Activation of mTORC1 signaling in gastric X/A-like cells induces spontaneous pancreatic fibrosis and derangement of glucose metabolism by reducing ghrelin production

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ABSTRACT

Background: Pancreatic fibrosis is a pathophysiological process associated with excessive deposition of extracellular matrix in pancreas, leading to reduced insulin secretion and derangement of glucose metabolism. X/A-like cells, a group of unique endocrine cells in gastric oxyntic mucosa, produce and secret ghrelin to influence energy balance. Whether gastric X/A-like cells affect pancreatic fibrosis and subsequent glucose homeostasis remains unclear.

Methods: We established a Ghre-cre transgene in which the cre enzyme is expressed in X/A-like cells under the control of ghrelin-promoter. TSC1fl/ox mice were bred with Ghre-cre mice to generate Ghre-TSC1−/− (TG) mice, within which mTORC1 signaling was activated in X/A-like cells. Pancreatic fibrosis and insulin secretion were analyzed in the TG mice.

Findings: Activation of mTORC1 signaling by deletion of TSC1 gene in gastric X/A-like cells induced spontaneous pancreatic fibrosis. This alteration was associated with reduced insulin expression and secretion, as well as impaired glucose metabolism. Activation of mTORC1 signaling in gastric X/A-like cells reduced gastric and circulating ghrelin levels. Exogenous ghrelin reversed pancreatic fibrosis and glucose intolerance induced by activation of mTORC1 signaling in these cells. Rapamycin, an inhibitor of mTOR, reversed the decrease of ghrelin levels and pancreatic fibrosis.

Interpretation: Activation of mTORC1 signaling in gastric X/A-like cells induces spontaneous pancreatic fibrosis and subsequently impairs glucose homeostasis via suppression of ghrelin.

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1. Introduction

Fibrosis is a pathophysiological process characterized by excessive deposition of extracellular matrix (ECM). Extracellular matrix proteins are synthesized in fibroblasts and degraded by metalloproteases. When balance between biosynthesis and degradation is broken, extracellular matrix will deposit in different tissues, such as heart, liver, pancreas, and kidney [1]. The main pathophysiological change shared by fibrosis in different tissues is the activation of fibroblasts. Activation of fibroblasts can be induced by a variety of pathways ranging from sterile inflammation, aberrant stimulation of transforming growth factor-β (TGFβ) signaling, parenchymal injury, to microvascular dysfunction and hypoxia [2]. Uncontrolled fibrosis impairs organ function. For example, mice with pancreatic fibrosis demonstrate impairment in islet mass function characterized by decrease in early-phase insulin secretion.

Pancreatic fibrosis, a characteristic histological feature of chronic pancreatitis, develops as a result of abnormal activation of pancreatic stellate cells (PSCs) and deposition of ECM proteins. Although reversible at its early stages, end-stage of pancreatic fibrosis often demonstrates exocrine and endocrine insufficiency [3–6], leading to the malabsorption and pancreatic diabetes [7,8]. At the cellular levels, activation of PSCs is proposed as the patho-mechanism for fibrogenesis in pancreas. A variety of endogenous and exogenous factors may activate the PSCs. Endogenous molecules include inflammatory cytokines: tumor necrosis factor α (TNFα) and interleukins, as well as growth factors such as transforming growth factor-β1 (TGFβ1) and platelet derived growth factor (PDGF), which are derived locally from damaged acinar cells, neutrophils, macrophages, and PSCs. Ethanol and its metabolites acetaldehyde, as well as lipopolysaccharide are well-recognized exogenous factors. All these factors trigger oxidative stress within PSCs, leading to the increased expression of αSMA, procollagen, and TGFβ1.

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Antioxidants have thus been used to resolve chronic pancreatitis-related symptoms, but recovery of pancreatic tissue damage and function preservation are limited in humans. X/A-like cells are the second most abundant gastric endocrine cell type, accounting for 20–30% of the oxyntic endocrine cells. These cells produce and secrete ghrelin, a 28 amino acid peptide hormone [9,10], to influence a variety of physiological and pathological processes such as growth hormone secretion, food intake, glucose and lipid metabolism [11–14], as well as immunity and inflammation [15,16]. Ghrelin also alters the exocrine and endocrine functions of pancreas with conflicting reports, indicating that X/A-like cells communicate with pancreas via ghrelin to modulate the function of its parenchymal cells [17–23]. Whether X/A-like cells can signal the pancreas to alter the activation of PSCs and the homeostasis of pancreatic microenvironment remains unknown.

Here, we reported that mechanistic target of rapamycin complex 1 (mTORC1) in gastric X/A-like cells is critical for the integrity of pancreatic microenvironment. Deletion of tuberous sclerosis 1 (TSC1) driven by ghrelin promoter activates mTORC1 signaling in X/A-like cells. Activation of mTORC1 in these endocrine cells induces the development of pancreatic fibrosis in mice fed with either normal chow diet (NCD) or high fat diet (HFD). This spontaneous pancreatic fibrosis is associated with impairment of islet mass function and glucose intolerance. Further, activation of mTORC1 signaling in X/A-like cells suppresses ghrelin production. Supplementation use of exogenous ghrelin reverses the pancreatic fibrosis and glucose intolerance induced by activation of mTORC1 signaling in gastric X/A-like cells.

