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Research paper

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

Ruili Yu [a], Ziru Li [a,b], Shiying Liu [a], Bahetiyaer Huwatibieke [a], Yin Li [a], Yue Yin [a,*], Weizhen Zhang [a,*]

[a] School of Basic Medical Sciences, Peking University, Beijing 100191, China
[b] Department of Surgery, University of Michigan Medical Center, Ann Arbor, MI 48109-0346, USA

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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 *Ghrl-cre* transgene in which the cre enzyme is expressed in X/A-like cells under the control of ghrelin-promoter. *TSC1*$^{flox/flox}$ mice were bred with *Ghrl-cre* mice to generate *Ghrl-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.

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.

Abbreviations: ECM, extracellular matrix; TGFβ, transforming growth factor-β; PSC, spancreatic stellate cells; TNFα, tumor necrosis factor α; PDGF, platelet derived growth factor; mTORC1, mechanistic target of rapamycin complex 1; TSC1, tuberous sclerosis 1; NCD, normal chow diet; HFD, high fat diet; TG, *Ghrl-TSC1* −/−; WT, wild type.

* Corresponding authors at: Department of Physiology and Pathophysiology, School of Basic Medical Sciences, Peking University Health Science Center, Beijing 100191, China.
E-mail addresses: yueyin@bjmu.edu.cn (Y. Yin), weizhenzhang@bjmu.edu.cn (W. Zhang).

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Research in context

Evidence before this study

(1) X/A-like cells, a group of unique endocrine cells in gastric oxyntic mucosa, secret ghrelin to influence energy balance.
(2) There may exist a close relation between exocrine pancreas and endocrine pancreas. Hyperactivity of endocrine pancreas activates pancreatic stellate cells via insulin, leading to fibrosis response in pancreas. On the other hand, insufficiency of exocrine pancreas is associated with an impairment in insulin secretion and glucose metabolism.

Added value of this study

(1) Cell-specific manipulation of genes in gastric X/A-like cells allows us to explore the nature of these largely unknown endocrine cells.
(2) Activation of mTORC1 activity in gastric X/A-like cells induces sporadic pancreatic fibrosis, leading to subsequent impairment in insulin secretion. Our study furthers understandings about gut-pancreas communication.
(3) Pancreatic microenvironment is critical for insulin secretion and glucose homeostasis.

Implications of all the available evidence

Gastric mTORC1 may provide a novel target and strategy for the prevention or treatment of pancreatic fibrosis and diabetes.

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. Supplemental 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/6 J 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-TSC1* −/− mice and $TSC1^{flox/flox}$ 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 $g \cdot kg^{-1}$). 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 $nmol \cdot kg^{-1} \cdot d^{-1}$) 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 $mg \cdot kg^{-1} \cdot d^{-1}$) or DMSO was administered by intraperitoneal injection for 14 days. Glucose (3 $g \cdot kg^{-1}$) was administrated by oral gavage, while insulin (1 $U \cdot kg^{-1}$) 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 $g \cdot kg^{-1}$). 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 Gt(Rosa)26Sor^{tm1sor}/J mice, which carry the *loxP*-flanked DNA STOP sequence preventing expression of the downstream *LacZ* gene. In these *ROSA-Ghrl-cre* (RG) mice, *Ghrl-cre* positive cells express β-galactosidase because the STOP sequence flanking *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*$^{flox/flox}$ mice (Tsc1^{tm1Djk} from the Jackson Laboratory) to generate *Ghrl-TSC1* −/− transgene 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. H&E 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 Se, 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

