Published in final edited form as:

J Mol Med (Berl). 2015 October ; 93(10): 1085–1093. doi:10.1007/s00109-015-1308-9.

Gremlin is a Key Pro-fibrogenic Factor in Chronic Pancreatitis

Dustin Staloch^{#1}, Xuxia Gao^{#1,2}, Ka Liu^{#1}, Meihua Xu^{1,3}, Xueping Feng^{1,3}, Judith F. Aronson⁴, Miriam Falzon⁵, George H. Greeley⁶, Cristiana Rastellini⁶, Celia Chao⁶, Mark R. Hellmich⁶, Yanna Cao^{1,6}, and Tien C. Ko^{1,6}

¹Department of Surgery, The University of Texas Health Science Center-Houston, Texas

²Department of General Practice, Beijing Anzhen Hospital, Capital Medical University, Beijing, China

³Institute of Medical Sciences, Xiangya Hospital, Central South University, Changsha, Hunan, China

⁴Department of Pathology, University of Texas Medical Branch, Galveston, Texas

⁵Department of Pharmacology, University of Texas Medical Branch, Galveston, Texas

⁶Department of Surgery, University of Texas Medical Branch, Galveston, Texas

[#] These authors contributed equally to this work.

Abstract

The current study aims to identify the pro-fibrogenic role of Gremlin, an endogenous antagonist of bone morphogenetic proteins (BMPs) in chronic pancreatitis (CP). CP is a highly debilitating disease characterized by progressive pancreatic inflammation and fibrosis that ultimately leads to exocrine and endocrine dysfunction. While transforming growth factor (TGF)- β is a known key pro-fibrogenic factor in CP, the TGF- β superfamily members BMPs exert an anti-fibrogenic function in CP reported by our group recently. To investigate how BMP signaling is regulated in CP by BMP antagonists, the mouse CP model induced by cerulein was used. During CP induction, *TGF- β 1* mRNA increased 156-fold in two weeks, a BMP antagonist *Gremlin 1* (*Grem1*) mRNA levels increased 145-fold at three weeks, and increases in *Grem1* protein levels correlated with increases in collagen deposition. Increased *Grem1* was also observed in human CP pancreata compared to normal. *Grem1* knockout in *Grem1*^{+/-} mice revealed a 33.2% reduction in pancreatic fibrosis in CP compared to wild-type littermates. *In vitro* in isolated pancreatic stellate cells, TGF- β induced *Grem1* expression. Addition of the recombinant mouse *Grem1* protein blocked BMP2-

Send correspondence to: Tien C. Ko, MD, Department of Surgery, The University of Texas Health Science Center at Houston, 5656 Kelly, 30S62008, Houston, TX 77026, Phone: (713) 566-5098; FAX: (713) 566-4583, Tien.C.Ko@uth.tmc.edu; Yanna Cao, MD, MSc, Department of Surgery, The University of Texas Health Science Center at Houston, 6431 Fannin Street, MSB4.608, Houston, TX 77030, Phone: (713) 500-7224; FAX: (713) 500-7268, Yanna.Cao@uth.tmc.edu..

AUTHOR CONTRIBUTIONS

Dustin Staloch, Xuxia Gao, Ka Liu: Experimental design, data generation and interpretation, manuscript draft. Meihua Xu, Xueping Feng: Data generation. Judith F. Aronson, Miriam Falzon, George H. Greeley Jr., Cristiana Rastellini, Celia Chao, Mark R. Hellmich: Experimental design and data interpretation. Yanna Cao: Experimental design, data generation and interpretation, manuscript draft and submission. Tien C. Ko: Experimental design, data interpretation, manuscript draft and approval.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

induced Smad1/5 phosphorylation and abolished BMP2's suppression effects on TGF- β -induced collagen expression. Evidences presented herein demonstrate that Grem1, induced by TGF- β , is pro-fibrogenic by antagonizing BMP activity in CP.

