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Protection of cerulein-induced pancreatic fibrosis by pancreas-specific expression of Smad7

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ABSTRACT

Pancreatic fibrosis is the hallmark of chronic pancreatitis, currently an incurable disease. Pancreatitis fibrosis is caused by deposition of extracellular matrix (ECM) and the underlying pathological mechanism remains unclear. In addition to its broad biological activities, TGF-β is a potent pro-fibrotic factor and many *in vitro* studies using cell systems have implicated a functional role of TGF-β in the pathogenesis of pancreatic fibrosis. We analyzed the *in vivo* role of TGF-β pathway in pancreatic fibrosis in this study. Smad7, an intracellular inhibitory protein that antagonizes TGF-β signaling, was specifically expressed in the pancreas using a transgenic mouse model. Chronic pancreatitis was induced in the mouse with repeated administration of cerulein. Smad7 expression in the pancreas was able to significantly inhibit cerulein-induced pancreatic fibrosis. Consistently, the protein levels of collagen I and fibronectin were decreased in the Smad7 transgenic mice. In addition, α-smooth muscle actin, a marker of activated pancreas stellate cells, was reduced in the transgenic mice. Taken together, these data indicate that inhibition of TGF-β signaling by Smad7 is able to protect cerulein-induced pancreatic fibrosis *in vivo*.

1. Introduction

Transforming growth factor beta (TGF-β) is a multipotent cytokine that plays important roles in the regulation of cell growth, cell differentiation, apoptosis, immune response, and cancinogenesis [1,2]. In addition, TGF-β has been shown to be a potent pro-fibrotic factor that is implicated in the pathogenesis of fibrosis in the liver, pancreas, lung, and other organs [3–5]. TGF-β ligand initiates its downstream signaling by binding to type I and type II receptors, followed by activation of downstream Smad proteins. Three classes of Smad proteins are involved in the signal transduction and regulation of TGF-β family receptors: receptor-regulated Smad (R-Smad), Co-mediator Smad (Co-Smad or Smad4), and inhibitory Smad (I-Smad) [6]. R-Smads are phosphorylated by the serine/theronine kinase domain of type I receptor. Activated R-Smad and Smad4 form a protein complex that is translocated into the nucleus and regulates the transcription of target genes. Smad7 belongs to the I-Smad subfamily which negatively regulates TGF-β signaling by competing with R-Smads for receptor binding [7].

The mechanism of chronic pancreatitis is obscure and the disease cannot be cured at present. Chronic pancreatitis is characterized by the gradual loss of exocrine and endocrine cells with the development of fibrosis. The degradative process is thought to be caused by the injury of epithelium and the resulting recruitment of inflammatory cells [8]. Along with the disappearance of exocrine compartment, mainly acinar cells, the increasingly formed extracellular matrix (ECM) takes over the place, leading to fibrosis [9].

The mechanism of fibrosis is poorly understood. Recent evidence suggests that TGF-β promotes pancreatic fibrogenesis. TGF-β activates pancreas stellate cells (PSCs), which are thought to be the culprit of fibrosis [10,11], in both autocrine and paracrine manners [9,12]. The activated PSCs secret ECM such as type I collagen, fibronectin, laminin, and MMPs [13–15]. It has been shown that α-smooth muscle actin (α-SMA) is a marker of PSCs. Transgenic mice overexpressing TGF-β in islet cells developed exocrine pancreas fibrosis [16]. Consistently, TGF-β expression was observed in acinar cells adjacent to fibrotic areas in human chronic pancreatitis tissues [10].