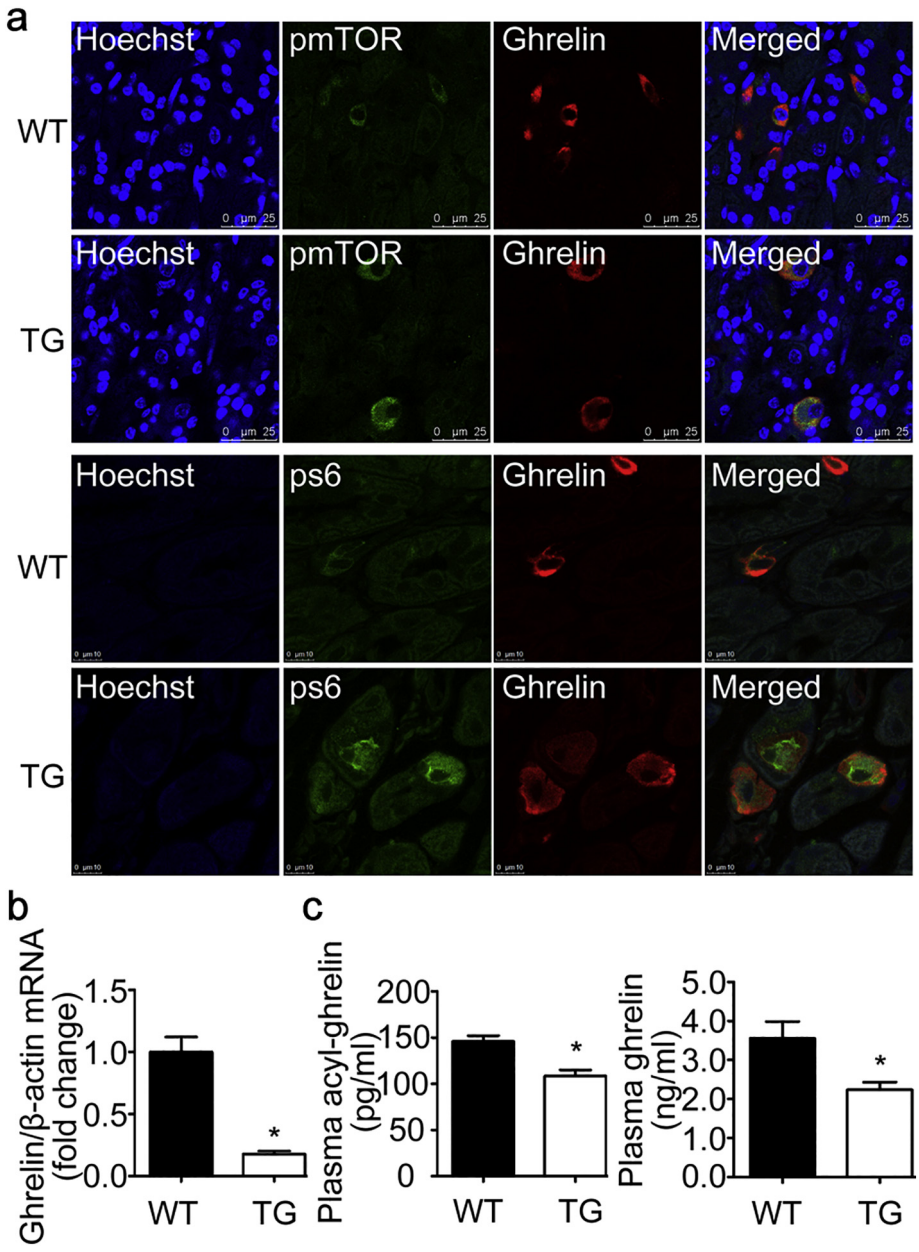

Fig. 1. *Ghrl-TSC1* −/− mice. *Ghrl-TSC1* −/− transgenic mice were generated by cross-breeding *Ghrl-cre* transgenes with *TSC1*$^{flox/flox}$ mice. Validated *Ghrl-TSC1* −/− mice and wild-type littermates were fed with normal chow diet (NCD) for 16 weeks. Data was presented as mean ± SEM. $^{*}P < 0.05$ 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 co-localization 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.

to wild-type littermates (Fig. 3a). Glucose tolerance test showed that glucose metabolism was significantly impaired in *Ghrl-TSC1* −/− mice (Fig. 3b). However, insulin sensitivity remained unaltered (Fig. 3c). In *Ghrl-TSC1* −/− mice, levels of phosphorylated AKT in liver or adipose tissue were similar with wild-type littermates, even increased in skeletal muscle (Fig. 3d). These observations suggest that glucose intolerance in *Ghrl-TSC1* −/− mice is not caused by insulin resistance.

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 littermates (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 littermates (Fig. 4d). Averaged islet area was reduced, whereas number of small islets increased (Fig. 4d).