Keywords

Bone morphogenetic protein antagonists; Gremlin; pancreatic fibrosis; cerulein; pancreas stellate cells

INTRODUCTION

Pancreatitis is an extremely painful disease with severe complications. It is a major contributor to the economic burden of gastrointestinal diseases in the United States, with estimated costs in 2004 at \$3.7 billion [1]. Following an initial acute pancreatitis admission, 16% of patients progress to chronic pancreatitis (CP); this number increases to 38% following a second admission [2]. As fibrosis progresses, organ dysfunction ensues as acinar and islet cells are lost. CP is also a major risk factor for pancreatic cancer, a diagnosis with survival rates less than 7%. Pancreatic cancer is currently the 4th leading cause of cancer deaths in the United States and is projected to surpass colorectal cancers to become the 2nd leading cause of cancer deaths by 2030 [3]. However, currently there are no effective therapies to stop or reverse CP progression [4].

During CP progression, pancreatic stellate cells (PSCs) are activated by pro-fibrogenic cytokines and differentiate into myofibroblast-like cells elaborating excessive extracellular matrix (ECM) deposition and leading to fibrosis [5]. As a key pro-fibrogenic cytokine in CP, transforming growth factor (TGF)- β binds to the transmembrane receptors on PSCs, subsequently phosphorylates Smad2/3, and leads to PSC activation and ECM production. In contrast, bone morphogenetic proteins (BMPs), a major subgroup of the TGF- β superfamily, are anti-fibrogenic in the lungs, kidneys, and liver [6-9]. They act by binding cell surface receptors and phosphorylating Smad1/5/8. Recently, we reported that in the pancreas, BMP signaling constitutes an opposing mechanism to TGF- β 's pro-fibrogenic function; BMP signaling is impaired in CP, which is associated with enhanced TGF- β signaling, resulting in disease progression [10, 11]. However, the mechanisms that regulate BMP signaling in CP are unknown. Gremlin (specifically Grem1), an endogenous BMP antagonist, is the product of *Grem1*, a highly conserved member of the DAN/cerebus family glycoprotein important in development [12]. Its expression is increased in fibrotic diseases of the lungs [7], kidneys [13], and liver [14] as well as various malignancies [15]. *In vitro*, TGF- β stimulates Gremlin expression in bronchial and kidney epithelial cells [7, 16]. Knockdown of *Grem1* *in vivo* by siRNA inhibits ECM accumulation in a mouse model of diabetic nephropathy [17]. Depletion of *Grem1* expression protects *Grem1*^{+/-} mice from kidney fibrosis [18]. These findings suggest that Grem1 is required for the development of organ fibrosis.

In the current study, we found that *Grem1* was elevated following an elevation of TGF- β 1 in CP. We hypothesize that TGF- β -induced Grem1 blocks BMP signaling and function, which composes a novel mechanism for CP progression. This study thus aimed to test if Grem1 expression in the pancreas promotes pancreatic fibrosis during CP progression. We found

that *Grem1* knockout in *Grem1*^{+/-} mice attenuated pancreatic fibrosis in CP compared to wild-type littermates. *In vitro* in isolated PSCs, TGF- β induced *Grem1* expression, and *Grem1* blocked BMP signaling and anti-fibrogenic function. Our data indicate that *Grem1* is pro-fibrogenic by antagonizing BMP activity in CP. Thus strategies to block *Grem1* may represent innovative therapies for CP.

MATERIALS AND METHODS

Reagents

Cerulein, a cholecystokinin analog and secretagogue, was obtained from Bachem Americas, Inc. (Torrance, CA). Direct Red 80 and picric acid for Sirius red staining was purchased from Sigma-Aldrich Corporate (St. Louis, MO). Recombinant human TGF- β 1 and BMP2, and mouse *Grem1* proteins were obtained from R&D Systems, Inc. (Minneapolis, MN), and diluted in a vehicle solution (0.1% BSA, 4 mM HCl). The antibody against *Grem1* for immunohistochemistry and immunofluorescence was from R&D Systems (Catalog number AF956), and the antibody against *Grem1* for Western blotting was from Abgent, Inc. (San Diego, CA). Phospho(p)Smad1/5 and Smad1/5 were from Cell Signaling Technology, Inc. (Billerica, MA), collagen type I, alpha 1 (Col1a1) was from Abcam (Cambridge, MA), and GAPDH was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). HRP conjugated secondary antibodies were from Bio-Rad Laboratories (Hercules, CA).