Recent studies have revealed that inhibition of TGF-β signaling results in improvement of fibrosis. Inhibition of TGF-β by a neutralizing antibody reduced ECM production in cerulein-induced pancreatitis in rats [17]. Overexpression of a dominant-negative mutant form of TGF-β type II receptor also reduced pancreatic fibrosis [18,19]. Taken together, these studies have revealed that TGF-β contributes to fibrogenesis in pancreas and inhibition of TGF-β signaling has a beneficial role to improve pancreatic fibrogenesis. However, whether or not Smad7 is implicated in pancreatic fibrogenesis has not been investigated. We hypothesize that Smad7 could decrease pancreatic

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fibrosis by interrupting TGF-β signaling. We analyzed this hypothesis using a transgenic mouse with pancreas-specific expression of Smad7. As described in this study, we found that overexpression of Smad7 is able to ameliorate pancreatic fibrosis induced by chronic administration of cerulein.

2. Materials and method

2.1. Animals

The Smad7 transgenic mice were generated as previously described [20]. All transgenic mice were maintained by crossing with C57BL/6J strain mice.

2.2. Identification of transgenic mice

Genomic DNA was extracted from a 2-mm tail biopsy: The tissues were incubated with 50 mmol/L NaOH at 100 °C for 1 h, neutralized with 1 M Tris–HCl (pH 8.0). The samples were centrifuged and the supernatant was used in PCR genotyping with primers 5′-CTTTGTACTTTCATGTCACCTGTGC-3′ and 5′-CGCCGGACGAGCGCA-GATCGTT-3′.

2.3. Tissue harvest, protein extraction, and Western blotting analysis

Pancreas and other tissues were removed and washed with cold PBS, homogenized, and solubilized in cold RIPA buffer (50 mM Tris–HCl at pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, and 1 mM EDTA) containing protease inhibitor cocktail (Sigma-Aldrich, Saint Louis, MO, USA). Proteins from various tissues were resolved by SDS/PAGE, and transferred to PVDF membranes (Millipore, Billerica, MA, USA). The Myc-tagged Smad7 were detected by Western blotting using a mouse anti-Myc antibody (Santa Cruz, Santa Cruz, CA, USA).

2.4. Mouse experimental protocol

Female and male mice of 2-month-old were treated with cerulein to induce chronic pancreatitis as previously described [21]. Briefly, 50 μg/kg cerulein (BACHEM California Inc., Torrance, CA, USA) was injected intraperitoneally every hour for 5 consecutive times. Such injection was repeated for 3 days per week up to a total of 8 weeks. Saline was injected as control. Mice were sacrificed four days after the final cerulein treatment. The pancreas was swiftly removed and fixed in Bouin's solution (75% picric acid saturated aqueous solution, 25% of 40% formaldehyde, and 5% glacial acetic acid).

2.5. Histological analysis

Mouse pancreas specimens were fixed in Bouin's solution overnight at room temperature and embedded in paraffin using standard techniques. The paraffin-embedded sections (5 μm) were stained with hematoxylin/eosin (H&E) to determine the histopathological features.

2.6. Immunohistochemistry and quantitative analysis

The immunohistochemistry was done on 5 μm sections using SABC (mouse/rabbit IgG) kit according to the manufacturer's instructions (Boster, Wuhan, Hubei, China). The primary antibodies used were as follows: rabbit anti-collagen I antibody (1:500, Abcam, Cambridge, MA, USA), rabbit anti-α-SMA antibody (1:1000, Abcam), Mouse anti-fibronectin antibody (1:500, Abcam). Densitometric analysis of α-SMA protein expression was performed manually. In brief, three fields per α-SMA-stained pancreatic sections (5 sections for each experimental group) were randomly selected at 200× magnification by an examiner blinded to the treatment groups. The α-SMA positive cells were examined to determine the relative level of α-SMA staining. Fibronectin staining was used to analyze fibrosis area. Four non-overlapping fields per fibronectin-stained pancreatic sections (at least 3 sections for each experimental group) were randomly selected at 200× magnification by an investigator who was unaware of the sample identity. The fibrosis area was calculated by the image processing software ImageJ (developed by NIH and downloaded from http://rsbweb.nih.gov/ij/). Briefly, the images were converted to HSB stack with saturation set at 155 as MinThreshold and 255 as MaxThreshold to segment the fibronectin-stained area. The percentage of area within the threshold in the whole image was measured as the fibrosis area.

