Sfrp5 mediates glucose-induced proliferation in rat pancreatic β-cells

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Abstract

The cellular and molecular mechanisms of glucose-stimulated β-cell proliferation are poorly understood. Recently, secreted frizzled-related protein 5 (encoded by *Sfrp5*; a Wnt signaling inhibitor) has been demonstrated to be involved in β-cell proliferation in obesity. A previous study demonstrated that glucose enhanced Wnt signaling to promote cell proliferation. We hypothesized that inhibition of SFRP5 contributes to glucose-stimulated β-cell proliferation. In this study, we found that the *Sfrp5* level was significantly reduced in high glucose-treated INS-1 cells, primary rat β-cells, and islets isolated from glucose-infused rats. Overexpression of SFRP5 diminished glucose-stimulated proliferation in both INS-1 cells and primary β-cells, with a concomitant inhibition of the Wnt signaling pathway and decreased cyclin D2 expression. In addition, we showed that glucose-induced *Sfrp5* suppression was modulated by the PI3K/AKT pathway. Therefore, we conclude that glucose inhibits *Sfrp5* expression via the PI3K/AKT pathway and hence promotes rat pancreatic β-cell proliferation.

Key Words
- *Sfrp5*
- glucose
- β-cell
- proliferation

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Introduction

The β-cell mass is dynamic and can change rapidly on insulin demand during insulin resistance to maintain normoglycemia (Finegood *et al.* 1995, Bonner-Weir 2000, Accili 2001). Reductions in the β-cell mass are associated with type 2 diabetes in humans (Butler *et al.* 2003). The β-cell mass is regulated by a complicated balance of cell proliferation, neogenesis, apoptosis, and hypertrophy (Rhodes 2005). The proliferation of β-cells is considered to be a major component in maintaining the β-cell mass (Dor *et al.* 2004, Teta *et al.* 2007). However, the identity of the factors that control the β-cell mass remain elusive. Elucidating the factors and mechanisms responsible for pancreatic β-cell mass maintenance is crucial for the development of specific drug treatments for type 2 diabetes.

Several mitogens have been implicated in β-cell proliferation, including glucose, insulin-like growth factor 1 (IGF1), placental lactogens (PL), prolactin (PRL), glucagon-like peptide 1 (GLP1), hepatic growth factor (HGF), and parathyroid hormone-related protein (PTHrp) (Chick 1973, Brelje *et al.* 1993, Hugl *et al.* 1998, Buteau *et al.* 2003, Cozar-Castellano *et al.* 2006, Vasavada *et al.* 2007). Glucose is a potent β-cell mitogen that increases β-cell proliferation both *in vitro* and *in vivo* (Chick 1973). *In vitro*, glucose stimulates the proliferation of several rodent insulinoma cell lines and primary rodent islets

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(Assmann *et al*. 2009, Metukuri *et al*. 2012, Zhang *et al*. 2012). In rat and mouse models, glucose infusion *in vivo* also results in an approximately 50% increase in β-cell proliferation (Bonner-Weir *et al*. 1989, Alonso *et al*. 2007, Zhang *et al*. 2012). Glucose has been proposed to upregulate the expression of insulin receptor substrate 2 (IRS2) through increased cytosolic calcium levels and then activate the PI3K/AKT pathway to induce β-cell proliferation (Jhala *et al*. 2003, Heit *et al*. 2006, Porat *et al*. 2011). Other studies also reported the glucose-induced expression of cell-cycle regulators, including cyclin D2, and CDK4, and increased ChREBP transcriptional activity (Metukuri *et al*. 2012). Despite these observations, the mechanism mediating the mitogenic effect of glucose on β-cells is not fully understood.

Secreted frizzled-related protein 5 (SFRP5) is a member of the secreted frizzled-related protein family. SFRP5 sequesters WNT proteins in the extracellular space and prevents them from binding to their receptors, thereby inhibiting the activity of the Wnt signaling pathway (Kawano & Kypta 2003). SFRP5 was one of the most extensively studied proteins in adipose tissue (Ouchi *et al*. 2010, Mori *et al*. 2012). Recently, *Sfrp5* has been reported to be downregulated in the pancreatic islets of cafeteria diet-fed rats and involved in β-cell proliferation during the expansion of the β-cell mass in obesity through the regulation of Wnt signaling activity (Rebuffat *et al*. 2013). In our previous study, we performed Affymetrix microarrays on islets from a glucose-induced β-cell proliferation rat model, establishing by intravenous infusion of 50% glucose into rat. We observed a 64% decrease in *Sfrp5* expression in the islets from glucose-infused rats (Supplementary Fig. 1, see section on supplementary data given at the end of this article). The downregulation of *Sfrp5* activates Wnt signaling, which has been suggested to be a possible modulator of β-cell proliferation (Rulifson *et al*. 2007, Chocarro-Calvo *et al*. 2013). A previous study demonstrated that glucose enhanced Wnt signaling to promote cancer cell proliferation (Mao *et al*. 2013). In this study, we sought to clarify whether *Sfrp5* was involved in the adaptive β-cell expansion induced by glucose through the inhibition of the Wnt signaling pathway.