Animals and *in vivo* CP model

Swiss Webster mice were purchased from Harlan Laboratories, Inc. (Indianapolis, IN), and B6.129P2-*Grem1*^{tm1/Rmh}/J mice (*Grem1* heterozygous knockout, *Grem1*^{+/-}) were obtained through Jackson laboratory (Bar Harbor, Maine, USA). Colonies of *Grem1*^{+/-} were maintained through crossing male *Grem1*^{+/-} mice and female wild-type C57BL/6J mice (Jackson Laboratory); offspring were genotyped by PCR [12]. Male and female mice were utilized for experiments at the age of 8-10 weeks. All animal experiments were performed in accordance with Animal Welfare Committees of The University of Texas Health Science Center at Houston and the University of Texas Medical Branch at Galveston.

Mice were randomized into either a CP or control group. CP was induced by repetitive intraperitoneal injections of cerulein (50 μ g/kg, 5 hourly injections/day, 3 days/week) for up to 8 weeks as previously reported [10, 11]. Control mice were given saline injections of the same volume and frequency. At day 3-4 following completion of cerulein or saline injections, the mice were euthanized and the pancreata were harvested for analysis.

Quantitative PCR (qPCR)

Total RNA was extracted from pancreatic tissue samples of the mice or cells, and reversely transcribed to cDNA using RETROscript kit (Life Technology Co., Grand Island, NY). qPCR was performed using TaqMan gene expression master mix and specific gene probe sets as previously described [19]. The probe sets of mouse *TGF- β 1* (Mm01178820_m1), *Grem1* (Mm00488615_s1), *Noggin* (Mm01297833_s1), *Chordin1* (Mm00473158_m1), *Col1a1* (Mm00801666_g1), and *18s* (Hs99999901_s1) (Life Technology Co., Grand Island,

NY) were used in the study. The specific signals acquired were normalized to the signals acquired from *I8s*.

Human pancreas samples

Following the guidelines of the University of Texas Medical Branch at Galveston, the human pancreata were obtained from cadavers and from discarded pancreatic tissue from surgical resection.

Immunohistochemistry analysis

Pancreas sections (5 μm -thick) were prepared for immunohistochemical analysis (IHC) of Grem1 with ABC and DAB kits from Vector Laboratories, Inc. (Burlingame, CA, USA) according to the manufacturer's instructions and as previously described [10, 11]. Following staining, 5-8 random, non-overlapping fields per section were captured for analysis. Two-three investigators blinded to sample identity evaluated the staining images according to the criteria previously published for human samples [20-22] or for mouse pancreas samples [10, 11].

Quantitative analysis of pancreatic fibrosis

Sirius red staining was performed on pancreas sections for analysis of collagen deposition [23]. A minimum of 15-20 non-overlapping fields were captured from each section. Sirius red-stained area was quantified with NIS-Elements Br 3.0 software. Stained areas were calculated as a percentage of total tissue area analyzed and expressed as fold of control [10].

Western blotting

Western blotting was conducted as previously described [19]. Protein lysates were prepared from mouse pancreas and cells in 1x lysis buffer (Cell Signaling Technology, Billerica, MA) and protein concentration was measured with a protein assay dye (Bio-Rad Laboratories, Hercules, CA). Blots were probed with indicated primary antibodies, bound primary antibodies were detected with horseradish peroxidase-conjugated secondary antibodies, and signals were visualized with enhanced chemiluminescent reagents (Thermo Scientific).

Isolation and culture of PSCs

Mouse primary PSCs (mPSCs) were isolated from mouse pancreata (8-10 weeks old) using an outgrowth method [11, 24]. Isolated mPSCs were pooled from 3-4 mice. The PSCs were seeded and cultured at 37°C in a humidified incubator (containing 95% O₂ and 5% CO₂). PSCs were grown to 60-80% confluence, starved for 18-20 h in medium supplemented with 0.1% serum before respective treatments.

Immunofluorescence

The PSCs were treated, alone or in combination, with respective proteins, or vehicle. After 48 h, PSCs were fixed with 4% paraformaldehyde for 15 min. Immunofluorescence (IF) was performed using specific antibodies as previously described [11].

