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ORIGINAL ARTICLE

Wingless-type MMTV integration site family member 5a is a key inhibitor of islet stellate cells activation

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Keywords
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ABSTRACT

Aims/Introduction: Type 2 diabetes mellitus is a chronic metabolic disorder characterized by islet β-cell dysfunction, which might result from the activation of islet stellate cells (ISCs). Our recent study showed that a specific population of ISCs is prone to be activated in type 2 diabetes mellitus accompanied by reduced secretion of insulin. The wingless-type MMTV integration site family member 5a (Wnt5a)/frizzled-5 signaling pathway might play an important role in this process. The present study aimed to explore the effects of Wnt5a on the activation of ISCs isolated from *db/db* mice.

Materials and Methods: ISCs were isolated from *db/db* mice and matched *db/m* mice. Immunohistochemistry and western blotting analysis were applied for the determination of Wnt5a expression. Exogenous Wnt5a and lentivirus containing the target gene Wnt5a short hairpin ribonucleic acid were used as a molecular intervention. The experiment of transwell and wound healing was used to evaluate the migration of the isolated ISCs.

Results: Our data showed that the expression of Wnt5a and frizzled-5 was decreased in the ISCs isolated from *db/db* mice compared with *db/m* mice. Both the exogenous Wnt5a and the overexpression of Wnt5a could inhibit the outgrowth rate of ISCs from islets, and its viability, migration and α smooth muscle actin expression. These changes were associated with the inactivation of the Smad2/3 signaling pathway in a frizzled-5-dependent manner.

Conclusions: Our observations revealed a potential role of Wnt5a in preventing ISC activation. The maintenance of quiescent ISCs might be a desirable outcome of therapeutic strategies for diabetes mellitus.

INTRODUCTION

Type 2 diabetes mellitus is one of the most prevalent metabolic disorders characterized by insulin resistance, β-cell dysfunction or both[1]. Fibrosis of islets plays a prominent role in the progression of β-cell dysfunction. Emerging evidence suggested that the pathophysiological transformation of pancreatic stellate cells (PSCs) in both biological character and phenotype might contribute to the islet fibrosis in type 2 diabetes mellitus[2]. Our recent studies showed that a type of stellate cell located inside islet stellate cells (ISCs) is are similar, but not identical to, the classical PSCs. These cells are prone to being activated, leading to islet fibrosis and, consequently, reducing insulin secretion in type 2 diabetes mellitus. Based on the spatial localization of ISCs, we have verified that the activation of ISCs contributes to islet fibrosis and eventually induces the β-cell dysfunction in type 2 diabetes mellitus[3–6]. However, there is still very little knowledge about the causation and mechanism involved in the activation of ISCs.

This paper revealed a potential role of Wnt5a in preventing ISC activation. The maintenance of quiescent ISCs might be a desirable outcome of therapeutic strategies for diabetes mellitus.

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Wingless-type MMTV integration site family member (Wnt) signaling pathways are a group of signal transduction pathways that contribute to diverse physiological processes, including proliferation, survival, polarity, migration, cell fate determination and self-renewal[7]. Previous studies have shown that Wnt signaling takes part in the development of the endocrine pancreas and regulates mature β-cell functions, such as insulin secretion, β-cell survival and proliferation[8–11]. Wnt signaling might also be associated with the metabolic disorders of obesity and diabetes[12,13].

Wnt5a is involved in several non-canonical Wnt signaling pathways through binding to its receptor frizzled-5 (Fzd5), and acts as a critical modulator in several developmental processes and diverse pathogenic diseases, such as cancer, inflammatory diseases and metabolic disorders[14–17]. Wnt5a protein is also known to regulate various cellular functions, such as proliferation, differentiation, migration, adhesion and invasion[15,18–20]. Previous studies have shown that Wnt5a/Fzd5 is essential to islet formation and β-cell migration in the development of the pancreas in vertebrates[21]. A recent clinical study showed that serum Wnt5a was significantly decreased in newly diagnosed type 2 diabetes mellitus patients compared with normal controls[22]. However, the effects of Wnt5a on the activation of ISCs has not yet been fully clarified.

Considering the effect of stimulation of quiescent ISCs on the insulin secretion of β-cells through Wnt5a, and the clinical findings described above, we hypothesized that Wnt5a might be a key modulator of ISC-mediated dynamic homeostasis of the islet function. Therefore, further elucidating the association between the Wnt5a protein and ISC activation, especially in regard to the suppression of ISCs activation, might be beneficial to identifying potential therapeutic targets for the prevention of type 2 diabetes mellitus.