In the current study, we found that *Sfrp5* levels were significantly reduced in high glucose-treated INS-1 cells, primary rat islets, and islets isolated from rats infused with glucose for 24 h. Overexpression of SFRP5 diminished glucose-stimulated proliferation in both INS-1 cells and primary β-cells, and was correlated with the inhibition of the Wnt signaling pathway. Moreover, glucose-induced *Sfrp5* suppression was modulated by the PI3K/AKT pathway. We concluded that glucose reduced the *Sfpr5* expression level via PI3K/Akt signaling and activated Wnt signaling to promote β-cell proliferation. This result could represent one of the intracellular mechanisms underlying glucose-stimulated β-cell proliferation.

Materials and methods

Glucose infusion animal model

Male Sprague–Dawley rats (approximately 8 weeks of age, 200–250 g) were obtained from the Shanghai Experimental Animal Center, Chinese Academy of Sciences. The infusion into the animals was performed as described previously (Bonner-Weir *et al*. 1989). Briefly, adult male Sprague–Dawley rats were catheterized in the jugular vein by surgery. After a 3 to 5-day recovery period, the rats received infusions of 0.45% saline ($n=10$) or 50% glucose ($n=10$) with 500 μg/mL bromodeoxyuridine (Sigma-Aldrich) at a constant rate of 2 mL/h for 24 h. The animals were allowed free access to regular chow and water during the infusion period. Blood samples were drawn from the tail veins. Blood glucose concentrations were measured using a portable blood glucose meter (Accu-Chek, Roche). After 24 h of infusion, a group of the infused animals was killed to isolate the islets. The remaining animals were killed for histological analyses. All procedures were performed in accordance with the principles of the Guide for the Care and Use of Experimental Animals of Shanghai Jiaotong University School of Medicine and were approved by the Animal Care Committee at Shanghai Jiaotong University.

Islet purification and culture

The islets were isolated from male Sprague–Dawley rats by common ductal collagenase infusion and separated by density gradient centrifugation (Zhang *et al*. 2012). Freshly isolated rat islets were transferred to a 6 cm dish and cultured overnight in DMEM containing 5.6 mM glucose, 10 mM HEPES, 10% FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified 5% CO_2 atmosphere before the next experiment.

Cell culture

INS-1 cells (passage 23–35) purchased from the CAMS Cell Culture Center (Beijing, China) were grown in RPMI 1640 medium containing 11.1 m glucose and supplemented with 10 mM HEPES, 10% FBS, 2 mM L-glutamine, 1 mM sodium

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pyruvate, 5 μl/L β-mercaptoethanol, 100 IU/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified 5% CO_2 atmosphere. The cells were subcultured when they reached 80% confluence.

Adenovirus infection and plasmid transfection

The *Sfrp5* adenovirus was a gift from Dr Jiqiu Wang (Shanghai Jiaotong University). The control green fluorescent protein (GFP) adenovirus was purchased from Boshang Biotech Company (Shanghai, China). INS-1 cells were plated in 6-well plates 1 day before infection. When the cell reached 70–80% confluence, each well was infected with adenovirus at an MOI of 20. For isolated rat islets, adenovirus was added at an MOI of 500. PcDNA3.1-ca*Akt* plasmid transfections were conducted using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Proliferation assay

For INS-1 cells, proliferation was assessed by 5-ethynyl-2′-deoxyuridine (EdU) incorporation using the Click-iT EdU Flow Cytometry Assay Kit (Invitrogen). INS-1 cells were plated in a 6-well plate at a density of 3×10^5 cells/well in the growth medium. The next day, the cells were synchronized overnight with 2.8 mmol/L glucose and 0.2% BSA. For EdU incorporation, the cells were subsequently stimulated for 24 h in medium supplemented with various concentrations of glucose and 0.2% BSA. The EdU solution was added to the medium during the final 4 h of the 24 h incubation period. Detection of EdU incorporation was determined by flow cytometry using a BD FACSCalibur flow cytometer (Becton Dickinson Biosciences).