Statistical analysis

Data are expressed as mean $\pm$ standard error (SEM). The P value < 0.05 is considered significant. *In vitro* experiments were repeated 2-3 times and similar results were obtained. Differences between two groups were analyzed using Student's *t*-test. Differences among multiple groups were analyzed using ANOVA with Holm-Sidak *post hoc* test.

RESULTS

***Grem1* mRNA expression increases along with increased *TGF- β 1* expression in CP**

As *TGF- β* is reported to influence expression of BMP antagonists in other tissues, investigation began in a mouse model of CP with a survey of pancreatic *TGF- β 1* and BMP antagonist mRNA expression (Fig. 1). *TGF- β 1* mRNA levels were dramatically elevated during CP induction at 1 week (136-fold), 2 weeks (156-fold), and 3 weeks (35-fold), decreased following the recovery period. Increased *TGF- β 1* mRNA levels were associated with increased expression of *Grem1*, *Noggin*, and *Chordin1* mRNAs following CP induction. *Grem1* expression demonstrated the greatest induction of the investigated BMP antagonists, peaking at 145-fold following 3 weeks of treatment. While expression of *Noggin* and *Chordin1* peaked before *Grem1* expression. The predominant elevation of *Grem1* over *Noggin* and *Chordin1* suggests that *Grem1* may be the major BMP antagonist responsible for blocking BMP signaling during the development of CP.

Elevated *Grem1* protein levels correlate with fibrosis in cerulein-induced CP mouse model

To investigate the temporal relationship of *Grem1* expression in CP and progression of fibrosis, cerulein injections in the mouse model were performed through 8 weeks. Saline injections were given in control mice. Sirius red staining was conducted to quantify collagen deposition (Fig. 2) along the time course for correlation with *Grem1* elaboration by IHC (Fig. 3). Collagen area was unchanged in control mice but trended upward in CP mice throughout the time course, with significant increases versus time-matched control beginning at 4 weeks treatment (Fig. 2). *Grem1* expression was increased significantly in CP mice after 1 week of treatment, peaked at 4 weeks, and remained elevated through 8 weeks; expression in the control group remained minimal throughout (Fig. 3A, 3B). Western blotting of *Grem1* supports the IHC findings, demonstrating that *Grem1* protein levels increased in CP mice at 2 and 4 weeks, compared to the controls (Fig. 3C, 3D). These data demonstrate that *Grem1* protein expression is upregulated in the CP mouse model during the development of fibrosis.

***Grem1* protein expression increases in human CP**

It has been previously reported that Gremlin expression is upregulated in fibrotic disease of the lungs, kidneys, and liver [7, 13, 14, 16]. We, therefore, set about correlating our mouse model findings in the pancreas with human disease via investigation of human CP pancreata. Analysis of immunohistochemistry showed that *Grem1* expression was low in normal pancreata but significantly upregulated in the pancreata of CP patients (Fig. 4). Furthermore, pSmad1/5 IHC with the same set of human samples showed a moderate reduction of pSmad1/5 in CP versus the normal pancreas ($P>0.05$). These indicate that *Grem1* is

upregulated in human CP, which corroborates mouse model findings and validates the study as clinically relevant in investigating Grem1 in pancreatic fibrosis in CP.

Grem1 haplodeficiency attenuates cerulein-induced fibrosis

Numerous factors may be involved in maintenance of tissue architecture in response to pancreatic injury during CP induction. To identify the Grem1-dependent portion of pancreatic fibrosis in CP *in vivo*, a mutant mouse line with heterozygous *Grem1* knockout (*Grem1*^{+/-}) underwent CP induction as homozygous *Grem1* knockout is lethal and thus not available. Adult *Grem1*^{+/-} mice were phenotypically normal compared to wild-type (WT) littermates prior to CP induction. Sirius red staining showed no difference in collagen deposition between saline control *Grem1*^{+/-} and WT; following CP induction for 4 weeks, however, collagen deposition in *Grem1*^{+/-} mice was reduced by 33.2% compared to WT (Fig. 5). These indicate a pro-fibrogenic role of Grem1 in the pancreas.