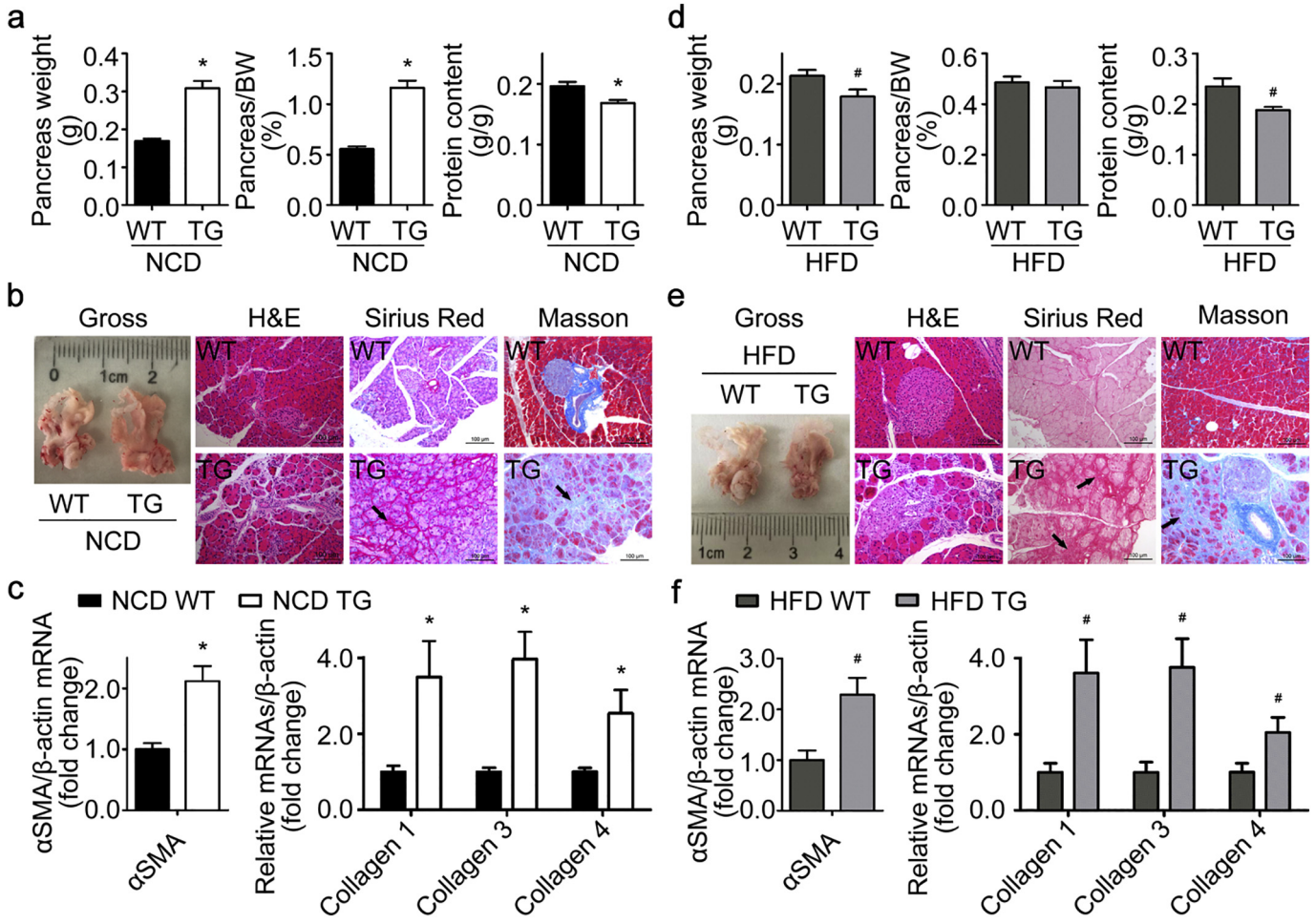

Fig. 2. Spontaneous pancreatic fibrosis in *Ghrl-TSC1* −/− mice. Four-week-old *Ghrl-TSC1* −/− mice and wild-type littermates were fed with normal chow diet (NCD) or 60% high fat diet (HFD) for 12 weeks. Results were expressed as mean ± SEM. *P < 0.05 vs. NCD WT. #P < 0.05 vs. HFD WT. n = 11 for NCD 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.

3.5. Alteration of genes related to matrix degradation in Ghrl-TSC1 −/− mice

Transforming growth factor-β (TGFβ) and inflammation are classical pathways leading to fibrosis [1]. To further explore the cause of spontaneous pancreatic fibrosis in *Ghrl-TSC1* −/− mice, we next examined 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 phosphorylation of Smad2 and Smad3, the downstream targets of TGFβ signaling. mRNA levels of inflammatory cytokines remained unaltered in pancreas of *Ghrl-TSC1* −/− mice (Fig. 5b). On the other hand, RNAseq analysis revealed a significant reduction in degradation of the extracellular 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 metalloproteinase 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 concept, 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 reversed the reduction of plasma acyl-ghrelin levels in *Ghrl-TSC1* −/− mice. Consistent with the previous reports showing the orexigenic effect of ghrelin [9], decreased ghrelin level of *Ghrl-TSC1* −/− mice was associated with reduced food intake, while administration of exogenous ghrelin reversed the reduction of food intake (Fig. S8). These observations 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 significant improvement in pancreatic fibrosis evidenced by decreased collagen deposition detected by H&E, Sirius Red, and Masson staining (Fig. 6b), as well as down-regulation 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 reduction 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 indicated. As shown in Fig. 6g, ghrelin treatment downregulated mRNA levels of collagen I and fibronectin. A decreased expression of αSMA was observed after ghrelin treatment (Fig. 6h). mRNA levels of MMP9