For isolated rat islets, the proliferation ratio was measured by the Click-iT EdU Image Assay Kit (Invitrogen). Isolated rat islets were dispersed with 0.25% trypsin/EDTA (Gibco) and plated on coverslips treated with poly-l-lysine. Then, the dispersed rat islet cells were cultured for 24 h in media with 5.6 mmol/L glucose supplemented with 10% FBS. Next, steps were performed similar to those described for the INS-1 cells except that EdU was added for the last 24 h.

Real-time PCR

Total RNA was extracted from INS-1 cells and isolated islets using the TRIzol reagent (Invitrogen) according to the manufacturer's protocol. After quantification using a spectrophotometer, 2 μg of total RNA were used for reverse transcription with Superscript III (Invitrogen) with random hexamer primers. Quantitative PCR amplification and detection were performed with the SYBR Premix Ex Taq Mixes (TaKaRa, Kyoto, Japan) on an ABI Prism 7000 Sequence Detection System (Applied Biosystems). The specific primers used for real-time PCR were as follows: *Sfrp5* (forward: 5′-GCCCAG AAGAAGAAGAAGCTGC-3′; reverse: 5′-TCTTCTT-GTCCCAGCGGTAGAC-3′) and β-actin (forward: 5′-AGGC CCCTCTGAACCCTAAG-3′; reverse: 5′-GGAGCGCGTAA CCCTCATAG-3′). The expression levels were normalized to β-actin. The results were analyzed using the $2-\Delta\Delta^{Ct}$ method.

Western blot

The concentrations of proteins in the islets and INS-1 cell lysates were quantitated by BCA protein assay (Pierce). Nuclear and cytoplasmic lysates were prepared with the NE-PER Kit (Pierce) according to the manufacturer's instructions. Protein samples (20 μg) were separated by electrophoresis with an 8% SDS-PAGE gel and transferred onto a polyvinylidene fluoride membrane, followed by immunoblotting according to the protocol outlined by Cell Signaling Technology. The blotted membrane was developed with ECL Advance (Cell Signaling) and imaged with a LAS-4000 Super CCD Remote Control Science Imaging System (GE). The relative abundance was quantified by densitometry using Quantity One 4.6.2 software (Bio-Rad). The following antibodies were used: mouse anti-SFRP5 (1:500, Santa Cruz Biotech), mouse anti-cyclin D2 (1:700, Abcam), rabbit anti-pAKT(473) (1:1000, Cell Signaling), rabbit anti-AKT (1:1000, Cell Signaling), mouse anti-tubulin (1:20,000, Sigma-Aldrich), and mouse anti-actin (1:1000, Santa Cruz).

Immunocytofluorescence staining and β-cell replication analysis

The pancreas were fixed in 4% paraformaldehyde at 4°C overnight. The tissues were dehydrated, embedded in paraffin, and sectioned at 5 μm thickness. The primary antibodies used were as follows: rabbit anti-BrdU (1:50, Sigma-Aldrich), guinea pig anti-insulin (1:800, Dako), mouse anti-SFRP5 (1:50, Santa Cruz Biotech), and rabbit anti-glucagon (1:100, Santa Cruz Biotech). Incubations were performed overnight in a humidified chamber at 4°C. For BrdU/insulin staining, sections were treated with 1 M HCl at 37°C for 60 min before incubation