METHODS

Animals

C57BL/KsJ-db/db mice, *db/m* mice, Wistar rats and Goto-Kakizaki rats (8–12-week-old males) were purchased from the Model Animal Research Center of Nanjing University (Nanjing, China). This study protocol was reviewed and approved by the Animal Care and Use Committee according to institutional guidelines and national animal welfare.

Isolation and culture of mouse ISCs

Islets and ISCs were isolated from *db/db* mice, *db/m* mice, Wistar rats and Goto-Kakizaki rats, as described previously[23]. Isolated mouse islets were divided into groups of 50 for culture alone or with exogenous Wnt5a (0.05 μg/mL; Wnt5a; R&D Systems, Abingdon, UK) for 48 h, unless otherwise specified.

Oil red O staining

ISCs were culture alone or with exogenous Wnt5a (0.05 μg/mL) for 48 h, unless otherwise specified. Oil red O staining (Sigma, Carlsbad, CA, USA) was carried out as described previously[23].

Immunohistochemistry

Pancreases (12, at 36 weeks-of-age) were perfused and fixed in 4% paraformaldehyde in 0.1 mol/L phosphate-buffered saline for 24 h at 4°C, paraffin-embedded and sectioned (4 μm). Immunohistochemistry was carried out as described previously[23].

Immunofluorescence microscopy of Wnt5a and Fzd5

Immunofluorescence microscopy was carried out as described previously[23] to evaluate differences between ISCs isolated from mice and rats in the expression of Wnt5a, Fzd5 and α-smooth muscle actin (α-SMA; 1:200; Abcam, Cambridge, UK). All immunocytochemical analyses were carried out in triplicate.

Determination of cell viability

Cell viability was assessed directly by WST-8 assay as described previously. ISCs were either cultured for exogenous Wnt5a, overexpression of Wnt5a, sh-Fzd5−/− and sh-Fzd5−/−+ exogenous Wnt5a for 24 h, unless otherwise specified. All experiments were carried out in quintuplicate on three separate occasions (n = 15).

Determination of cell proliferation

Cell proliferation was assessed directly by Edu assay. ISCs were cultured alone or with exogenous Wnt5a (0.05 μg/mL) for 48 h, unless otherwise specified. All experiments were carried out in quintuplicate on three separate occasions (n = 15).

Detection of cell migration

Wound healing

ISCs were seeded in six-well culture plates and grown for 48–72 h to reach confluence. ISCs were cultured as described above. After the cells were starved in serum-free medium overnight, wound healing assays were carried out as described previously[23]. The wound area was measured by tracing the wound margin and calculated using an image analysis program (Image J, National Institutes of Health, Bethesda, Maryland, USA).

Transwell assay

ISCs were seeded in transwells (BD 353097; 8-mm pores), and cultured as described above; then, transwell assays were carried out as described previously[23].

Apoptosis assay

A Caspase-Glo 3/7 (Caspase-Glo®; Promega, Madison, Wisconsin, USA) apoptosis detection kit was used to analyze cell apoptosis as described previously[24].

Western blotting analysis

Islets, ISCs, MPC-83 (mouse pancreatic acinar cancer cell line) and Min6 cells were harvested, and washed with cold phosphate-buffered saline and lysed with ice-cold lysis buffer supplemented with protease inhibitors (Roche, Basel, Switzerland) as described previously[23]. The primary antibodies used were as