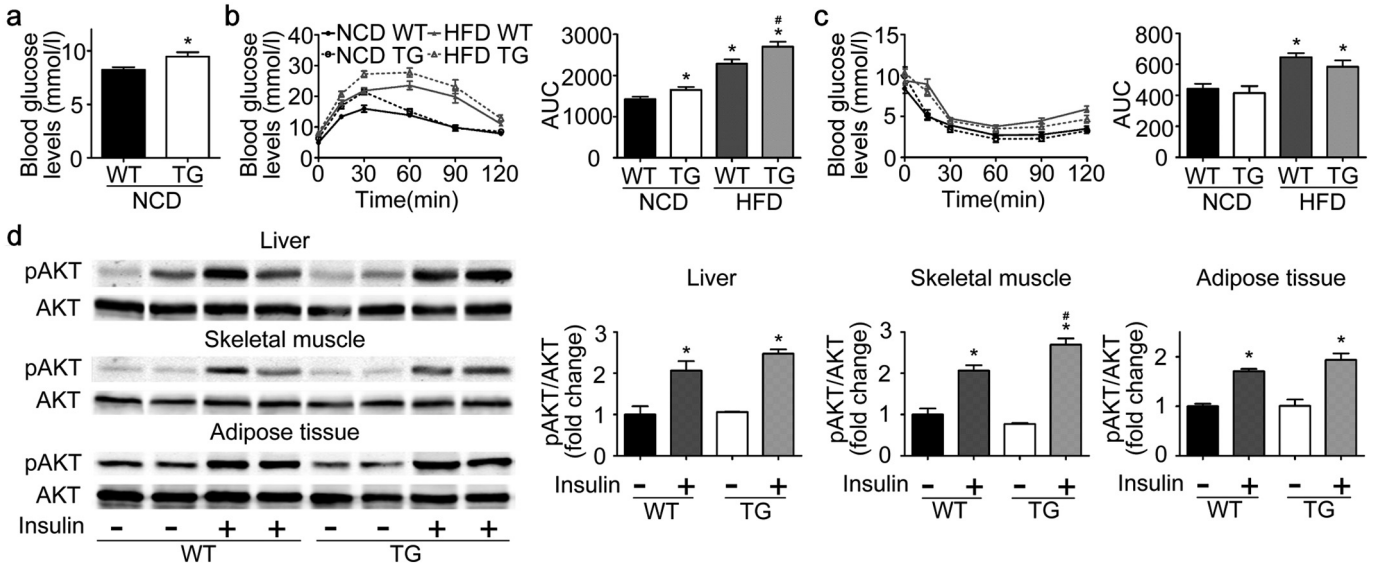

Fig. 3. Impaired glucose metabolism in *Ghrl-TSC1* −/− mice. Four-week-old *Ghrl-TSC1* −/− mice and wild-type littermates were fed with normal chow diet (NCD) or 60% high fat diet (HFD) for 12 weeks. Data was expressed as mean ± SEM. *P < 0.05 vs. WT NCD or WT saline. #P < 0.05 vs. WT HFD or WT insulin. n = 11 for NCD or 12 for HFD. a. Basal glucose levels in blood. b. Glucose tolerance test and the area under curve. c. Insulin tolerance test and the area under curve. d. Levels of pAKT and AKT in liver, skeletal muscle, and adipose tissue. Shown were representative western blots from HFD-induced obese mice received intraperitoneal injection of insulin (1 U/kg) or saline for 15 min. β-actin was used as loading control. Signal intensity was quantified using Image J software.

was markedly increased upon ghrelin treatment, whereas metalloproteinase inhibitor TIMP1 reduced significantly (Fig. 6g). Ghrelin did not alter the phosphorylated levels of mTOR or S6 (Fig. 6h), 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 $mg \cdot kg^{-1} \cdot d^{-1}$), 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. 7e and f). 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 7TM 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

