full spectrum PanIN lesions and low-frequency progression to invasive and metastatic adenocarcinoma (37), although the tumorigenic effect of the Kras mutation in pancreatic cancer development is variable depending on the targeting promoters used in the mouse models (27, 38). Inactivation of p16INK4a alone was not able to produce any neoplastic lesion in the pancreas. However, the activated Kras mutation is able to cooperate with p16INK4a deficiency to induce progressive and metastatic pancreatic carcinoma, indicating a role of Kras in tumor initiation and p16INK4a in tumor promotion (39). The tumor-promoting activity of p16INK4a is further supported by the finding that c-myc expression in the pancreas can induce cancer formation only in the presence of p16INK4a deficiency (40). In addition, the tumor promotion function of p53 was evidenced by the finding that p53 deficiency is able to accelerate tumor development in the TGF-α transgenic mouse (41).

The in vivo function of Smad4 in pancreatic cancer formation using mouse models is limited by the findings that homozygous Smad4-deficient mice die before day 7.5 of embryogenesis (42, 43). Heterozygous Smad4-deficient mice, however, fail to yield any form of pancreatic pathology (42, 43). Our findings that inhibition of TGF-β signaling by Smad7 is able to induce PanIN lesions in the pancreas would argue for a unique function of TGF-β in the early initiation of pancreatic cancer, in addition to its tumor promotion activity postulated from human mutation studies (6, 34, 36). In other words, our results would put TGF-β into the same category as Kras mutations that are believed to major initiating events during carcinogenesis in the pancreas. This proposed tumor initiation activity of TGF-β can be tested, for example, by crossing the Smad7 transgenic mouse with the p16INK4a-deficient mouse to determine whether or not TGF-β disruption can cooperate with loss of p16INK4a to recapitulate the full spectrum of pancreatic cancer progression.

In summary, our studies demonstrated that disruption of TGF-β is able to induce early PanIN lesions in the mouse pancreas. This animal model would greatly aid in understanding the tumor initiation and/or tumor promotion function of TGF-β blockade, a long sought culprit in pancreatic cancer formation due to the extensive mutations of Smad4 and other TGF-β-signaling components in pancreatic cancers. Because this animal model is associated with early pancreatic premalignant lesions, it would serve as a unique tool to facilitate mechanistic studies of early stage pancreatic cancers as well as designs of strategies for early detection and early therapy for the preinvasive lesions of pancreatic cancers.

Materials and Methods
Generation of Transgenic Mice. The transgene was constructed by standard recombinant DNA techniques by fusing a rat elastase I promoter/enhancer fragment (~205/8 bp) with a myc-tagged rat Smad7 cDNA, as shown in Fig. 1. The elastase I enhancer/promoter plasmid was a gift kindly provided by R. Macdonald and G. Swift (Texas Southwestern Medical Center, Dallas). It has been found that this region of the elastase I gene is able to direct specific gene expression in both the endocrine and exocrine pancreas (15, 16). The linearized transgene was used in microinjection into fertilized mouse eggs at the Indiana University Transgenic/Knockout facility according to National Institutes of Health (NIH) animal guidelines. All transgenic mice were generated with the C57 strain and maintained with the DBA2 strain.

Characterization of Transgenic Mice. Genomic DNA was extracted from a 2-mm tail biopsy with a Genomic DNA Extraction Kit (Promega). Mice were genotyped by both Southern blotting analysis and PCR. For Southern blotting, the genomic DNA were digested with BamHI, separated on agarose gel, and transferred to the Hybond N membrane (Amersham). The membranes were subjected to hybridization with a 32P-labeled DNA probe specific
for the transgene, followed by autoradiography. Samples yielding a 3-kb hybridized band were considered positive for the transgene. For PCR genotyping, 100 ng of aliquot of the genomic DNA was used in a PCR mixture containing the following primer pairs: 5'-CCTCTTCTCCTCCATGTCACCCG-3' and 5'-CCGCCGAGGAGGCGACATCGTT-3'. The positive samples yielded a 620-bp product.

**Tissue Harvest, Protein Extraction, and Western Blot Analysis.** Animals were euthanized with a lethal dose of CO2 per institutional guidelines. Pancreata and other tissues/organisms were removed and immediately washed with cold 1× PBS, homogenized, and solubilized in cold RIPA buffer (50 mM Tris-HCl pH 7.4/1.4% Nonidet P-40/0.25% sodium deoxycholate/150 mM NaCl/1 mM EDTA) containing complete proteinase inhibitor mixture and β-glycero-phosphate (Sigma). Proteins from the various tissues were resolved by SDS-PAGE, and transferred to poly(vinylidene difluoride) (PVDF) membranes (Millipore). The myc-tagged Smad7, actin, phosphorylated Smad2, and total Smad2 were detected by Western blotting by using mouse anti-myc antibody (Roche Applied Science, Indianapolis), rabbit anti-actin antibody (Santa Cruz Biotechnology), rabbit anti-phosphorylated-Smad2 antibody (Cell Signaling Technology), and mouse anti-Smad2 antibody (Santa Cruz Biotechnology), respectively.

**Histological Analysis.** Mouse pancreas specimens were fixed in 4% paraformaldehyde solution in PBS overnight at 4°C and embedded in cold RIPA buffer (50 mM Tris-HCl pH 7.4/1.4% Nonidet P-40/0.25% sodium deoxycholate/150 mM NaCl/1 mM EDTA) containing complete proteinase inhibitor mixture and β-glycero-phosphate (Sigma). Proteins from the various tissues were resolved by SDS-PAGE, and transferred to poly(vinylidene difluoride) (PVDF) membranes (Millipore). The myc-tagged Smad7, actin, phosphorylated Smad2, and total Smad2 were detected by Western blotting by using mouse anti-myc antibody (Roche Applied Science, Indianapolis), rabbit anti-actin antibody (Santa Cruz Biotechnology), rabbit anti-phosphorylated-Smad2 antibody (Cell Signaling Technology), and mouse anti-Smad2 antibody (Santa Cruz Biotechnology), respectively.

**Immunofluorescence Staining and Immunohistochemistry.** Mouse pancreas specimens were fixed in 4% paraformaldehyde solution in PBS overnight at 4°C and embedded in paraffin by using standard techniques. The paraffin-embedded organs were removed and fixed in 4% paraformaldehyde solution in PBS overnight at 4°C and embedded in paraffin by using standard techniques. The paraffin-embedded sections (5 μm thickness) were air-dried and stained with Hoechst 33342. For paraffin-embedded sections, immunohistochemistry was done on 5-μm tissue sections by using the Histostain-Plus kit according to the manufacturer’s instructions (Invitrogen). Before staining, antigen retrieval procedures were performed by incubation with 0.5% pepsin (Sigma) in 5 mM HCl for 20 min at 37°C. The primary antibodies used were as follows: mouse anti-myc antibody (1:200; Roche Applied Science), rabbit anti-phosphorylated-Smad2 antibody (1:1,000; Cell Signaling Technology), mouse anti-PCNA antibody (1:3,000; Sigma), mouse anti-cytokeratin-19 (TROMA III) antibody developed by Dr. Rolf Kiemler was obtained from the Developmental Studies Hybridoma Bank under the auspices of the National Institute of Child Health and Human Development and maintained by The University of Iowa, Department of Biological Sciences. This work was supported by research grants from the American Cancer Society (P00-273-01-MO), the National Institute of Diabetes and Digestive and Kidney Diseases (RO1 DK55991), the Chinese Academy of Sciences (Bairen Plan), the National Natural Science Foundation of China (30470870 to Y.C.).

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