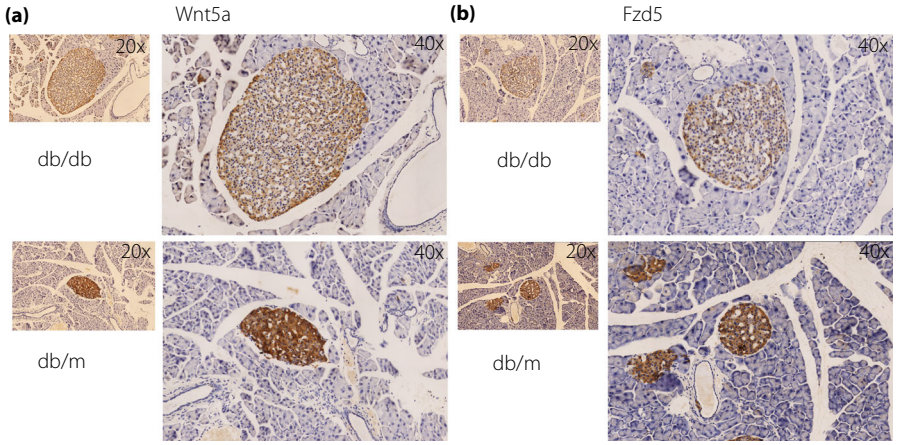

Figure 1 | Expression of (a) wingless-type MMTV integration site family member 5a (Wnt5a) and (b) frizzled-5 (Fzd5) in the pancreases of *db/db* mice and *db/m* mice. Wax-embedded sections of *db/db* and *db/m* mouse pancreases showing Wnt5a and Fzd5 expression by immunohistochemistry (magnification: ×20, ×40).

follows: Wnt5a (1:1,000; Abcam), Fzd5 (1:1,000; Abcam), β-catenin (1:1,000; Abcam), Ror2 (1:1,000; Abcam), P-CamKII (1:1,000; Abcam), α-SMA (1:1,000; Abcam), Smad2/3, P-Smad2/3 and β-Actin (1:2,000; Sigma).

Short hairpin ribonucleic acid preparation and targeting gene knockdown

The mouse genes, Wnt5a and Fzd5 were selected from GenBank. Specific short hairpin ribonucleic acids (shRNAs) and control shRNA were synthesized by GENECHEM (Shanghai, China). A Basic Local Alignment Search Tool search was carried out of the National Center for Biotechnology Information database to ensure that the shRNA constructs targeted only mouse overexpression of Wnt5a (Wnt5a +/+) and knockdown of Fzd5 (Fzd5–/–). The lentiviruses containing the targeted gene shRNA were collected and used to transfect ISCs according to the manufacturer's instructions.

Statistical analysis

The results are expressed as the mean ± standard error of the mean for quantitative data. The statistical significance of multiple comparisons was determined using Bonferroni's *t*-test with SAS (SAS Institute, Cary, NC, USA), and differences were considered significant when $P < 0.05$.

RESULTS

Expression of Wnt5a and Fzd5 in the islets and the isolated ISCs

Pancreatic sections were fixed in 4% paraformaldehyde in phosphate-buffered saline for immunohistochemistry testing. Staining of sections for Wnt5a and Fzd5 protein was carried out as shown in Figure 1. Lightly stained Wnt5a+ and Fzd5+ cells were shown in pancreases from hyperglycemic *db/db* mice. In contrast, pancreases from *db/m* mice contained several heavily immunostained Wnt5a+ and Fzd5+ cells throughout the pancreas. Both the immunohistochemistry and western blot analysis showed that the expression of Wnt5a+ and Fzd5+ in the islets and ISCs isolated from hyperglycemic *db/db* mice were significantly decreased compared with those isolated from *db/m* mice (Figures 1 and 2). The expression of Wnt5a and fzd5 was found in both MPC-83 cells and Min6 cells (Figure 2). These

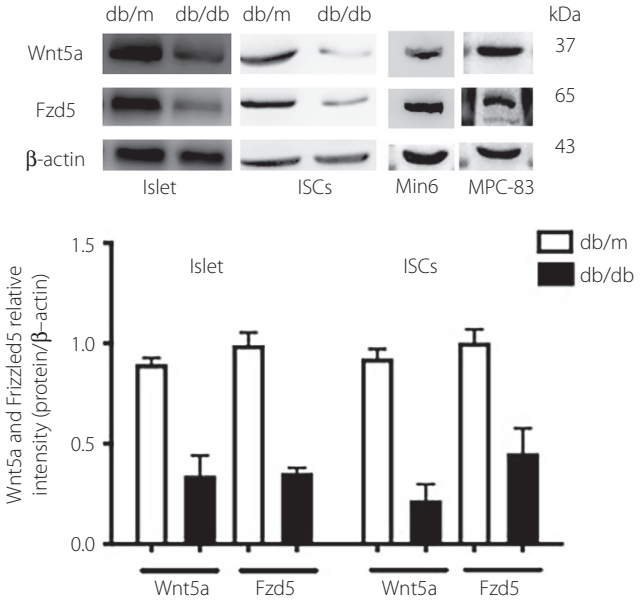

Figure 2 | Expression of wingless-type MMTV integration site family member 5a (Wnt5a) and frizzled-5 (Fzd5) in the islets, islet stellate cells (ISCs), Min6 and MPC-83. Quantification of Wnt5a and Fzd5 protein levels by western blotting in islet, ISCs, Min6 and MPC-83. The data are expressed as the mean ± standard error ($n = 3$). *$P < 0.05$, **$P < 0.01$, *db/db* ISCs compared with *db/m* ISCs.