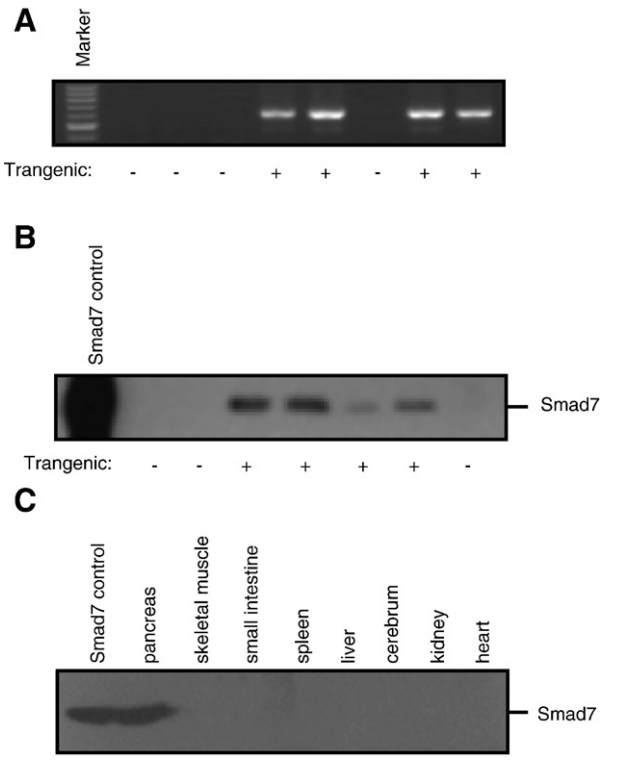

Fig. 1. Characterization of Smad7 transgenic mice. (A) Representative data showing the result of genomic PCR with mouse tails. The samples positive for Smad7 transgene are marked in the bottom. The DNA molecular size marker is also shown in the picture. (B) Western blotting analysis with pancreas of the mice. The samples with detectable expression of the Myc-tagged Smad7 are labeled in the bottom. The Smad7 control was from cell lysate with overexpression of a Myc-tagged Smad7. (C) Pancreas-specific expression of exogenous Smad7 in the transgenic mouse. Different tissues were isolated from a Smad7 transgenic mouse as indicated and protein preparation from these tissues was used in Western blotting with an anti-Myc antibody.

3. Results and discussion

In order to determine whether or not Smad7 plays a role in pancreatic fibrosis, we generated a transgenic mouse model with pancreatic expression of Smad7 using the strategy as previously described by us [20]. The plasmid used for the transgenic mouse contains a −205/+8 bp rat elastase I gene promoter/enhancer, a Smad7 cDNA with a Myc-tag at its N terminus, and an SV40 poly(A) tail [20]. The plasmid was linearized and microinjected into the zygote with C57BJ/6L (female) and CBA (male) background. From 155 offspring, we found that 24 mice carried the Smad7 transgene and only two of them expressed the exogenous *Smad7* gene. These two founders were crossed with mice of C57BJ/6L background to generate Smad7 transgenic mice for the study. The transgenic mice were identified by genomic PCR (Fig. 1A) to determine the presence of the Smad7