TGF- β induces Grem1 in mPSCs

We have demonstrated that the peak elevation of *TGF- β 1* during CP induction *in vivo* precedes *Grem1* (Fig. 1), allowing for induction of Grem1 expression by TGF- β . To examine whether TGF- β has a direct effect on Grem1 expression in PSCs *in vitro*, mPSCs were isolated and treated with TGF- β 1 (1 ng/ml) for either 24 h for evaluation of mRNA levels by qPCR or 48 h for measurement of protein levels by Western blotting and IF. Treatment with TGF- β 1 increased *Grem1* mRNA levels by 1.8-fold (Fig. 6A) and Grem1 protein levels by 2.3-fold in Western blotting (Fig. 6B). The blots were probed with the Grem1 antibody with and without pre-incubation of the recombinant Grem1 protein. Optical densities of the reported Grem1 protein band at the size around 25 kD reduced to 52% in the PSC samples and to 45% in the recombinant Grem1 protein when probed with the Grem1 antibody pre-incubated with the recombinant protein, compared to the Grem1 antibody alone (Fig. 6C), demonstrating a partial blocking of the signals in the presence of the recombinant protein. These findings confirmed Grem1 protein size around 25 kD as reported. Treatment with TGF- β 1 induced Grem1 expression in mouse PSCs (Fig. 6D). Thus our data indicate a direct induction of Grem1 by TGF- β in mPSCs.

Grem1 blocks the anti-fibrogenic effects of BMP2 in mPSCs

We reported recently that BMP signaling is anti-fibrogenic in the pancreas during CP induction [10, 11]. In this study, we attempted to test our hypothesis that elevated Grem1 expression in CP imposes antagonism of the anti-fibrogenic BMP signaling, thereby enhancing pancreatic fibrosis. First, the dose effects of Grem1 on BMP2-induced Smad1/5 signaling were evaluated by Western blotting (Fig. 7A). BMP2 induced a 16.2-fold increase of pSmad1/5. This effect was inhibited 62% by the recombinant Grem1 at 50 ng/ml and abolished at 500 ng/ml. Thus Grem1 dose of 500 ng/ml was used in later experiments. Western blotting was then conducted to evaluate Smad1/5 signaling following treatment of mPSCs with TGF- β 1, BMP2, and/or Grem1. Treatment with TGF- β 1 did not significantly induce pSmad1/5 levels or affected BMP2-induced pSmad1/5. Grem1 blocked BMP2-induced pSmad1/5 (Fig. 7B). To investigate effects of BMP signaling inhibition on fibrosis, mRNA and protein expression of Col1a1 were conducted following similar treatments as a

functional readout of fibrosis. TGF- β increased *Colla1* mRNA expression by 2.0-fold, and BMP2 blocked TGF- β -induced *Colla1* mRNA expression. Addition of Grem1 restored *Colla1* mRNA expression otherwise attenuated by BMP2, with *Colla1* mRNA expression similar to TGF- β treatment alone (Fig. 7C). The same pattern was also observed on *Colla1* protein expression by IF (Fig. 7D). Together, these findings demonstrate a pro-fibrogenic effect of Grem1 in the pancreas through antagonism of BMP signaling.

DISCUSSION

Chronic pancreatitis stems from a variety of initial insults to the exocrine pancreas, after which counterbalancing signaling normally serves to maintain pancreatic function and structure tip excessively toward a pro-inflammatory and pro-fibrogenic milieu. Via human and genetic knockout animal studies *in vivo* and *in vitro*, our findings demonstrate that Grem1 is upregulated in the diseased pancreas and promotes fibrosis by blocking anti-fibrogenic BMP signaling and function.

Among BMP antagonists, Gremlin has been reported to play a pro-fibrogenic role in the kidneys, lungs and liver [7, 13, 14, 16]. This is indeed the case in our study that the levels of *Grem1* are predominantly elevated and sustained in CP in comparison with other BMP antagonists, *Noggin* and *Chordin1* examined (Fig. 1). The evidences of Grem1's pro-fibrogenic function provided in our study through human CP and an animal CP model further our knowledge of the pathogenic role of Grem1 in fibrotic diseases.