observations were confirmed by the immunofluorescence microscopy measurements (Figures S1 and S2), in which both Wnt5a and Fzd5 immunoreactivities were higher in ISCs isolated from Wistar rats than in ISCs from Goto-Kakizaki rats. Taken together, these data show that the activation of ISCs in diabetic mice/rats with hyperglycemia were decreased compared with that of normoglycemic controls.

Exogenous or overexpression of Wnt5a inhibits the activation of ISCs isolated from *db*/*db* mice

We used shRNA-mediated overexpression of Wnt5a and knockdown of Fzd5 in *db*/*db* ISCs (Figure S3). As shown in Figure 3a, the outgrowth rate of ISCs isolated from *db*/*db* mice was much higher than *db*/*m* controls. Furthermore, we also observed a series of changes in the ISCs from *db*/*db* mice, such like the loss of Oil red O-stained fat droplets (Figure 3b), enhanced cell migration (Figure 4), increased cell viability and proliferation (Figure 5a, Figure S4), and elevated expression of α-SMA. However, either overexpression of Wnt5a or addition of exogenous Wnt5a (0.05 μg/mL) could reverse all the changes described above (Figures 3–5). Figure 5b shows that preculture for 48 h in the presence of 0.05 μg/mL Wnt5a had no effect on levels of caspase 3/7 activity in the absence of a cocktail of inflammatory cytokines (interleukin-1β, interferon-γ and tumor necrosis factor-α). In view of the combination of Wnt5a and its receptor Fzd5 being fundamental to the Wnt signaling pathway, *db*/*db* ISCs had lower levels than control ISCs of β-catenin, Ror2 and P-CamKII, as shown in Figure 6a. Incubation of *db*/*db* ISCs with exogenous Wnt5a caused a significant increase of β-catenin and P-CamKII, with a light increase in the levels of Ror2 (Figure 6a). The downregulation of Fzd5 through shRNA (Fzd5−/−) abolished the effects of exogenous Wnt5a in α-SMA (Figure 6b). Thus, these results show that Wnt5a suppresses the activation of ISCs through its receptor, Fzd5; and by this pathway, decreases the development of fibrosis of islets in type 2 diabetes mellitus.

Wnt5a inhibits the activation of ISCs through Smad2/3 signaling

To determine the potential intracellular signaling cascades involved in the Wnt5a/Fzd5 system regulation of ISC activation, western blotting was used to identify specific signaling elements. ISCs isolated from hyperglycemic *db*/*db* mice maintain higher levels of phosphorylation and Smad2/3 activation than those of normoglycemic *db*/*m* controls (Figure 6b). Co-culture with exogenous Wnt5a resulted in a significant reduction in the phosphorylation of Smad2/3 in the ISCs isolated from hyperglycemic *db*/*db* mice. In contrast, total Smad2/3 expression has no change by treatment with exogenous Wnt5a, showing that the effects of Wnt5a were on the activation of the intracellular signaling pathways. Upregulation of Wnt5a promotes the decreases of Smad2/3 phosphorylation (Figure 6), but Fzd5