2. Materials and methods

2.1. Main reagents

Rapamycin and DMSO were obtained from Sigma-Aldrich (St Louis, MO, USA). Ghrelin peptide was purchased from Phoenix Pharmaceuticals Inc. (Burlingame, CA). Rabbit anti-phospho-mTOR (ser235/236), rabbit anti-mTOR, rabbit anti-phospho-S6 (ser235/236), rabbit anti-S6, rabbit anti-phospho-AKT (ser473), rabbit anti-AKT, rabbit anti-insulin, rabbit anti-phospho-Smad2 (ser465/467), mouse anti-Smad2, rabbit anti-phospho-Smad3 (ser423/425), mouse anti-GAPDH, and mouse anti-β-actin antibodies were obtained from Cell Signaling Technology (Beverly, MA). Rabbit anti-αSMA and mouse anti-ghrelin antibodies were purchased from Abcam (Cambridge, UK). IRDye-conjugated affinity purified anti-rabbit, anti-mouse IgGs were purchased from Rockland (Gilbertsville, PA). Goat anti-rabbit fluoresceinisothiocyanate-conjugated IgG, goat anti-mouse Texas Red-conjugated IgG, and goat anti-insulin A (C-12) antibody were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Trizol reagent and the reverse transcription (RT) system were from Promega Inc. (Madison, WI).

2.2. Animals and treatment

2.2.1. Animals

Animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 8023, revised 1978). All experimental protocols were approved by the Animal Care and Use Committee of Peking University (Permit Number: LA2012–60). Ghrl-TSC1−/− (TG) mice and wild-type littermates (WT) were generated by crossing homozygous Ghrl-cre transgenes with TSC1^flox/flox mice (Jackson Laboratory, Bar Harbor, ME) [24]. Both Ghrl-cre and TSC1^flox/flox lines are C57BL/6J strain. Female TSC1^flox/flox mice were intercrossed with male Ghrl-cre mice to generate Ghrl-cre;TSC1^flox−/− mice. Male Ghrl-cre;TSC1^flox−/− mice were then backcrossed with female TSC1^flox/flox parents to generate Ghrl-cre;TSC1^flox−/− mice and TSC1^flox/tm, wild-type littermates. Mice were housed in standard plastic rodent cages and maintained in a regulated environment (24 °C, 12-h light and 12-h dark cycle with lights on at 7:00 AM). Regular chow and water were available ad libitum unless specified otherwise.

2.2.2. Diets

Four-week-old male mice were assigned to receive standard normal chow diet (NCD, D12450H; Research Diets) or a high-fat diet (HFD, 60% fat, D12492; Research Diets) for 12 weeks.

2.2.3. Surgery and implantation of osmotic minipumps

Mice were anesthetized with pentobarbital (0.06 g·kg$^{-1}$). Through a 1 cm incision in the back skin, mice were implanted subcutaneously with an Alzet osmotic minipump (model 1002) filled with vehicle or acyl-ghrelin (11 nmol·kg$^{-1}$·d$^{-1}$) for 14 days. Before implantation, pumps were filled with the test agent and placed in a Petri dish with sterile 0.9% saline at 37 °C for at least 4 h before implantation to prime the minipumps.

2.2.4. Administration of rapamycin, glucose, and insulin

Rapamycin (1 mg·kg$^{-1}$·d$^{-1}$) or DMSO was administered by intraperitoneal injection for 14 days. Glucose (3 g·kg$^{-1}$) was administrated by oral gavage, while insulin (1 U·kg$^{-1}$) were intraperitoneally injected. Glucose or insulin tolerance tests were performed as described previously [25].

2.3. Cell culture

Human stellate cells were cultured in F12 high-glucose Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 15% FBS (U.S. Biotechnologies) and 100 units/ml penicillin and 100 units/ml streptomycin (Invitrogen). Cells were passaged weekly after trypsin-EDTA detachment. Cultured cells were treated with ghrelin or rapamycin for 3, 6, 12, 24, and 36 h, then harvested for mRNA or protein extraction.
2.4. Tissue sample preparation and immunofluorescent staining

C57BL/6 J mice were deeply anesthetized using pentobarbital (0.07 g·kg⁻¹). The stomach and pancreas were quickly removed and rinsed thoroughly with PBS, then fixed in 4% paraformaldehyde (wt/vol.), dehydrated, embedded in wax, and sectioned at 6 μm. Paraffin embedded sections were dewaxed, re-hydrated, and rinsed in PBS. After boiling for 10 min in 10 mmol/l sodium citrate buffer (pH 6.0), sections were blocked in 1% BSA (wt/vol.) in PBS for 1 h at room temperature, then incubated overnight with primary antibody. Tissue sections were then incubated at room temperature for 1 h with secondary antibody. Controls included substituting primary antibody with rabbit IgG or mouse IgG. Photomicrographs were taken under a confocal laser-scanning microscope (Leica, Germany).

2.5. Western blot analysis and quantitative RT-PCR

Cultured cells or snap-frozen pancreata from mice of desired genotypes were homogenized in RIPA buffer. Protein extracts were prepared, separated by SDS-PAGE, transferred to PVDF membrane, and immunoblotted as previously described using the antibodies indicated [26]. Total RNA was isolated from cultured cells or pancreatic tissues using Trizol and further purified with an RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Reverse transcription and quantitative PCR were performed as previously described [26]. Primer sequences are provided in the supplementary material, Table S1.

2.6. Statistical analysis

All values are expressed as mean ± SEM. Statistical differences were evaluated by two-way ANOVA and Newman-Student-Keuls test. Comparisons between two groups involved use of the Student t-test. P < 0.05 denotes statistical significance.