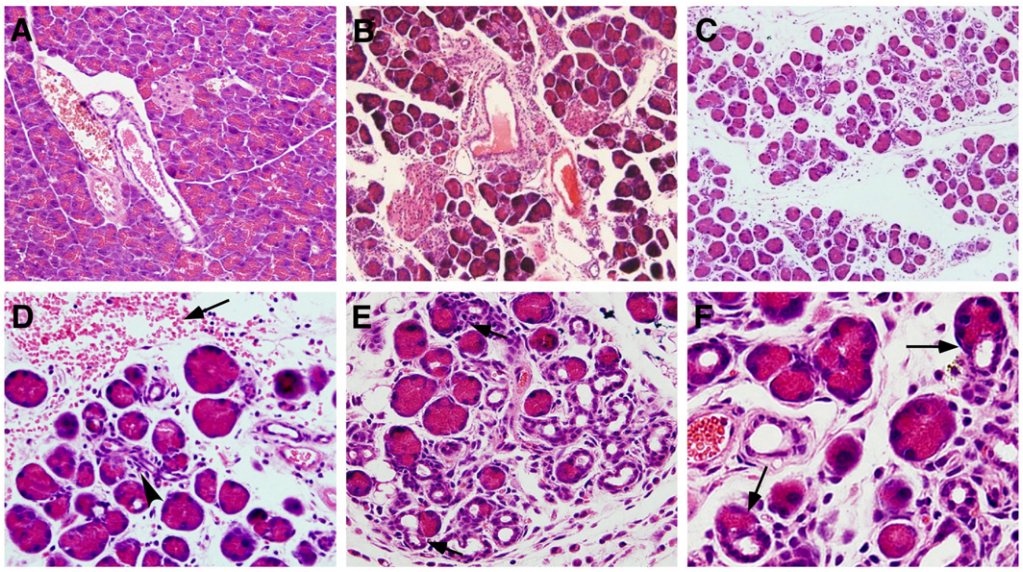

Fig. 2. Pathological changes in pancreas after repetitive injections of cerulean. Wild type mice were treated with saline (A) or cerulein for 4 weeks (B), or cerulein for 8 weeks (C to F). Note that pancreatic fibrosis occurred after 4 weeks' cerulein treatment, and was aggravated after 8 weeks' cerulein administration. The characteristics of chronic pancreatitis were apparent, including atrophy of acinar cells (B and C), hemorrhage (D, arrow) and inflammatory cell infiltration (E, arrowhead), and acinar-to-ductal metaplasia (E and F, arrows). Magnification: ×100 for A, B and C, ×200 for D, E, and ×400 for F.

transgene, and Western blotting to determine the expression of exogenous Smad7 with a Myc tag (Fig. 1B). Consistent with previous report [20], the exogenous Smad7 was specifically expressed in the pancreas, but not in other tissues (Fig. 1C).

We induced chronic pancreatitis by repeated induction of acute pancreatitis as previously reported [19,21]. Considering that premalignant ductal lesions occurs the Smad7 transgenic mice 6 months [20], we chose 2-month-old mice for induction of chronic pancreatitis. Injection of cerulein for 4 weeks provoked loss of acinar cells and ECM deposition in wild type mice (Fig. 2). Pancreatic fibrosis occurred after another 4 weeks' injection with the hallmarks of chronic pancreatitis (Fig. 2C), accompanied by hemorrhage and inflammatory cell infiltration (Fig. 2D). We also observed acinar-to-ductal metaplasia (Fig. 2E and F), another important pathological change in which acinar cells lose their characteristic zymogen granules and acquire the features of duct cells. Taken together, these data suggest that repeated administration of cerulein was able to induce formation of chronic pancreatitis in the mouse.

We next determined whether exogenous expression Smad7 is able to affect chronic pancreatitis induced by cerulein. We compared the pathological changes of pancreas in 6 wild type mice and 23 Smad7 transgenic mice. The representative results are shown in Fig. 3. We found that exogenous expression of Smad7 had a protective role in the development of chronic pancreatitis. The loss of acinar cells in Smad7 transgenic mice was much less than the wild type animals. Moreover, some areas in Smad7 transgenic mice were intact without acinar atrophy. As collagen I is the main component of fibrosis, we used immunohistochemistry to determine the expression level of collagen I in these mice. As shown in Fig. 4A, the staining of collagen I was reduced in Smad7 transgenic mice in comparison with the wild type