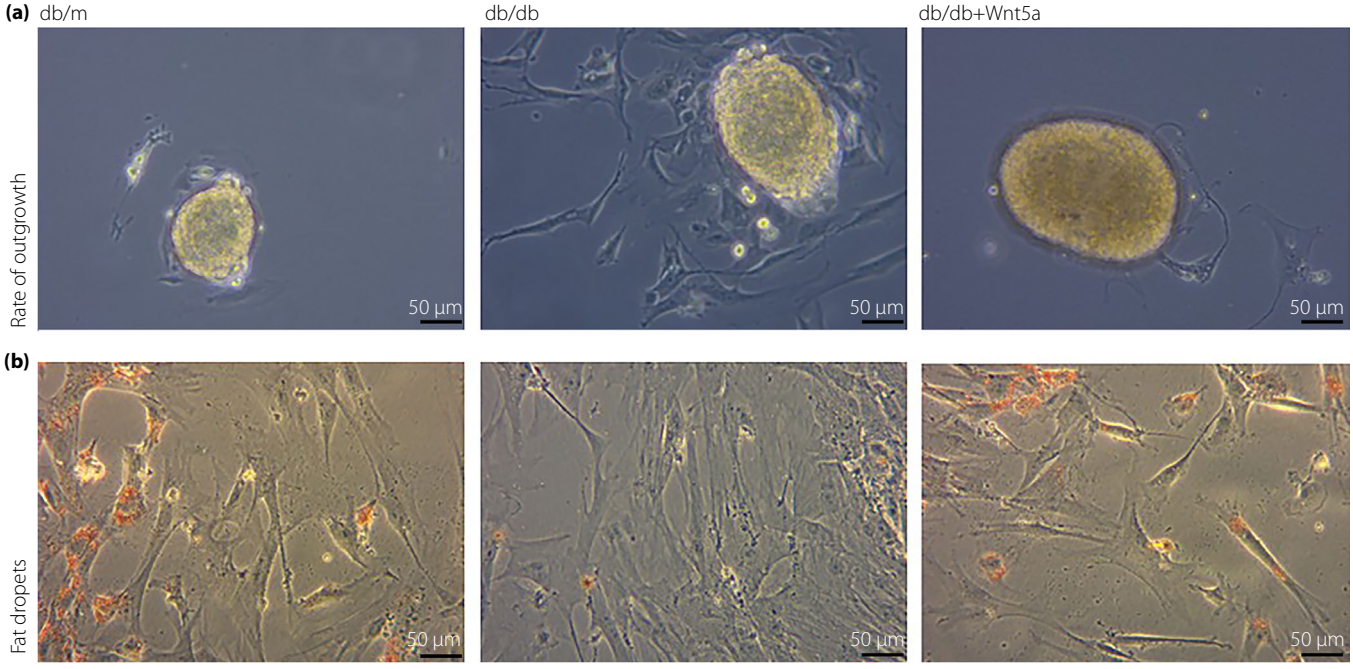

Figure 3 | Wingless-type MMTV integration site family member 5a (Wnt5a) inhibits the rate of outgrowth and decreases the loss of lipid droplets in *db*/*db* islet stellate cells (ISCs). (a) Light microscopy micrographs show faster rates of ISC outgrowth from diabetic islets. Wnt5a inhibited ISC activation, as determined by a significantly decreased rate of outgrowth from the islets. Scale bar, 50 μm. (b) Oil red O staining of lipid droplets in the cytoplasm of ISCs shows faster activation and loss of lipid droplets in diabetic ISCs. Wnt5a inhibited ISC activation, as determined by a significant decrease in the loss of lipid droplets. Scale bar, 50 μm.