3. Results

3.1. Genetic activation of mTORC1 signaling in gastric X/A-like cells and its effect on ghrelin levels

In order to manipulate the gene expression specifically in gastric X/A-like cells, we first generated a transgene: Ghrl-cre. A similar strategy as published before was used to establish Ghrl-cre transgenic mouse colonies in which the cre enzyme is specifically expressed in gastric X/A-like cells driven by the ghrelin promoter [27]. Briefly, a ghrelin BAC construct containing the 59.36 kb sequence upstream the ATG code was used. The first 29-bp of the ghrelin coding sequence was replaced by the coding sequence of iCre gene followed by an SV40 polyadenylation signal (pA). The construct was microinjected into pronuclei of fertilized one-cell stage embryos of mice with pure C57BL/6 J genetic background. First generation pups of distinct founders were screened for transgenic lines with high levels of cre gene. Out of seven transgenic lines, we identified and validated a transgenic line with selective expression of cre gene in the stomach but not in the hypothalamus and pancreas, two other tissues in which ghrelin has been reported to be present (Fig. S1a). Transgenic lines with abundance of cre mRNA in stomach were bred with Gr(\textit{Rosa})26Sor\textit{mice} / mice, which carry the loxp-flanked DNA STOP sequence preventing expression of the downstream \textit{LacZ} gene. In these \textit{ROSA-Ghrl-cre} (RG) mice, Ghrl-cre positive cells express β-galactosidase because the STOP sequence flanking \textit{LacZ} gene is removed by cre enzyme. The efficiency and specificity of cre enzyme were validated by detection of β-galactosidase. No positive signal was shown in the hypothalamus (data not shown). Strong β-galactosidase signal was detected in ghrelin-positive X/A-like cells in gastric mucosa (Fig. S2a), whereas β-galactosidase in pancreatic islet were age-dependent. Sporadic β-galactosidase was detected in neonatal islet, while its expression was absent after 8 weeks (Fig. S2b). There was no difference in body weight (Fig. S3a), food intake (Fig. S3b), tissue weight (Fig. S3c), gastric morphology (Fig. S3d), plasma ghrelin (Fig. S3e), or basal glucose levels (Fig. S3f) between Ghrl-cre mice and wild-type littermates. The validated Ghrl-cre transgenic mice were intercrossed with Tsc1\textsuperscript{-}\textsuperscript{fl/fl} mice (Tsc1\textsuperscript{tm1Djk} from the Jackson Laboratory) to generate Ghrl-TSC1−/− mice in which TSC1 gene, an upstream suppressor of mTORC1, is deleted and mTORC1 signaling subsequently activated. Colonies in which genotyping confirmed the deletion of TSC1 exons 17 and 18 were selected and maintained. Ghrl-TSC1−/− mice (TG, n = 11) demonstrated increased gastric pmTOR and pS6 (Fig. 1a), indicating the activation of mTORC1 signaling. Levels of S6 phosphorylation remained unaltered in adult pancreatic islets (Fig. S4a), indicating that mTORC1 signaling is not activated in the transgene. This alteration was associated with a significant decrease in gastric ghrelin mRNA (Fig. 1b), plasma levels of acyl-ghrelin, and total ghrelin (Fig. 1c) relative to wild-type littermates (WT) (\(P < 0.01, n = 11\)). Average area of ghrelin-positive X/A-like cells in stomach increased significantly (Fig. S5a), whereas the number of these cells remained no change between two groups (Fig. S5b).

3.2. Spontaneous pancreatic fibrosis induced by activation of mTORC1 signaling in gastric X/A-like cells in lean and obese mice

A significant increase in pancreas weight was found in Ghrl-TSC1−/− mice fed with normal chow diet, which was accompanied with decreased protein content in pancreas (Fig. 2a). Gross examination of pancreas revealed a firm morphology and diffusive sclerosis (Fig. 2b) in Ghrl-TSC1−/− mice relative to wild-type littermates. HE staining showed a diffusive increase in fibrotic tissue, a significant decrease in acinar gland and islet size in the transgene (Fig. 2b). The increase in fibrosis was confirmed by Sirius Red and Masson staining (Fig. 2b). Consistently, pancreatic mRNA levels of αSMA, as well as collagen I, III, and IV were markedly increased in Ghrl-TSC1−/− mice (Fig. 2c).

The effect of activation of mTORC1 signaling in gastric X/A-like cells on pancreatic fibrosis was also examined in obese mice. Four-week-old Ghrl-TSC1−/− mice and wild-type littermates were fed with a 60% high fat diet for 12 weeks to induce obesity. Pancreas weight and protein content (Fig. 2d) were significantly reduced in obese Ghrl-TSC1−/− mice relative to wild-type littermates. A greater sclerosis of pancreas was observed in Ghrl-TSC1−/− mice relative to wild-type littermates (Fig. 2e). Compared with lean mice, activation mTORC1 signaling in gastric X/A-like cells induced more severe collagen deposition in pancreas of obese mice as evidenced by H&E, Sirius Red, and Masson staining (Fig. 2e and Fig. S6a). mRNA levels of αSMA and collagen I, III, IV in pancreas were significantly increased in obese Ghrl-TSC1−/− mice relative to wild-type littermates (Fig. 2f and Fig. S6b). These results indicate that activation of mTORC1 signaling in gastric X/A-like cells induces more severe pancreatic fibrosis in obese mice.

3.3. Derangement of glucose metabolism induced by activation of mTORC1 signaling in gastric X/A-like cells

Wild-type mice fed with a 60% high fat diet for 12 weeks demonstrated a significant increase in body weight relative to animals fed with normal chow (Fig. S7a). Ghrl-TSC1−/− mice were resistant to high fat diet-induced obesity (Fig. S7a), with fat mass significantly less than wild-type littermates (Fig. S7b) and lean mass more than wild-type littermates (Fig. S7c). As shown in Fig. S7d and S6, food intake was slightly reduced in Ghrl-TSC1−/− mice. These results suggest that activation of mTORC1 signaling in gastric X/A-like cells inhibits food intake and reduces body weight. To examine its effect on glucose homeostasis, we measured circulating levels of glucose. Surprisingly, basal glucose level in Ghrl-TSC1−/− mice was markedly increased relative
to wild-type littersmates (Fig. 3a). Glucose tolerance test showed that glucose metabolism was significantly impaired in Ghrl-TSC1−/− mice and wild-type littersmates were fed with normal chow diet (NCD) for 16 weeks. Data was presented as mean ± SEM. *P < 0.05 vs. WT. n = 11. a. Validation of mTORC1 activation in X/A-like cells. mTORC1 activity was measured by phosphorylation of mTOR and S6 using immunofluorescent staining. X/A-like cells were identified by ghrelin immunoreactivity. Shown is the colocalization of pmTOR or pS6 (green) with ghrelin (red) in mouse stomach. Nuclei were stained with Hoechst dye (blue). b. mRNA levels of gastric ghrelin analyzed by RT-qPCR. β-actin was used as internal control. c. Plasma levels of acyl-ghrelin and total ghrelin.