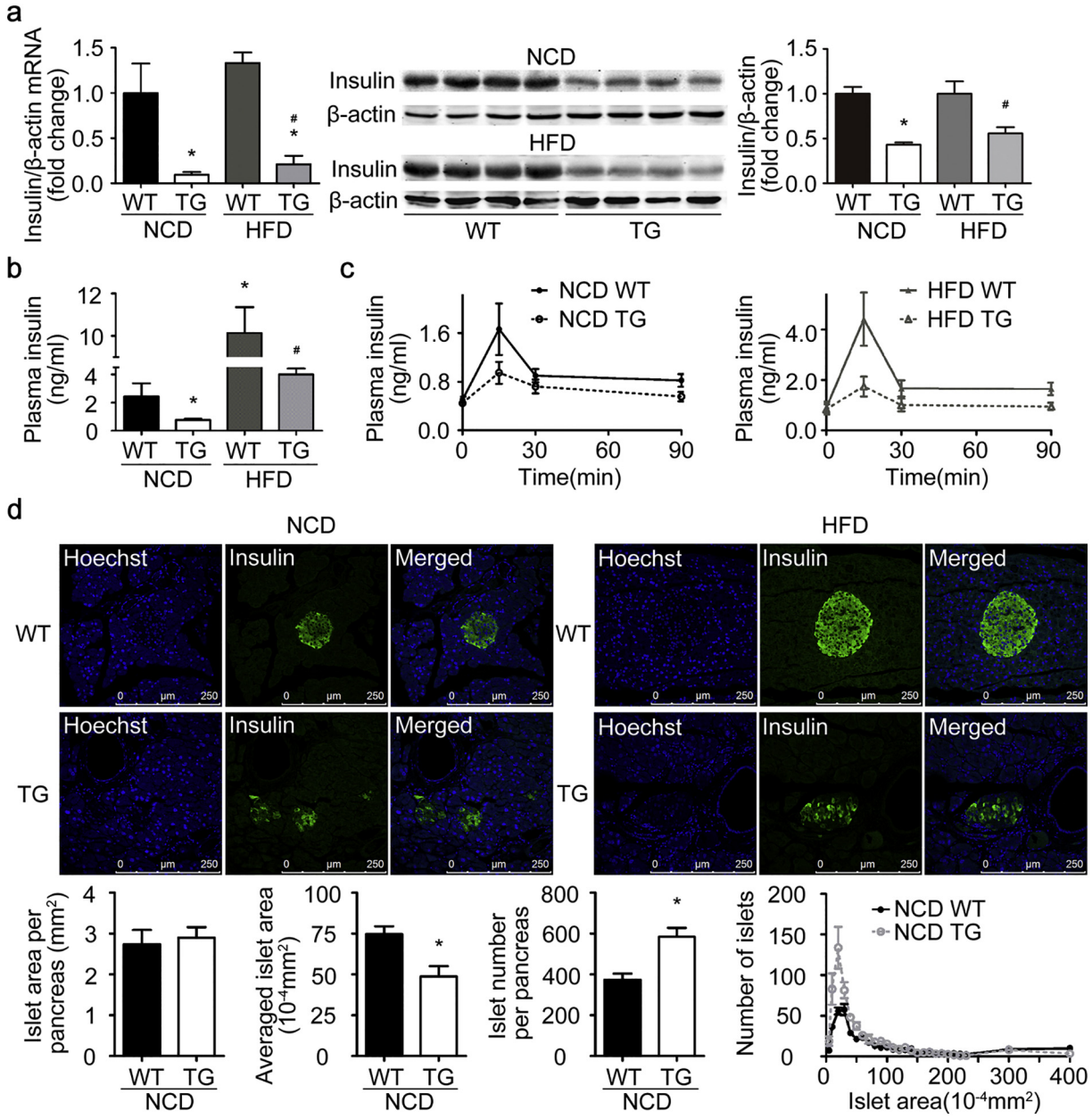

Fig. 4. Decreased insulin expression and secretion in *Ghrl-TSC1* −/− mice. Four-week-old *Ghrl-TSC1* −/− mice and wild-type littermates were fed with normal chow diet (NCD) or 60% high fat diet (HFD) for 12 weeks. Data was expressed as mean ± SEM. *P < 0.05 vs. NCD WT. #P < 0.05 vs. HFD WT. n = 11–12 unless indicated otherwise. β-actin was used as internal control. a. Levels of insulin mRNA and protein. b. Basal plasma levels of insulin. c. Plasma insulin levels during oral glucose tolerance test. $n = 8$. d. Insulin immunoreactivity (green) in mouse pancreas. And nuclei were stained with Hoechst dye (blue). Islet area and number were measured and calculated. Sections were obtained at 100-um intervals from each pancreas. All sections of each pancreas were stained with H&E and observed with a Leica microscope. The islet area, islet number, and average size of islets were determined using the Image J software.

that the function of ghrelin in preventing hypoglycemia under famine condition requires the expression of β1ARs 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 *Ghrl-cre* transgenic lines in C57BL/6 J genetic background. Out of seven lines, we have identified and validated a *Ghrl-cre* line in which *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 $TSC1^{flox/flox}$ mice to generate transgenic mice: *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 *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 *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 *Ghrl-TSC1* −/− transgenic mice. Secondly, inhibition of mTORC1 activity by rapamycin demonstrates no effect on the fibrosis-related genes in cultured