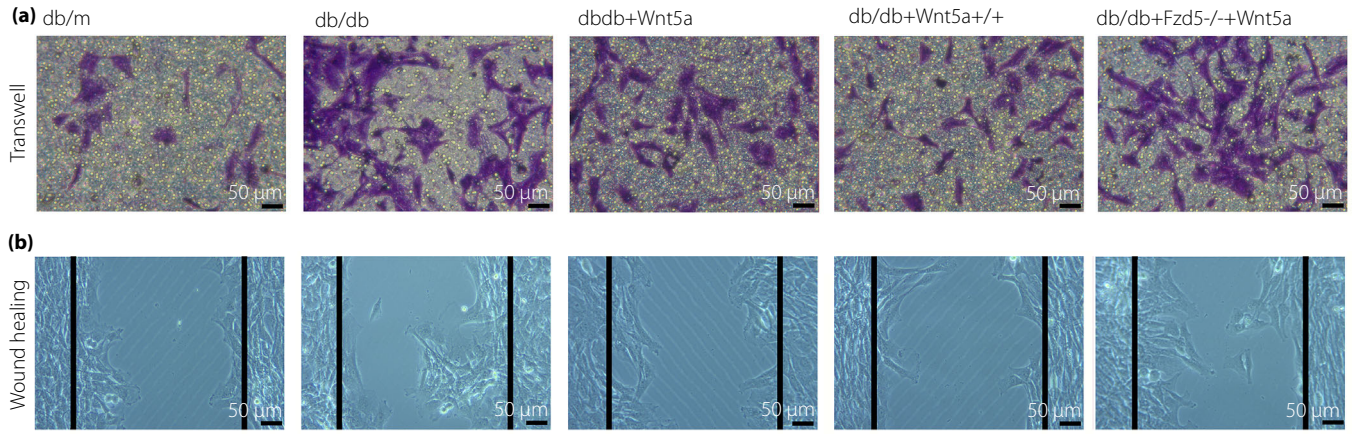

Figure 4 | Wingless-type MMTV integration site family member 5a (Wnt5a) inhibits the migration of islet stellate cells (ISCs) isolated from *db/db* mice. (a) Transwell assays for migration rates show more diabetic ISCs migrating across the wound. Wnt5a inhibited their activation, as determined by a significant decreased in migration. Scale bar, 50 μm. (b) Wound healing assays for migration rates show greater diabetic ISC migration. Wnt5a inhibited ISC activation, as determined by a significant decrease in migration. Scale bar, 50 μm.

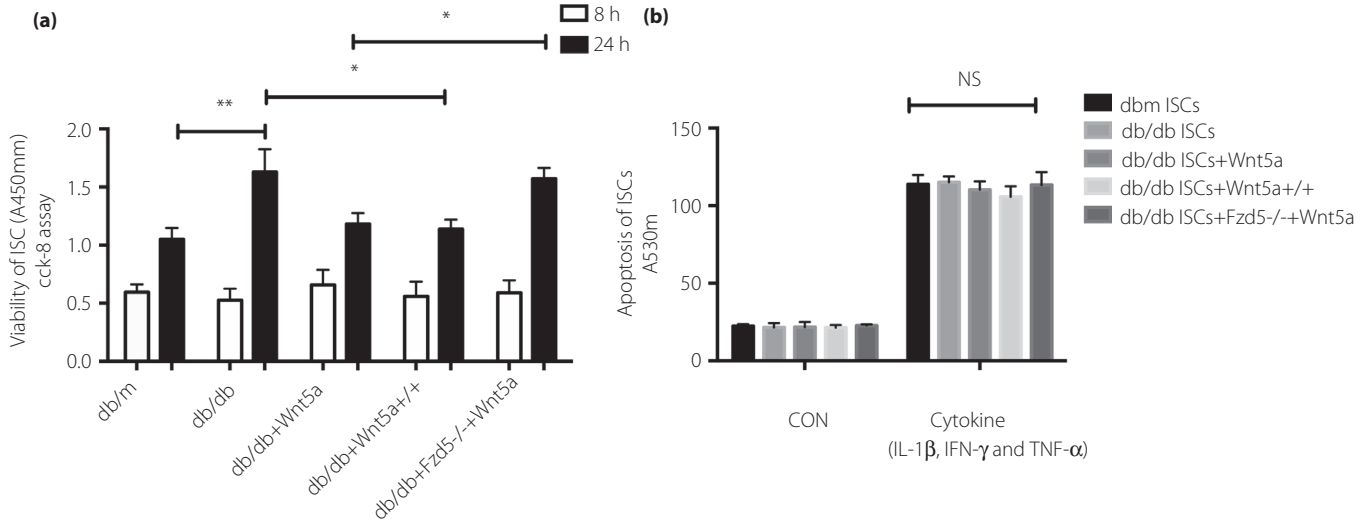

Figure 5 | Wingless-type MMTV integration site family member 5a (Wnt5a) inhibits islet stellate cells (ISCs) viability in *db/db* mice with no change in apoptosis. (a) CCK-8 assay of ISC viability. ISC viability was activated faster in diabetic ISCs than in *db/m* ISCs. Wnt5a inhibited ISC activation, as determined by a significant decreased in viability. The data are expressed as the mean ± standard error ($n = 15$); $^{*}P < 0.05$, $^{**}P < 0.01$. (b) Apoptosis assay in ISCs. The data are expressed as the mean ± standard error ($n = 3$); $^{*}P < 0.05$, $^{**}P < 0.01$.

downregulation could block the effects of exogenous Wnt5a on Smad2/3 signaling. Taken together, these observations suggested that Wnt5a inhibits the Smad pathways in ISCs from hyperglycemic *db/db* mice through Fzd5.