3.4. Reduction of insulin expression and secretion in Ghrl-TSC1−/− mice

Glucose levels are mainly influenced by insulin sensitivity in insulin targeted organs and circulating insulin levels secreted from the pancreatic islets. To further explore the mechanism responsible for the derangement of glucose metabolism in Ghrl-TSC1−/− mice, we examined insulin expression and secretion. As shown in Fig. 4a, activation of mTORC1 signaling in gastric X/A-like cells caused a significant decrease in insulin mRNA and protein levels in pancreas. This alteration was associated with a significant reduction in plasma insulin levels under basal condition in Ghrl-TSC1−/− mice relative to wild-type littersmates (Fig. 4b). Glucose stimulated insulin secretion was also reduced in Ghrl-TSC1−/− mice (Fig. 4c). Insulin is synthesized and secreted by islet β-cells. Consistently, a decrease in the number of insulin positive β-cells was observed in Ghrl-TSC1−/− mice relative to wild-type littersmates (Fig. 4d). Averaged islet area was reduced, whereas number of small islets increased (Fig. 4d).
3.5. Alteration of genes related to matrix degradation in 
Ghrl-TSC1−/− mice

Transforming growth factor-β (TGFβ) and inflammation are classi-
cal pathways leading to fibrosis [1]. To further explore the cause of
spontaneous pancreatic fibrosis in 
Ghrl-TSC1−/− mice, we next exam-
ined the expression of TGFβ and inflammatory cytokines in pancreas of
Ghrl-TSC1−/− mice and wild-type littermates fed with NCD for 16
weeks. As shown in Fig. 5a, activation of mTORC1 signaling in gastric
X/A-like cells demonstrated no effect on TGFβ mRNA level or the phos-
phorylation of Smad2 and Smad3, the downstream targets of TGFβ sig-
naling. mRNA levels of inflammatory cytokines remained unaltered in
pancreas of 
Ghrl-TSC1−/− mice (Fig. 5b). On the other hand, RNAseq
analysis revealed a signi-
ficant reduction in degradation of the extracel-
lar matrix and activation of matrix metalloproteinases in the pancreas
of 
Ghrl-TSC1−/− mice (Fig. 5c). This
finding was further confirmed
by quantitative RT-PCR analysis showing that mRNA expression of metal-
loproteinase MMP9 in pancreas was significantly decreased, whereas
TIMP1, a tissue inhibitor of metalloproteinase, increased in 
Ghrl-TSC1−/− mice relative to wild-type littermates (Fig. 5d).

3.6. Ghrelin-mediated effects

Since gastric and circulating ghrelin was significantly reduced in
Ghrl-TSC1−/− mice, we propose that reduction of ghrelin contributes
to the spontaneous pancreatic fibrosis in these animals. To test this con-
cept, exogenous acyl-ghrelin was continuously infused for 2 weeks into
Ghrl-TSC1−/− mice and wild-type littermates fed with a 60% high fat
diet for 12 weeks. As shown in Fig. 6a, infusion of exogenous ghrelin re-
versed the reduction of plasma acyl-ghrelin levels in 
Ghrl-TSC1−/− mice. Consistent with the previous reports showing the orexigenic ef-
fect of ghrelin [9], decreased ghrelin level of 
Ghrl-TSC1−/− mice was as-
sociated with reduced food intake, while administration of exogenous
ghrelin reversed the reduction of food intake (Fig. S8). These observa-
tions indicate that activation of mTORC1 in X/A-like cells reduces food
intake by the direct function of ghrelin. The restoration of ghrelin levels
was associated with a signi-
ficant improvement in pancreatic
fibrosis evidenced by decreased collagen deposition detected by H&E, Sirius Red,
and Masson staining (Fig. 6b), as well as a decreased expression of αSMA, collagen I, III, and IV
mRNAs in pancreas (Fig. 6c). Exogenous ghrelin also increased expression of MMP9 in pancreas of 
Ghrl-TSC1−/− mice (Fig. 6d). Infusion of exogenous ghrelin significantly attenuated the re-
duction of plasma insulin levels in 
Ghrl-TSC1−/− mice (Fig. 6e), leading
to the improvement of glucose metabolism (Fig. 6f). To further confirm
the effect of ghrelin on pancreatic fibrosis, we treated human pancreatic
stellate cells with acyl-ghrelin (10−8 mol/l) or saline for 6 h or time in-
dicated. As shown in Fig. 6g, ghrelin treatment downregulated mRNA
levels of collagen 1 and fibronectin. A decreased expression of αSMA was observed after ghrelin treatment (Fig. 6h), mRNA levels of MMP9

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**Fig. 2.** Spontaneous pancreatic fibrosis in 
Ghrl-TSC1−/− mice. Four-week-old 
Ghrl-TSC1−/− mice and wild-type littermates were fed with normal chow diet (NCD) or 60% high fat diet (HFD) for 12 weeks. Results were expressed as mean ± SEM. *P < 0.05 vs. NCD WT. †P < 0.05 vs. HFD WT. n = 11 for NCD or 12 for HFD. a. d. Pancreas weights and protein contents in pancreas of mice fed with NCD (a) or HFD (d). b. e. Morphology of pancreas in mice fed with NCD (b) or HFD (e). Shown were gross morphology, H&E, Sirius Red, and Masson staining of pancreas. Arrows identify the representative bands of fibrosis. c. f. mRNA levels of relative genes in pancreas of mice fed with NCD (c) or HFD (f) analyzed by RT-qPCR. β-actin was used as internal control.
was markedly increased upon ghrelin treatment, whereas metalloprotease inhibitor TIMP1 reduced significantly (Fig. 6g). Chretin did not alter the phosphorylated levels of mTOR or S6 (Fig. 6b), indicating that ghrelin does not induce mTORC1 signaling in pancreatic stellate cells.