Concentrations of BMP2 and Grem1 utilized in current study were chosen based on the TGF- β concentration (1 ng/ml) we have reported as a stimulant of fibrosis *in vitro* [11]. Accordingly, the BMP2 concentration used is an optimal concentration to abolish TGF- β 's effects on ECM production. The dose of the recombinant Grem1 at 500 ng/ml used in this study was necessary to abolish BMP2-induced Smad1/5 phosphorylation and BMP's anti-fibrogenic effects. This Grem1 concentration is also relevant to natural levels of Gremlin as reported in human serum as 508 ng/ml and in human plasma as 349 ng/ml [25, 26]. Furthermore, Grem1 at 500 ng/ml did not induce phosphorylation of VEGFR2 in mPSCs (data not shown). However, off-target effects of Grem1 via other receptors cannot be excluded.

Others have postulated mechanisms by which Gremlin drives fibrosis in other organ systems, identifying a central role for Gremlin in hypoxia-induced pulmonary hypertension by antagonism of anti-fibrogenic BMP signaling [27] and in chronic kidney disease [13]. We have also established through a BMPR2 knockout study that BMP signaling is important in protecting against pancreatic fibrosis [10]. Furthermore, our current studies demonstrate a pro-fibrogenic role for Grem1 in driving pancreatic fibrosis via blockade of BMP signaling and function. Our studies indicate a connection between pro-fibrogenic TGF- β signaling and anti-fibrogenic BMP via Grem1 in pancreatic fibrosis.

Understanding of Gremlin's role in physiology and diseases is evolving, and it is increasingly implicated not only in ECM regulation but also in vascular homeostasis. It is of growing interest in tumor biology, not only for its upregulation in numerous carcinomas and

promotion of cancer cell survival and expansion [28–30], but also for its novel role as a vascular endothelial growth factor type 2 receptor (VEGFR2) agonist [31]. What role of VEGFR2-mediated effects may have played in our study is unknown, and study will seek to further this understanding. As evidence for Gremlin's role in chronic fibrotic processes and tumor biology continues to grow more robust, our study suggests a potential link between pancreatitis and pancreatic cancer via Grem1. Thus investigation of Grem1-targeting therapies potentially serves both development for CP therapy and prevention of pancreatic cancer.

In summary, evidence reported here demonstrates Grem1 expression in the pancreas is dynamic and induced in the diseased state. It promotes fibrosis by blocking BMP signaling to dampen anti-fibrogenic signal transduction. Grem1 may represent a novel target for stemming fibrotic disease progression in human subjects. Given the normally low expression of Grem1 in the pancreas, future work may seek to specifically block Grem1 synthesis and activity and/or enhance BMP signaling through pharmaceutical therapies. If such therapies are developed, they may be applicable as early interventions to prevent CP progression and its sequelae.

ACKNOWLEDGMENTS

The authors thank E. Figueroa, S. Schuenke, and K. Martin in the Department of Surgery, University of Texas Medical Branch for manuscript and figure preparation and submission.

GRANTS

This study was supported by a grant from the National Institutes of Health (P01 DK35608).

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Figure 1. CP increases expression of pancreatic TGF- β 1 and BMP antagonists

Pancreata were harvested from C57BL/6 mice following 1, 2, or 3 weeks CP induction with cerulein or after 5 weeks recovery following 3 weeks CP induction (3w+5w recovery). Total RNAs were extracted and converted to cDNAs. mRNA expression levels of *TGF- β 1*, *Grem1*, *Noggin*, and *Chordin1* were measured by qPCR. The specific signals were normalized against *18S* rRNA and expressed as fold of respective 0 week expression levels (mean $\pm$ SEM). n=5-9/group. * P <0.05 compared with respective 0 week using one-way ANOVA and multiple comparison test (Dunn's method).

Figure 2. Collagen deposition increases in mouse pancreata in a time-dependent manner following CP induction

Pancreata were harvested from Swiss Webster mice following up to 8-week CP induction with cerulein or saline control (CON). **A.** Images of Sirius red staining for collagen deposition. Original magnification= 400X. **B.** Quantification. Data are expressed as fold of CON, and presented as mean $\pm$ SEM. n=4-6 mice/group. * P <0.05 compared to time-matched controls using Student's t -test.