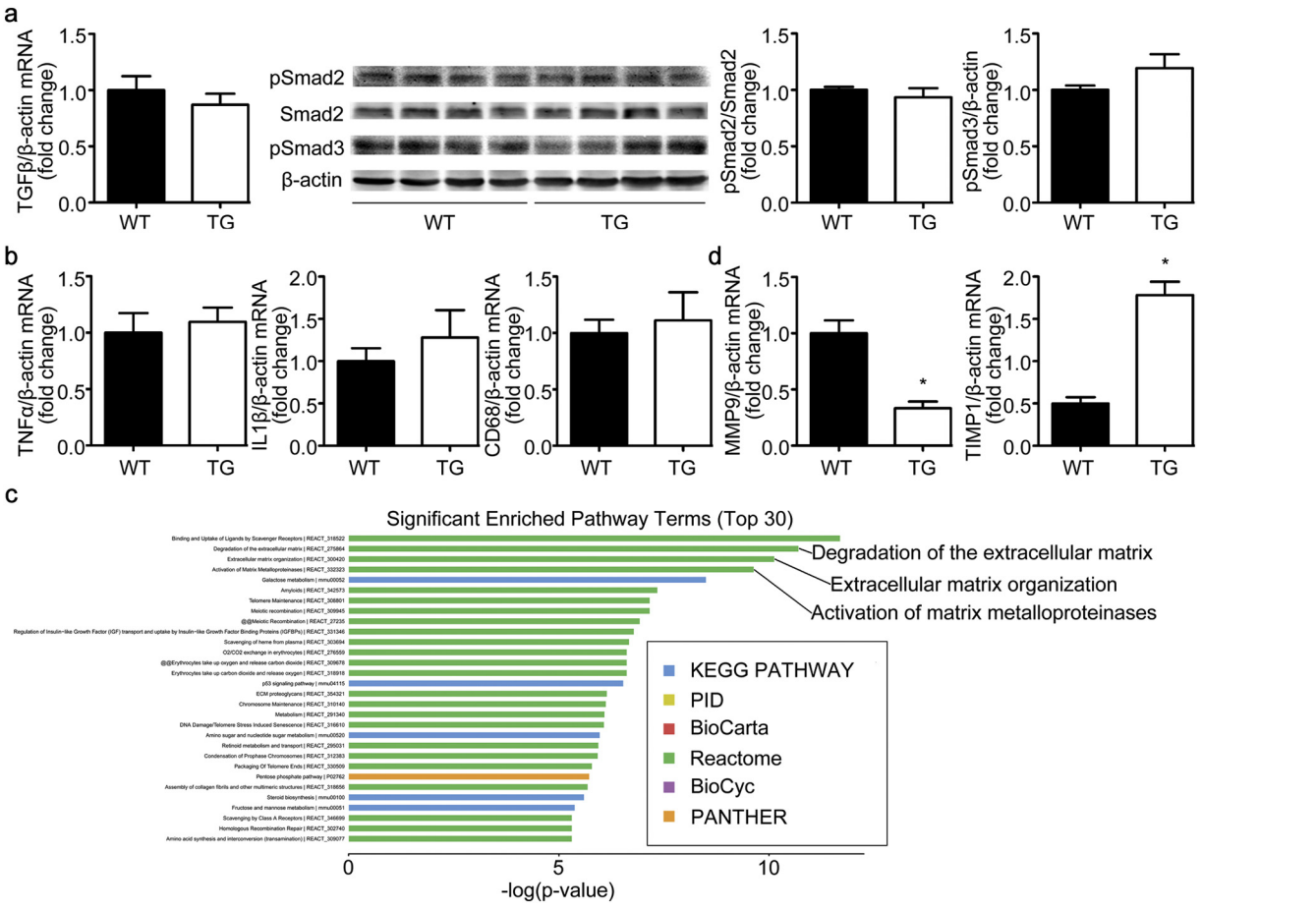

Fig. 5. Analysis of fibrosis-related genes in pancreas. *Ghrl-TSC1* −/− mice and wild-type littermates were fed with normal chow (NCD) for 16 weeks. Pancreatic total RNA and protein were extracted. mRNA was analyzed by RT-qPCR. Proteins were analyzed by western blots. β-actin was used as internal control. Data was expressed as mean ± SEM. *P < 0.05 vs. WT. n = 11. 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.

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 transgenes. 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 transgene. 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. It is worth of noting that additional molecules may also contribute to the effect of mTORC1 signaling in X/A-like cells on the pancreatic microenvironment. Indeed, X/A-like cells also produce nesfatin-1, which is identified in these cells, but in different vesicles than ghrelin, and often exerts opposing metabolic functions to ghrelin. Our previous studies have demonstrated that activation of mTORC1 signaling increases the production of nesfatin-1 in a manner opposite to the ghrelin. Consistently, increase of nesfatin-1 has been detected in *Ghrl-TSC1* −/− mice in which mTORC1 signaling is activated specifically in X/A-like cells (data not shown). However, until the receptor for nesfatin-1 is characterized, it is not possible to efficiently block the action of nesfatin-1, and thus to confirm that increase of nesfatin-1 contributes to increase of pancreatic