### 3.7. Rapamycin reverses the spontaneous pancreatic fibrosis

If mTORC1 signaling in gastric X/A-like cells contributes to the spontaneous pancreatic fibrosis, inhibition of mTORC1 signaling would be expected to reverse the spontaneous pancreatic fibrosis in Ghrl-TSC1−/− mice. Our study demonstrates this concept. Intraperitoneal injection of rapamycin (1 mg·kg−1·d−1), a well-characterized mTORC1 inhibitor, for two weeks significantly improved the pancreatic fibrosis in Ghrl-TSC1−/− mice. Relative to the control dimethylsulfoxide (DMSO), rapamycin significantly reduced the fibrosis in pancreas detected by Sirius Red staining (Fig. 7a), mRNA levels of αSMA, collagen I, III, and IV were significantly reduced, whereas MMP9 mRNA markedly increased (Fig. 7b). The increase of pancreatic weight in Ghrl-TSC1−/− mice was also reversed by rapamycin (Fig. 7c). Associated with the improvement in pancreatic fibrosis, insulin expression, and plasma insulin levels were significantly increased by rapamycin treatment in Ghrl-TSC1−/− mice (Fig. 7d). Consistent with previous reports demonstrating that systemic rapamycin impairs glucose metabolism, glucose stimulated insulin secretion and glucose tolerance were not improved in the transgene treated with rapamycin (Fig. S6a and S6b). Further, rapamycin significantly attenuated the activation of mTORC1 signaling evidenced by reduction of pS6, the downstream target of mTORC1, in X/A-like cells of Ghrl-TSC1−/− mice (Fig. 7e). Interestingly, rapamycin also reversed the decrease of gastric and circulating ghrelin in Ghrl-TSC1−/− mice (Fig. 7f). To exclude the direct effect of rapamycin on fibrogenesis, we treated human pancreatic stellate cells with rapamycin (1 nmol/l) for 12 h or time indicated. As shown in Fig. 7g and h, rapamycin treatment completely inhibited the phosphorylation of mTOR and S6, but demonstrated no effect on expression levels of collagen I, fibronectin, and αSMA. All these observations suggest that inhibition of mTORC1 by rapamycin has no direct effect on pancreatic stellate cells, rather it improves pancreatic fibrosis indirectly by increasing the production of ghrelin in X/A-like cells.

### 4. Discussion

The major finding of the present study is that activation of mTORC1 signaling in gastric X/A-like cells induces spontaneous pancreatic fibrosis, leading to reduced insulin secretion and subsequent impairment in glucose metabolism. This conclusion is supported by following distinct observations: 1) Deletion of TSC1 activates mTORC1 signaling in gastric X/A-like cells, leading to spontaneous pancreatic fibrosis, which is followed by reduced insulin secretion and derangement of glucose metabolism; 2) Inhibition of mTORC1 signaling by rapamycin improves pancreatic fibrosis and reverses decrease of insulin levels in Ghrl-TSC1−/− mice; 3) Activation of mTORC1 signaling in gastric X/A-like cells suppresses gastric and circulating ghrelin; 4) Conversely, inhibition of mTORC1 signaling by rapamycin reverses reduction of ghrelin levels in Ghrl-TSC1−/− mice; 5) Infusion of exogenous ghrelin improves pancreatic fibrosis, with subsequent alleviation of decreased insulin levels and impaired glucose metabolism in Ghrl-TSC1−/− mice; 6) Ghrelin inhibits fibrosis in cultured pancreatic stellate cells, whereas rapamycin demonstrates no effect.

Gastric X/A-like cells, a distinct population that composes 20–30% of all endocrine cells in the oxyntic gland, produce two hormones: ghrelin and nesfatin-1. A variety of physiological functions have been demonstrated for these two hormones. These functions range from regulation of hormone release, food intake, glucose and lipid metabolism, to immunity and inflammation. Recent studies using the transgenic approach have significantly advanced our understanding on the nature of X/A-like cells. By cross-breeding the Ghrl-cre transgene with a lox-STOP-lox pertussis toxin line, Engelstoft et al. [28] have established a transgenic line in which pertussis toxin is selectively overexpressed in cells expressing ghrelin. Similarly, McFarlane et al. [29] have reported a transgenic mouse colony in which diphtheria toxin receptor is overexpressed in ghrelin cells. Using these transgenic mice, novel physiological functions related to the X/A-like cells are emerging. A comprehensive repertoire of 7TMR receptors and the corresponding Gα subunits have been identified to be present and functionally active for modulation of ghrelin secretion in the gastric X/A-like cells. Surprisingly, ablation of ghrelin cells shows an obvious hypoglycemia under calorie restriction condition, while demonstrating no effect on appetite, body weight, and diet-induced obesity [29]. Further experiments suggest...
that the function of ghrelin in preventing hypoglycemia under famine condition requires the expression of \(\beta_1\)ARs in ghrelin cells [30]. Although ghrelin is expressed in the fetal islets, a specific ghrelin BAC containing 59 kb upstream of the ghrelin Start codon and 104 kb downstream of the ghrelin Stop codon has been used to construct a ghrelin-GFP transgenic mice in which GFP is only expressed in the stomach and duodenum but not in fetal islets [27]. Using a similar strategy, we have generated \(\text{Ghrl-cre}\) transgenic lines in C57BL/6J genetic background. Out of seven lines, we have identified and validated a \(\text{Ghrl-cre}\) line in which \(\text{cre}\) gene is selectively expressed in stomach. Cre mRNA is abundant in the stomach but not in the hypothalamus and pancreas. We thus cross-bred this transgenic line with selective expression of cre gene in the stomach with TSC1\(^{fl/}\)fox/\(^{fl/}\)fox mice to generate transgenic mice: \(\text{Ghrl-TSC1}^{-/-}\). This transgenic line demonstrates a selective activation of mTORC signaling in gastric X/A-like cells but not in pancreas. Our studies using the \(\text{Ghrl-TSC1}^{-/-}\) transgenic mice reveal a novel physiological function for mTORC1 activity in gastric X/A-like cells in the maintenance of pancreatic microenvironment. Activation of mTORC1 signaling in gastric X/A-like cells leads to the spontaneous pancreatic fibrosis in both lean and obese animals. Blockade of mTORC1 signaling by rapamycin reduces the severity of pancreatic fibrosis in \(\text{Ghrl-TSC1}^{-/-}\) mice. The effect of rapamycin to reverse the pancreatic fibrosis in these animals appears due to its counteracting the mTORC1 signaling in gastric X/A-like cells. Firstly, rapamycin significantly attenuates the activation of mTORC1 signaling in these endocrine cells measured by the phosphorylation of S6 in \(\text{Ghrl-TSC1}^{-/-}\) transgenic mice. Secondly, inhibition of mTORC1 activity by rapamycin demonstrates no effect on the fibrosis-related genes in cultured...
pancreatic stellate cells. Lastly, ghrelin, which is a potent suppressor of fibrotic molecules such as αSMA, collagen I, and fibronectin, demonstrates no effect on mTORC1 signaling in cultured pancreatic stellate cells.