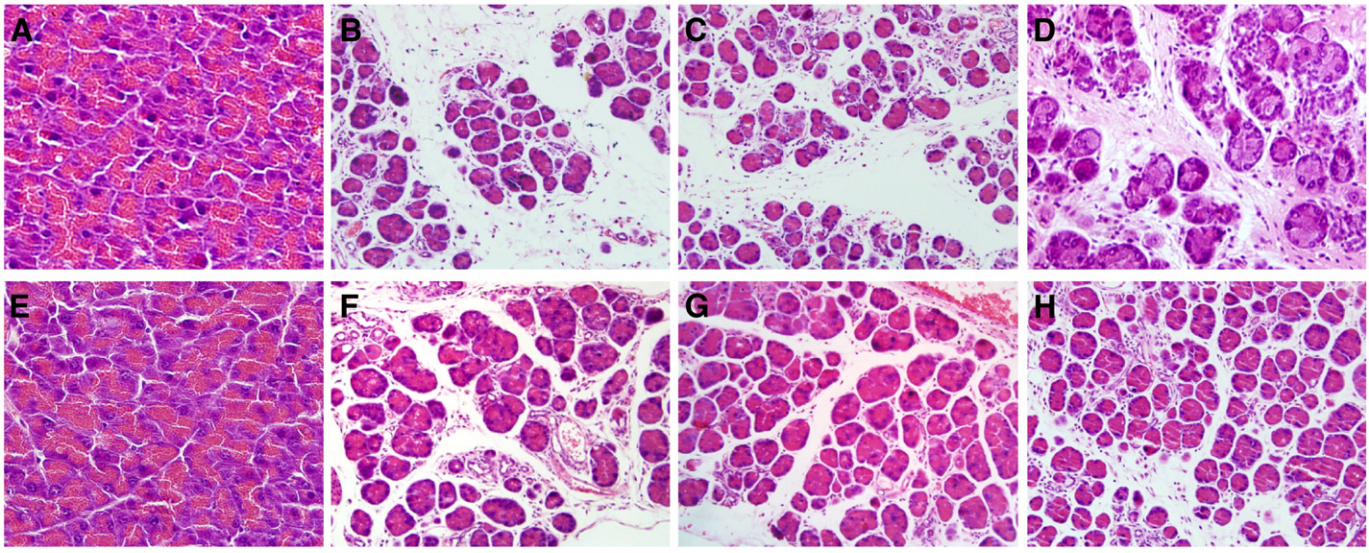

Fig. 3. Cerulein-induced chronic pancreatitis was ameliorated by exogenous expression of Smad7 in the pancreas. Wild type (A to D) and Smad7 transgenic mice (E to H) were treated with saline (A and E) or cerulein (C to E, F to H) for 8 weeks. Note that the overall pathological changes of chronic pancreatitis were markedly reduced in the Smad7 transgenic mice. Each picture represents the result from different mouse. Magnification: ×100.

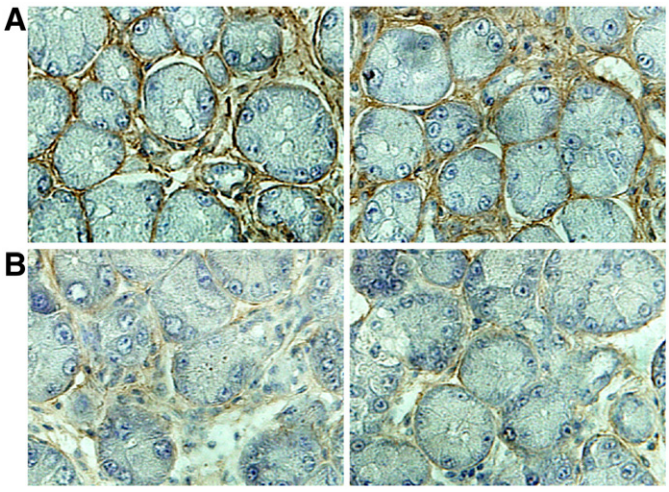

Fig. 4. Decreases of collagen I expression by Smad7 in the pancreas. Pancreatic sections from the wild type (A) and the Smad7 transgenic mice (B) treated with cerulein were used in immunohistochemistry (IHC) to investigate the expression levels of collagen I. Note that the level of collagen I was profoundly reduced in the Smad7 transgenic mice in comparison with the wild type animals. Representative pictures from different mice are shown. Magnification: ×400.