DISCUSSION

The present study aimed to investigate the effects of Wnt5a on ISCs activation and its intracellular signaling mechanism. Our data showed that exogenous Wnt5a could inhibit the outgrowth rate of ISCs in *db/db* mice, and lead to suppressed cell migration, decreased cell viability and reduced expression of α-SMA. All of these effects were associated with the inactivation of the Smad2/3 signaling pathway in a Fzd5-dependent manner. We hereby certify to the best of our knowledge, that this is the first report to determine the effects of Wnt5a on ISCs activation.

Type 2 diabetes mellitus is partially attributed to the dysfunction of β-cells in a diabetic environment[25]. Islet fibrosis accounts for a pivotal part in the progression of β-cell failure, because it can lead to β-cell apoptosis[26] and results in a decreased mass of functional β-cell[27]. There is emerging evidence that the activation of ISCs is essential for islet fibrosis to a certain extent[3–6]. The biological characteristics and phenotype

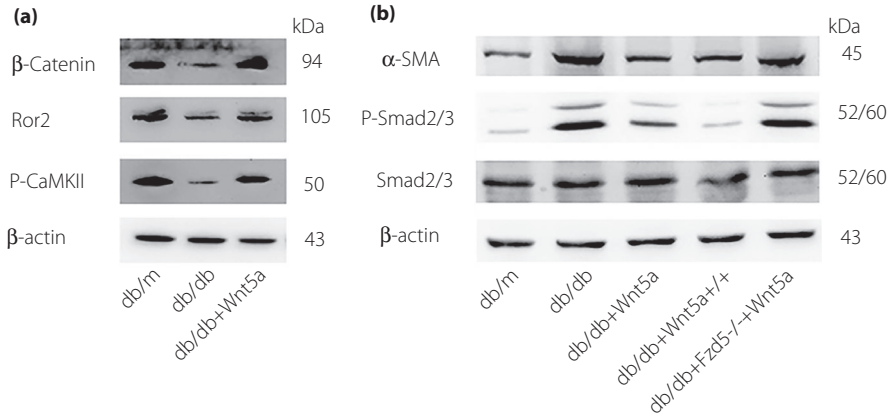

Figure 6 | Wingless-type MMTV integration site family member 5a (Wnt5a) activates the non-canonical Wnt5a pathway and suppress the Smad2/3 activation in islet stellate cells (ISCs). (a) Quantification of β-catenin, Ror2 and P-CamKII protein levels by western blotting of *db/m* ISCs, *db/db* ISCs and *db/db* ISCs treated with exogenous Wnt5a. (b) Western blotting of *db/m* ISCs, *db/db* ISCs and *db/db* ISCs treated with exogenous Wnt5a, overexpression of Wnt5a, Fzd5–/– and Fzd5–/–+ exogenous Wnt5a by and α-smooth muscle actin (α-SMA), Smad2/3 and P-Smad2/3 antibodies.

of the ISCs were modified in the condition of hyperglycemia both *in vitro* and *in vivo*. The elevated rate of outgrowth from the islets and loss of Oil red O-stained fat droplets, migration, viability and α-SMA expression contributed to the activation of diabetic ISCs, all of which are involved in the fibrotic transformation process. The disappearance of fat droplets inside islet stellate cells is an important marker of its activation. Adipogenic transcriptional regulation is essential for maintaining stellate cells in their quiescent with plenty of fat droplets. For instance, activated hematopoietic stem cells (HSCs), which have many biological features in common with ISCs, have decreased expression of peroxisome proliferator-activated receptor-γ. Ectopic expression of sterol regulatory element-binding transcription factor 1c or peroxisome proliferator-activated receptor-γ can reverse fully activated HSCs to quiescent HSCs[28]. In the previous experiments, we found that the ISCs transfected with the sterol regulatory element-binding transcription factor 1c gene were intracellularly re-enriched with lipid droplets, whereas the expression of α-SMA decreased, and extracellular matrix-related proteins, such as fibronectin and collagen type I expression, are also reduced. Retinol treatment can inhibit ISC activation, which is also confirmed in PSC-related studies[29]. It was shown that the activated-state ISC phenotype can be reversed. Taken together, the present data showed that ISCs were activated in response to the diabetic environment and promote islet fibrosis, and consequently, lead to the loss of functional β-cell mass, which was in accordance with our previous study[23]. The pancreatic regenerating protein 1 inhibited activation of ISCs through its receptor, EXTL3.