Levels of gastric and circulating ghrelin are significantly reduced in Ghrl-TSC1−/− transgenic mice. The reduced ghrelin production in Ghrl-TSC1−/− mice was associated with decreased lipogenesis and increased β-oxidation, leading to lower hepatic lipid content in both NCD and HFD animals. This observation is consistent with the pro-lipogenesis function of ghrelin in hepatocytes [13]. The observation that gastric and circulating ghrelin is significantly reduced in Ghrl-TSC1−/− transgenic mice indicates that ghrelin may function as a critical signal for gastric mTORC1 activity to maintain the integrity of pancreatic matrix. Activation of mTORC1 signaling suppresses the production of ghrelin, leading to the spontaneous pancreatic fibrosis.

This concept is supported by studies showing that infusion of exogenous ghrelin significantly improves the pancreatic fibrosis in these transgenic mice. Consistent with this proposal, several studies have demonstrated an anti-fibrotic effect of ghrelin in liver, heart, lung, kidney, and other tissues [31–37]. All these animal models involve chemical or microorganism injury which often triggers a significant inflammation, leading to a subsequent increase in fibrogenesis in these organs. In our animal model, no inflammation is detected in the pancreas. Our Ghrl-TSC1−/− transgenic mice may thus serve as a unique genetic model for pancreatic fibrosis. In these transgenic mice, mRNA levels of αSMA and collagen I, III, IV in pancreas increase significantly. This data suggests that activation of fibrogenesis contributes to the pancreatic fibrosis in Ghrl-TSC1−/− transgenic mice. In addition, reduction of extracellular matrix degradation further exacerbates the pancreatic fibrosis in the transgenic mice. By RNAseq analysis, we observed a significant reduction in signature for the degradation of pancreatic extracellular matrix and activation of matrix metalloproteinases. In particular, MMP9 is significantly reduced, whereas metalloproteinase inhibitor TIMP1 increased in Ghrl-TSC1−/− transgenic mice. Reconstitution of ghrelin significantly attenuates the pancreatic fibrosis by suppressing fibrogenesis while concurrently augmenting matrix degradation. Ghrelin appears to directly act on pancreatic stellate cells because treatment of these cells with ghrelin suppresses the expression of fibrotic matrix while increasing levels of MMP9. All these findings suggest that activation of mTORC1 signaling in X/A-like cells induces spontaneous fibrosis in pancreas by stimulating fibrogenesis and concurrently suppressing extracellular matrix degradation. This effect occurs through the reduction of ghrelin. Ghrelin is thus a critical regulator for the integrity of pancreatic microenvironment.

Fig. 5. Analysis of fibrosis-related genes in pancreas. Ghrl-TSC1−/− mice and wild-type littermates were fed with normal chow (NCD) for 16 weeks. Pancreatic total RNA and protein were extracted. mRNA was analyzed by RT-qPCR. Proteins were analyzed by western blots. a. Expression of TGFβ and its downstream molecules in pancreas. b. mRNA levels of inflammatory cytokine genes. c. Significant enriched pathways analyzed by RNAseq. d. Validation of MMP9 and TIMP1 expression in pancreas.
fibrosis in Ghrl-TSC1 −/− mice. In addition to ghrelin and nesfatin-1, there might exist other unknown secretory factors from gastric X/A-like cells, which contribute to the phenotype. Future investigation will focus on secretome profiling of Ghrl-TSC1 −/− mice to characterize these molecules.

Few attention has been focused on the functional relation between the endocrine and exocrine components of pancreas despite of their co-existence. From the physiological and clinical points of view, it is easier to study these two parts of pancreatic function separately. Thus, the exocrine pancreas has always been viewed as part of the gastroenterology domain, whereas the endocrine pancreas has been an area of interest for those studying diabetes. Hyperactivity of pancreatic endocrine has been reported to activate pancreatic stellate cells via insulin, leading to fibrosis response in pancreas [38]. On the other hand, insufficiency of pancreatic exocrine is associated with an impairment in insulin secretion and glucose metabolism [39–41]. These observations suggest that an interaction between pancreatic exocrine and endocrine. Our studies provide a perspective on this interaction. Pancreatic microenvironment may significantly alter the function of islet cells. Pancreatic fibrosis limits the secretion of insulin, leading to the subsequent derangement in glucose metabolism. Consistently, we have detected a significant impairment in the glucose tolerance in Ghrl-TSC1 −/− mice. Hyperactivity of pancreatic endocrine has been reported to activate pancreatic stellate cells via insulin, leading to fibrosis response in pancreas [38]. On the other hand, insufficiency of pancreatic exocrine is associated with an impairment in insulin secretion and glucose metabolism [39–41]. These observations suggest that an interaction between pancreatic exocrine and endocrine. Our studies provide a perspective on this interaction. Pancreatic microenvironment may significantly alter the function of islet cells. Pancreatic fibrosis limits the secretion of insulin, leading to the subsequent derangement in glucose metabolism. Consistently, we have detected a significant impairment in the glucose tolerance in Ghrl-TSC1 −/− mice.
transgenic mice. Islet mass, insulin expression as well as glucose stimulated release of insulin are significantly reduced in these transgenes. On the other hand, insulin sensitivity remains virtually unaltered.