animals, suggesting that the deposition of ECM was markedly reduced by overexpression of Smad7 in the pancreas. In addition, we analyzed the expression level of fibronectin, another major ECM component induced by TGF-β. As shown in Fig. 5, fibronectin was significantly

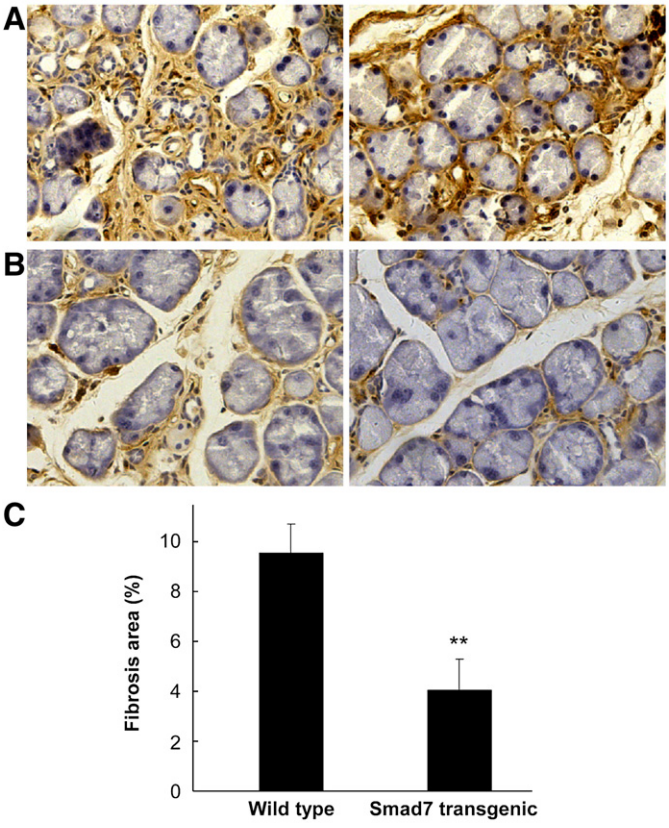

Fig. 5. Decreased fibronectin expression in Smad7 transgenic mice. Pancreatic sections from the wild type (A) and the Smad7 transgenic mice (B) treated with cerulein were used in IHC to examine the expression level of fibronectin, a marker of fibrosis. Representative pictures from different mice are shown. Magnification: ×200. Fibrosis area was measured by ImageJ's Analyze and Measure tool from NIH and is shown in C. ** indicated $p<0.01$ by Student's t-test.