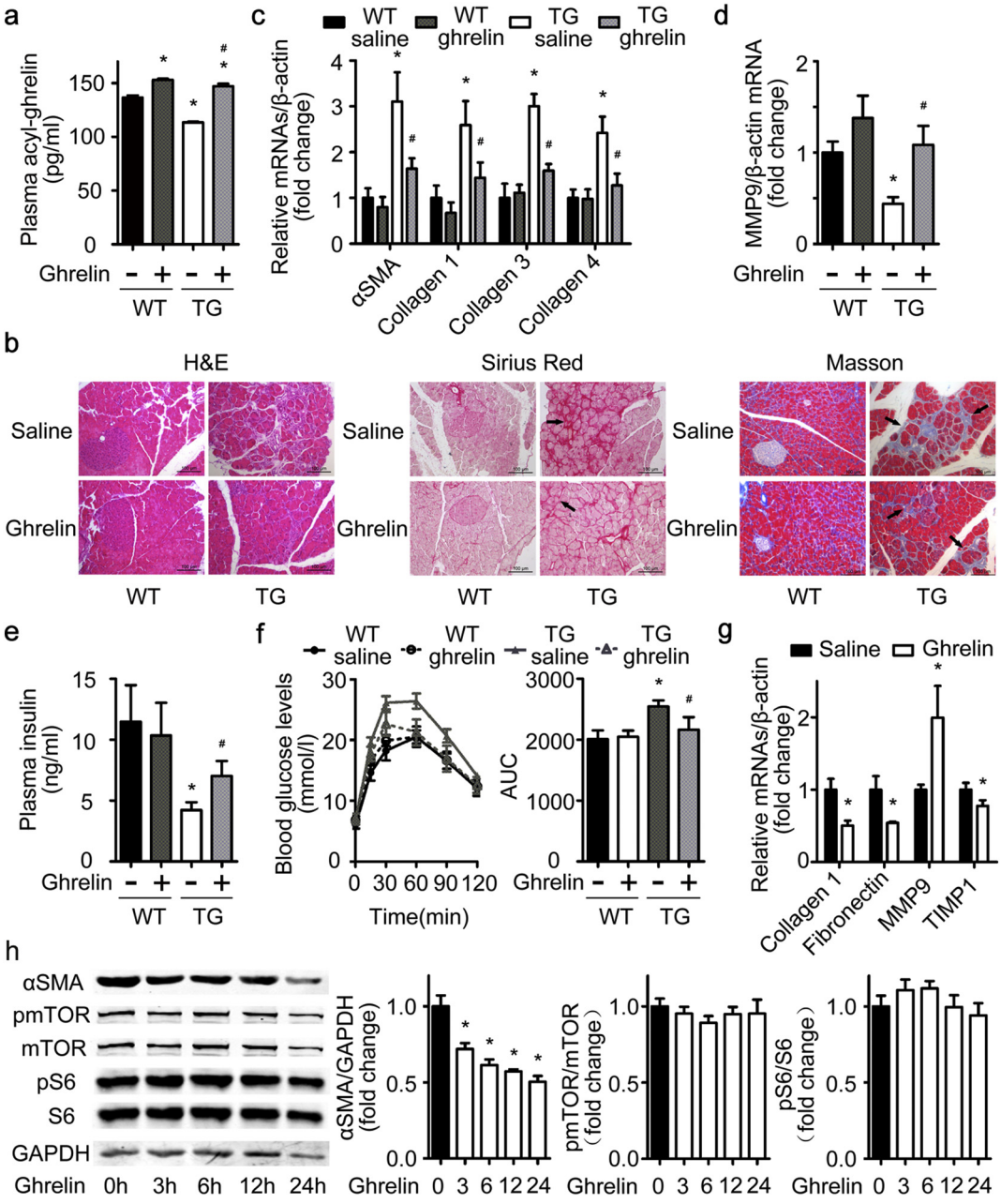

Fig. 6. Reversal effects of exogenous ghrelin. Four-week-old *Ghrl-TSC1* −/− mice and wild-type littermates were fed with 60% high fat diet (HFD) for 12 weeks. Acyl-ghrelin or saline was continuously infused (11 nmol·kg^{-1}·d^{-1}) for 2 weeks. Data was expressed as mean ± SEM. β-actin was used as internal control. a. Plasma acyl-ghrelin levels. b. H&E, Sirius Red staining, and Masson staining of pancreas. Arrows identify the representative bands of fibrosis. c. mRNA levels of fibrosis-relative genes in pancreas. d. Expression of MMP9 in pancreas. e. Basal plasma insulin levels. *P < 0.05 vs. WT saline. #P < 0.05 vs. TG saline. *n* = 9–11. f. Glucose tolerance test and the area under curve. *P < 0.05 vs. WT saline. #P < 0.05 vs. TG saline. n = 9–11. g. Direct effect of ghrelin on pancreatic stellate cells. Cultured human pancreatic stellate cells were treated with acyl-ghrelin (10^{-8} mol/l) or saline for 6 h. mRNA levels of collagen I, fibronectin, MMP9, and TIMP1 were analyzed by RT-qPCR. Experiments were repeated for three times. *P < 0.05 vs. Saline. h. Cultured human pancreatic stellate cells were treated with acyl-ghrelin (10^{-8} mol/l) or saline for time indicated. Shown were the representative western blots. Antibodies against αSMA, pmTOR, mTOR, pS6, and S6 were used. GAPDH was used as loading controls. Signal intensity was quantified and normalized with GAPDH. Experiments were repeated for three times. *P < 0.05 vs. Ghrelin 0 h.