Reconstitution of ghrelin levels reduces pancreatic fibrosis, which is associated with the improvement of insulin secretion and glucose tolerance. All these findings indicate that activation of mTORC1 signaling in...
X/A-like cells may impair insulin secretion and glucose metabolism by stimulating fibrosis in the pancreas.

There exist several limitations in our study. First, the molecular mechanism by which ghrelin suppresses the activation of pancreatic stellate cells remains unknown. Consistent with a number of previous reports showing the anti-fibrotic effect of ghrelin in liver, heart, lung, kidney, and other tissues, our data suggests that reduction in ghrelin production contributes to activation of pancreatic stellate cells, leading to the spontaneous fibrosis of pancreas in the Ghrl-/- mice. Further study should focus on revealing the molecular mechanism mediating the anti-fibrotic effect of ghrelin in pancreas. Second, the relevance of our finding to human beings requires further examination. Our finding that ghrelin suppresses fibrogenesis with concurrent stimulation of extracellular matrix degradation in human stellate cells shows a clinical relevance. Further study should focus on whether reduction of ghrelin or activation of mTORC1 signaling in gastric X/A-like cells could induce pancreatic fibrosis in human. Third, whether pancreatic fibrosis occurs in Ghrl-cre mice remains to be explored.

In summary, our study demonstrates that activation of mTORC1 signaling in gastric X/A-like cells induces spontaneous pancreatic fibrosis and impairs glucose homeostasis by suppressing ghrelin. These observations suggest mTORC1 signaling in gastric X/A-like cells is a critical driving signal from the gut to maintain the integrity of pancreatic micro-environment. mTORC1 signaling in X/A-like cells may thus provide an alternative strategy for the therapy of pancreatic fibrosis and its associated diabetes.

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Declarations of interests
All authors declare no conflict of interests.

Author contributions
Ruili Yu conducted and designed experiments, performed data analysis, and drafted the manuscript. Ziru Li, Shiyi Liu, Bahetiyaer Huwatibieke, and Yin Li assisted with the experiments. Yue Yin and Weizhen Zhang supervised the project, designed experiments, and edited the manuscript.

Guarantor statement
Weizhen Zhang, as corresponding author, had full access to all the data in the study and had final responsibility for the decision to submit for publication.