In recent years, many studies have shown that the Wnt signaling cascade is integral in numerous biological processes, including the development and progression of fibroproliferative diseases, such as pulmonary fibrosis[30–33], renal fibrosis[34–36], post-infarct remodeling[37] and hypertrophic scars[38,39]. A recent study reported that the non-canonical Wnt pathway is involved in the "antiadipogenic" activation of HSCs, and liver fibrosis could be inhibited by Wnt antagonism, which might be used as a new therapeutic target for liver fibrogenesis[40]. Our studies have showed that the non-canonical Wnt5a pathway is a key modulator of the activation of ISCs in a hyperglycemic environment. Previous results implicated a role of Wnt5a/Fzd5 signaling in proper insulin cell migration and islet formation during pancreatic development[21]. Clinical research also suggested that serum-secreted Fzd5, a secreted Wnt antagonist, was increased in patients with type 2 diabetes mellitus[22]. The present clinical study also identified that Wnt5a was significantly downregulated in newly diagnosed type 2 diabetes mellitus patients. An *in vitro* study showed that Wnt5a could increase the level of insulin when ISCs are co-cultured with islet β-cells[41]. In the present study, we showed that Wnt5a, Fzd5, β-catenin, Ror2 and P-CamKII were expressed in ISCs, albeit at a relatively high level under normoglycemic conditions. However, the expression of Wnt5a, Fzd5, β-catenin, Ror2 and P-CamKII were significantly decreased in ISCs isolated from diabetic *db/db* mice, which showed that the diabetic environment could downregulate their expression in ISCs.

In accordance with this result, the present study found that *db/db* ISCs activation was significantly inhibited after treatment with exogenous Wnt5a and the shRNA-induced overexpression of Wnt5. Thus, exposure of ISCs in a diabetic environment would lead to the loss of their ability to maintain the quiescent status and lead to the downregulation of the non-canonical Wnt5a pathway. Our observations showed that the Wnt5a/Fzd5 system inhibited the activation of ISCs and, subsequently, potential fibrotic transformation in a hyperglycemic environment. In regard to the downregulation of Fzd5 abolishing the effects of exogenous Wnt5a, we concluded that the effects of Wnt5a were mediated through its receptor, Fzd5. Thus, our

current study showed that Wnt5a acted through its receptor, Fzd5, as a suppressive factor of the ISCs activation and subsequent islet fibrosis in type 2 diabetes mellitus.

We had also showed that Smad2/3 signaling, the key pancreatic fibrosis parameter, was a switch molecule of ISC activation[23]. In the present study, we reported for the first time that endogenous and exogenous Wnt5a decreased the phosphorylation and the activation of Smad2/3, which is increased in diabetic *db*/*db* ISCs. Previous studies showed that Smad pathway activity increased in rat PSCs and mice ISCs, and subsequently induced extracellular matrix synthesis and elevated expression of α-SMA[23,42]. Wnt5a protein reduced the expression of α-SMA through the inhibition of the Smad pathway, and further reversed phospho-Smad2/3 levels to those of quiescent *db*/*m* ISCs, which was consistent with our demonstration that the Smad pathway was activated in diabetic ISCs. Although these *in vitro* findings support our hypothesis that Wnt5a is important for the inhibition of ISC activation in a diabetic environment, further experiments are required to investigate the role of Wnt5a in association with the activation of ISCs, such as biochip technology and transgenic models, and eventually to confirm the importance of this process in the pathogenesis of type 2 diabetes mellitus. Hence, more detailed intracellular signaling mechanisms of Wnt5a on the activation of ISCs need to be clarified.

In summary, the present study showed that Wnt5a could inhibit the activation of ISCs, which might consequently delay the development of islet fibrosis and provide new therapeutic targets for the prevention of type 2 diabetes mellitus.

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DISCLOSURE

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 | Expression of wingless-type MMTV integration site family member 5a and frizzled-5 in the islet stellate cells from rats.
Figure S2 | Expression of wingless-type MMTV integration site family member 5a and α-smooth muscle actin in the islet stellate cells from mice.
Figure S3 | Overexpression levels of wingless-type MMTV integration site family member 5a and knockdown for frizzled-5 in *db*/*db* islet stellate cells.
Figure S4 | Wingless-type MMTV integration site family member 5a inhibits islet stellate cells proliferation in *db*/*db* mice.