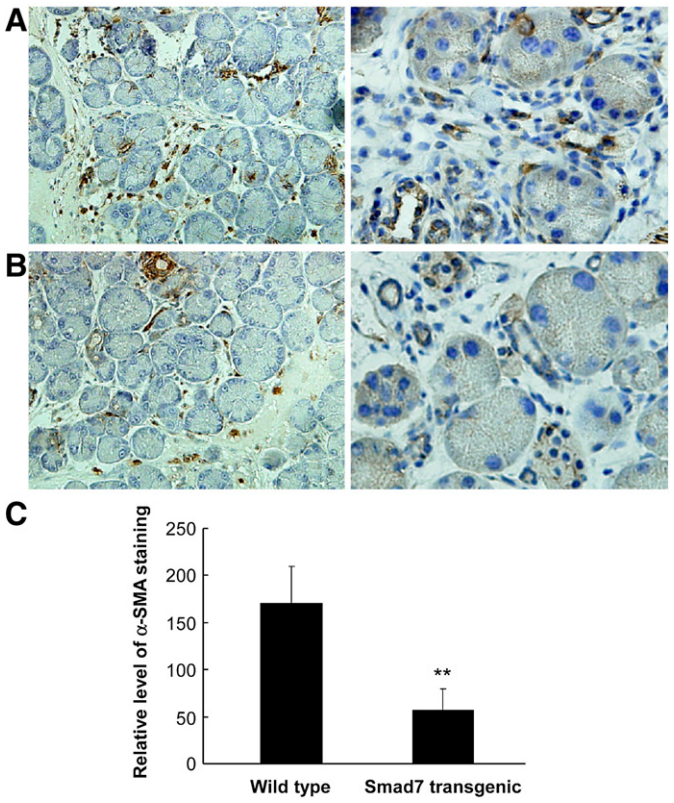

Fig. 6. Decreased PSC activation in Smad7 transgenic mice. Pancreatic sections from the wild type (A) and the Smad7 transgenic mice (B) treated with cerulein were used in IHC to examine the level of α-SMA, a marker of PSC activation. Representative pictures from different mice are shown. Magnification: ×200 for the left panels and ×400 for the panels. The relative level α-SMA staining as determined by densitometric analysis is shown in C. ** indicated $p<0.01$ by Student's t-test.

reduced in the pancreas when Smad7 was overexpressed. Therefore, these data provided additional evidence that Smad7 is able to inhibit ECM deposition in the pancreas.

We also analyzed PSCs in these mice, as PSC is the source of pancreatic fibrosis and TGF-β can cause ECM secretion by activating PSCs [11,12]. We examined the expression of α-smooth muscle actin (α-SMA), a marker of activated PSC in the pancreas after cerulein injections. Our data reveals that activated PSCs were greatly reduced in the Smad7 transgenic mice (Fig. 6). Thus the exogenous Smad7 appears to inhibit PSC activation through inhibition of TGF-β signaling and thereby reduce the extent of pancreatic fibrosis.

The molecular mechanism of chronic pancreatitis is not well understood. Partly because of this, there is currently no cure to pancreatitis except for pancreatectomy with limited success. Recent studies have indicated that TGF-β signaling is closely implicated in the development of fibrosis in chronic pancreatitis. Tissue trauma in chronic pancreatitis may stir inflammatory response which in turns summons inflammatory cells into the pancreas. The inflammatory cells secret inflammatory mediators including TGF-β, which activate PSCs to secrete ECM. If the inflammation in the pancreas persists, the increased ECM deposition would occupy the enlarged interlobular space, accompanied by atrophy and loss of acinar cells. Several groups have found that inhibition of TGF-β signaling leads to decrease of activated PSCs and pancreatic fibrosis [17–19]. As the major function of TGF-β is to activate PSCs [4], we postulate that the decreased staining of α-SMA by Smad7 overexpression (Fig. 6) is mainly due to a decrease of PSC activation, instead of reducing the number of PSCs. In addition, TGF-β has been shown to enhance deposition of ECM, a major mechanism underlying the fibrosis-promoting activity of TGF-β. We believe that the major effect of Smad7 in reducing pancreatic

fibrosis is via inhibiting TGF-β-induced ECM deposition, rather than accelerating the degradation of deposited ECM. Interestingly, Lee et al recently showed that the RECK, a novel membrane-anchored MMP inhibitor, can be maintained by TGF-β [22], indicating that TGF-β signaling may promote ECM accumulation by preserving the protease inhibitory activity of RECK. This is consistent with the finding that Smad7 was able to reverse the RECK-maintaining activity of TGF-β [22]. Nevertheless, our study provides another piece of evidence that inhibition of intracellular TGF-β signaling by Smad7 also reduces fibrosis and activation of PSCs in cerulein-induced chronic pancreatitis. Therefore, increasing Smad7 expression in the pancreas could potentially serve as new strategy to treat fibrosis in chronic pancreatitis.

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