Figure 3. Grem1 expression increases in mouse pancreata in a time-dependent manner following CP induction

Pancreata were harvested from Swiss Webster mice following up to 8-week CP induction with cerulein or saline control (CON). **A.** Images of immunohistochemistry using a goat anti-Grem1 antibody. IHC CON: Goat IgG isotype control. **B.** Quantification of Grem1 IHC. **C.** Images of Western blots. Pos: recombinant mouse Grem1 protein as a positive control. **D.** Quantification of Western blots. Grem1 levels are normalized against GAPDH, expressed as fold of CON, and presented as mean $\pm$ SEM. n=4-6 mice/group. * P <0.05 compared to time-matched controls using Student's t -test.

Figure 4. Grem1 expression increases in the pancreata of human CP

A. Paraffin sections from normal and CP human pancreatic tissue underwent immunohistochemical staining using a goat anti-Grem1 (arrow) antibody and a rabbit anti-pSmad1/5 (arrow head) antibody. IHC CON1: Goat IgG isotype control. IHC CON2: Rabbit IgG isotype control. **B.** Histoscores of Grem1 and pSmad1/5 were assigned to each of 5 different fields/slide as follows: 0 for no staining; 1+ for <30% cells positively stained; 2+ for 30-60% cells positively stained; and 3+ for >60% cells positively stained. Original magnification= 400X. Mean histoscores±SEM are presented for each group. n=4 normal pancreata; n=7 CP. * $P<0.05$ compared to normal pancreata using Student's *t*-test.

Figure 5. *Grem1* haplodeficiency in mice attenuates cerulein-induced pancreatic fibrosis
Representative images are shown for Sirius red staining of control (CON) and CP pancreatic paraffin sections from Wild-type (WT) and *Grem1*^{+/-} mice following 4-week CP induction with cerulein. Original magnification= 400X. Quantification of collagen area is expressed as a fold of WT control±SEM. n=6 mice/group. *P<0.05 compared to WT control, #P<0.05 compared to WT CP using one-way ANOVA and multiple comparison test (Holm-Sidak method).

Figure 6. TGF-β induces Grem1 in mPSCs

mPSCs were treated with either vehicle (Veh) or TGF-β1 (1 ng/ml) for 24 h for qPCR and for 48 h for IF and Western blotting. **A.** *Grem1* mRNA levels measured by qPCR, expressed as fold of Veh, and presented as mean±SEM from 3 independent sets of treatments. **B.** Images and quantification of Western blots. Grem1 levels are normalized against GAPDH, expressed as fold of Veh, and presented as mean±SEM. **C.** Western blots probed with Grem1 antibody or neutralized Grem1 antibody. +T: mPSCs treated with TGF-β1 for 48 h. rGrem1: recombinant mouse Grem1 protein as a positive control. **D.** Representative merged images of IF using antibodies against Grem1 (green) with DAPI nuclear staining (blue). Quantification of IF is expressed as fold of Veh and presented as mean±SEM. n=5-10 independent fields/slide. Original magnification= 200X. * $P<0.05$ compared to Veh using Student's *t*-test.

Figure 7. Grem1 blocks BMP2 signaling and anti-fibrogenic effects in mPSCs

A. Representative Western blot images and quantification of Grem1 dose curve study. mPSCs were pretreated with Grem1 (0, 50, 500 ng/ml) for 30 min followed by BMP2 treatment (50 ng/ml) for 30 min. **B.** Representative Western blot images and quantification. mPSCs were treated with Veh, TGF-β1 (1 ng/ml), BMP2 (50 ng/ml) for 30 min followed by TGF-β (1 ng/ml); or Grem1 (500 ng/ml) for 30 min followed by BMP2 (50 ng/ml) for 30 min and then TGF-β1 (1 ng/ml) for 30 min. pSmad1/5 levels are normalized against GAPDH and expressed as fold of Veh. **C.** The cells were treated as indicated for 24 h. *Col1a1* mRNA levels were measured by qPCR. **D.** The cells were treated as indicated for 48 h. Col1a1 protein levels were measured by IF. Data are presented as mean±SEM. n=3. **P*<0.05 compared to Veh, #*P*<0.05 compared to BMP2 or TGF-β treatment alone using one-way ANOVA and multiple comparison test (Holm-Sidak method).