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* −/−

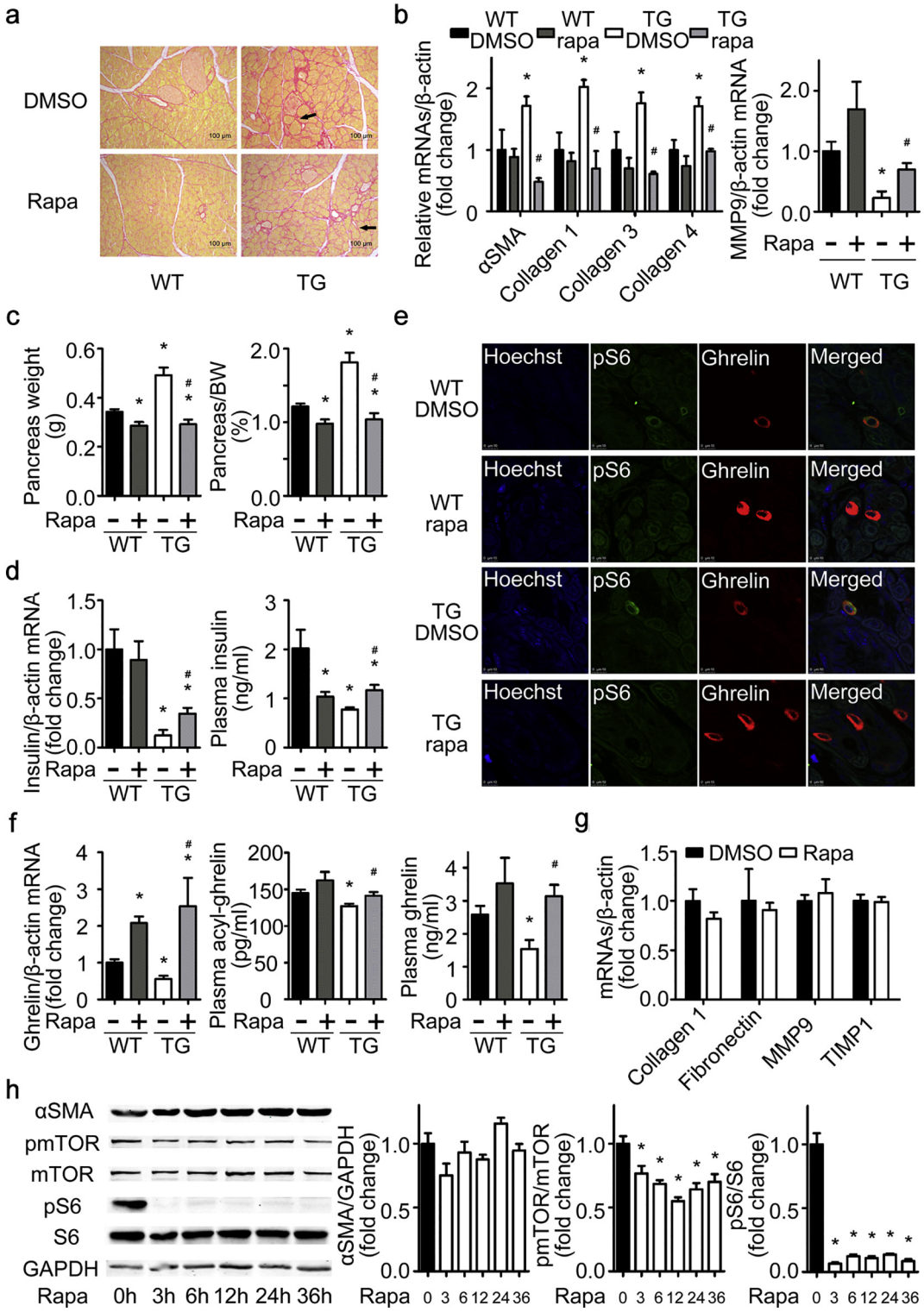

Fig. 7. Reversal effects of rapamycin. *Ghrl-TSC1* −/− mice and wild-type littermates fed with normal chow diet (NCD) for 16 weeks were used. Rapamycin (1 mg·kg^{-1}·d^{-1}) or DMSO was intraperitoneally injected for 2 weeks. a. Sirius Red staining of pancreas. Arrows identify the representative bands of fibrosis. b. mRNA levels of fibrosis-relative genes normalized to β-actin. c. Pancreas weights. d. Levels of insulin mRNA in pancreas and plasma insulin. e. Co-localization of pS6 (green) with ghrelin (red) in mouse stomach. Nuclei were stained with Hoechst dye (blue). f. Ghrelin mRNA in stomach, and plasma levels of acyl-ghrelin and total ghrelin. n = 8–12. *P < 0.05 vs. WT DMSO. #P < 0.05 vs. TG DMSO. g. Effects of rapamycin on pancreatic stellate cells. Cultured human pancreatic stellate cells were treated with rapamycin (1 nmol/l) or DMSO for 12 h. mRNA levels of collagen I, fibronectin, MMP9, and TIMP1 were analyzed by RT-qPCR. β-actin was used as internal control. Experiments were repeated for three times. h. Cultured human pancreatic stellate cells were treated with rapamycin (1 nmol/l) or DMSO for time indicated. αSMA, pmTOR, mTOR, pS6, and S6 were examined by western blots. GAPDH was used as internal control. Experiments were repeated for three times. *P> < 0.05 vs. Rapa 0 h.

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-TSC1*−/− 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 suggests 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 microenvironment. 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, Shiying 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.

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