References
[1] Rockey DC, Bell PD, Hill JA. Fibrosis—a common pathway to organ injury and failure. N Engl J Med 2015;372(12):1138–49.
[2] Zeisberg M, Kalluri R. Cellular mechanisms of tissue fibrosis. 1. Common and organ-specific mechanisms associated with tissue fibrosis. Am J Physiol Cell Physiol 2013; 304(3):216–25.
[3] Tran TC, Van T Hof G, Kazemier G, et al. Pancreatic fibrosis correlates with exocrine pancreatic insufficiency after pancreatectoduodenectomy. Dig Surg 2008;25(4):311–8.
[4] Kopelman H, Corey M, Gaskin K, Durie P, Weizman Z, Forstner G. Pancreatic fluid secretion and protein hyperconcentration in cystic fibrosis. N Engl J Med 1985;312(6): 441–5.
[5] Sheikhi S, Gudipaty L, De Leon DD, et al. Reduced β-cell secretory capacity in pancreatic-insufficient, but not pancreatic-sufficient. Cystic Fibrosis despite normal glucose tolerance. Diabetes 2017;66(1):134–44.
[6] Moran A, Becker D, Casella SJ, et al. Diabetes mellitus secondary to chronic pancreatitis and pancreatic cancer. Lancet Gastroenterol Hepatol 2016;31(12):2677–83.
[7] Stengel A, Täch C, Chiew J, et al. Ghrelin-a pleiotropic hormone secreted from endocrine X/A-like cells of the stomach. Front Neurosci 2012;6:1–16.
[8] Bardo E, Leiter AB, Kopin RS, Meller S, Solcia E. The “normal” endocrine cell of the gut: changing concepts and new evidences. Ann N Y Acad Sci 2004;1014:1–12.
[9] Heppner KM, Piechowksi CL, Müller A, et al. Both acyl and des-acyl ghrelin regulate adiposity and glucose metabolism via central nervous system ghrelin receptors. Diabetes 2014;63(1):122–31.
[10] Tschüpp M, Smiley DL, Heinam ML. Ghrelin induces adiposity in rodents. Nature 2000;407(6806):908–13.
[11] Li Z, Xu G, Qian Y, et al. Ghrelin promotes hepatic lipogenesis by activation of mTOR-PAR signaling pathway. Proc Natl Acad Sci U S A 2014;111(36):13613–8.
[12] Sun Y, Asnicar M, Saha PK, Chan L, Smith RG. Ablation of ghrelin improves the diabetic but not obese phenotype of Ob/Ob mice. Cell Metab 2006;3(3):379–86.
[13] Gortan Capperielli G, Zanetti M, Semoli A, et al. Unacylated Ghrelin Reduces Skeletal Muscle Reactive Oxygen Species Generation and Inflammation and Prevents High-Fat-Diet-Induced Hyperglycemia and Whole-Body Insulin Resistance in Diabetics. Diabetes 2016;65(4):874–86.
[14] Pereira JAD, da Silva FC, de Moraes-Vieira PMM. The impact of Ghrelin in metabolic disease: an immune perspective. J Diabetes Res 2017;2017:4527980.
[15] Li Y, Wu X, Zhao Y, Chen S, Oowyang C. Ghrelin acts on the dorsal vagal complex to stimulate pancreatic protein secretion. Am J Physiol Gastrointest Liver Physiol 2006;290(6):G1350–8.
[16] Zhang W, Chen M, Chen X, Segura BJ, Mulholland MW. Inhibition of pancreatic protein secretion by ghrelin in the rat. J Physiol 2001;537(Pt 1): 231–6.
[17] Liu RC, Cheng CH, Leung PS. The Ghrelin system in acinar cells: localization, expression, and regulation in the exocrinepancreatic tissues. 2007;35(3):e1–8.
[18] Gagno J, Baggio LL, Drucker DJ, Brubaker PL. Ghrelin is a Novel Regulator of GLP-1 Secretion. Diabetes 2015;64(5):1513–21.
[19] Dato V, Nakamoto M, Hantusch B, et al. Ghrelin is present in pancreatic alpha-cells of humans and rats and stimulates insulin secretion. Diabetes 2002;51(1):124–9.
[20] Heppner KM, Tong J. Mechanisms in endocrinology: regulation of glucose metabolism by the ghrelin system: multiplearmies and multiple actions. Eur J Endocrinol 2014;171(1):R21–32.
[21] Yada T, Dandie R, R Visa BS, et al. Ghrelin signalling in β-cells regulates insulin secretion and blood glucose. Diabetes Obes Metab 2014(Suppl. 1):111–7.
[22] Beiroowski B, Wong KM, Babetto E, Milbrandt J. mTORC1 promotes proliferation of immature Schwann cells and myelin growth of differentiated Schwann cells. Proc Natl Acad Sci U S A 2017;114(21):E4261–70.
[23] Lu Z, Gao L, Tang H, et al. Peripheral effects of nesfatin-1 on glucose homeostasis. PLoS One 2013;8(8):e71513.
[24] Xu G, Li Z, Ding L, et al. Intestinal mTOR regulates GLP-1 production in mouse L cells. Diabetologia 2015;58(8):1887–97.
[25] Sakata I, Nakano Y, Osborne-Lawrence S, et al. Characterization of a novel ghrelin cell reporter mouse. Regul Pept 2009;155(1–3):51–8.
[26] Engelstroft MS, Park WM, Sakata I, et al. Seven transmembrane G protein-coupled receptor repertoire of gastric ghrelin cells. Mol Med 2013;24(4–5):376–92.
[27] McFarlane MR, Brown MS, Goldstein JL, Zhao TJ. Induced ablation of ghrelin cells in adult mice does not decrease food intake, body weight, or response to high-fat diet. Cell Metab 2014;20(1):54–60.
[28] Mami BK, Osborne-Lawrence S, Vijayaraghavan P, Hepler C, Zigman JM. β1-Adrenergic receptor deficiency in ghrelin-expressing cells causes hypoglycemia in susceptible individuals. J Clin Invest 2016;126(9):3467–78.
[29] Yang C, Liu L, Liu K, et al. Ghrelin suppresses cardiac fibrosis of post-myocardial infarction failure rats by adjusting the active A-follistatin imbalance. Peptides 2018;56:27–35.
[30] Yao M, Zhang S, Yu F, Li H, Guo C, Fan X, Ghrelin Attenuates Liver Fibrosis through Regulation of TGF-β1 Expression and Autophagy. Int J Mol Sci 2015;16(9): 21911–30.
[31] Sun GX, Ding R, Li M, et al. Ghrelin attenuates renal fibrosis and inflammation of obstructive nephropathy. J Urol 2015;193(6):2107–15.
[32] Pei XM, Yung BY, Yip SP, Yang M, Benzie FJ, Siu PM. Desacyl ghrelin prevents doxorubicin-induced myocardial fibrosis and apoptosis via the GH/HS-independent pathway. Am J Physiol Endocrinol Metab 2014;306(3): E311–23.
[33] Tsoubouchi H, Yanagi S, Miura A, et al. Rikkunshito ameliorates cachexia associated with bleomycin-induced lung fibrosis in mice by stimulating ghrelin secretion. Nutri Res 2014;34(10):876–85.
[34] Eminiér AT, Aygün C, Konduk T, et al. The relationship between resistance and ghrelin levels with fibrosis in nonalcoholic fatty liver disease. J Res Med Sci 2014;19(11):1058–61.
[35] Angelo D, Reano S, Ferrara M, Agosti E, Graziani A, Filigheddu N. Antifibrotic activity of acylated and unacylated ghrelin. Int J Endocrinol 2015;2015:1–9.
[38] Yang J, Waldron RT, Su HY, et al. Insulin promotes proliferation and fibrosing responses in activated pancreatic stellate cells. Am J Physiol Gastrointest Liver Physiol 2016;311(4):G675–87.

[39] Lozinska L, Weström B, Prykhodko O, et al. Decreased insulin secretion and glucose clearance in exocrine pancreas-insufficient pigs. Exp Physiol 2016;101:100–12.

[40] Pierzynowski SG, Goncharova K, Gregory PC, et al. Experiments suggesting extra-digestive effects of enteral pancreatic amylase and its peptides on glucose homoeostasis in a pig model. Sci Rep 2017;7(1):4628.

[41] Pierzynowska KG, Lozinska L, Wolinski J, Pierzynowski S. The inverse relationship between blood amylase and insulin levels in pigs during development, bariatric surgery, and intravenous infusion of amylase. PLoS One 2018;13(6):e0198672.