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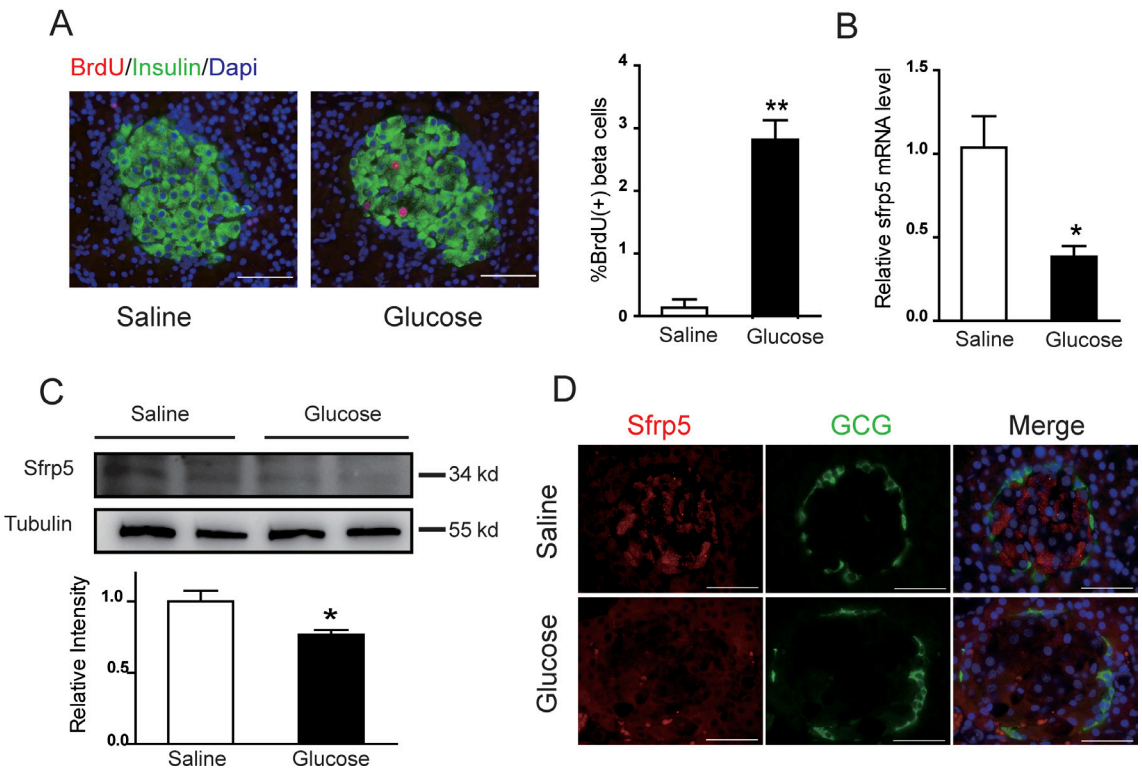

Figure 1
Glucose increases β-cell proliferation and reduces Sfrp5 expression in islets of glucose-infused rats (A) Immunofluorescence staining and analysis of BrdU (red) in pancreatic sections from rats infused with either saline or 50% glucose for 24 h (n = 3 rats per group). The percentage of BrdU and insulin double-staining cells were calculated. (B) Quantitative PCR analysis of *Sfrp5* mRNA levels in islets from rats infused with either saline or glucose for 24 h (n = 4–5 rats per group). (C) Western blot detection of SFRP5 protein levels in islets from rats infused with either saline or glucose for 24 h. SFRP5 band intensities were normalized based on the corresponding tubulin intensity (n = 3 rats per group). (D) Immunofluorescence staining for SFRP5 (red) and GCG (green) in pancreatic sections from rats infused with either saline or glucose for 24 h. Data are presented as means ± S.E.M. of independent experiments as indicated above, *$p < 0.05$, **$p < 0.01$ for differences between the two groups.

with the primary antibody. Secondary antibodies were used as follows: Alexa Fluor 488 donkey anti-guinea pig (1:500, Jackson Immunoresearch), Alexa Fluor 488 donkey anti-rabbit (1:500, Jackson Immunoresearch), and Alexa Fluor 594 donkey anti-mouse (1:500, Life Technologies). Images were captured using an Olympus Microscope system.

After immunelabeling with BrdU and insulin, pancreatic islets in paraffin-embedded sections were photographed at ×400 and assigned blinded filenames. The number of BrdU (+) β-cells was manually counted. At least 2000 β-cells were counted per animal using three sections that were separated by at least 100 μm. Three animals per group were analyzed.

Luciferase assays

INS-1 cells infected with *Sfrp5* adenovirus or GFP adenovirus were plated in 24-well plates 24 h before transfection. When the cells reached 70–80% confluence, they were transfected with plasmids (TopFlash and pRL-SV40) using Lipofectamine 2000 transfection reagent (Invitrogen). Six hours after transfection, the medium was changed to a medium supplemented with various concentrations of glucose and 0.2% BSA. Twenty-four hours later, the cells were lysed in 1XPLB, and luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega) as recommended by the manufacturer. All luciferase assay experiments were performed in triplicate.

Statistical analysis

Comparisons were made using ANOVA for multiple groups or Student's t-test for two groups. Data are presented as the means ± S.E.M. A value of $P<0.05$ was considered to be significant. Significance is indicated in the figures.

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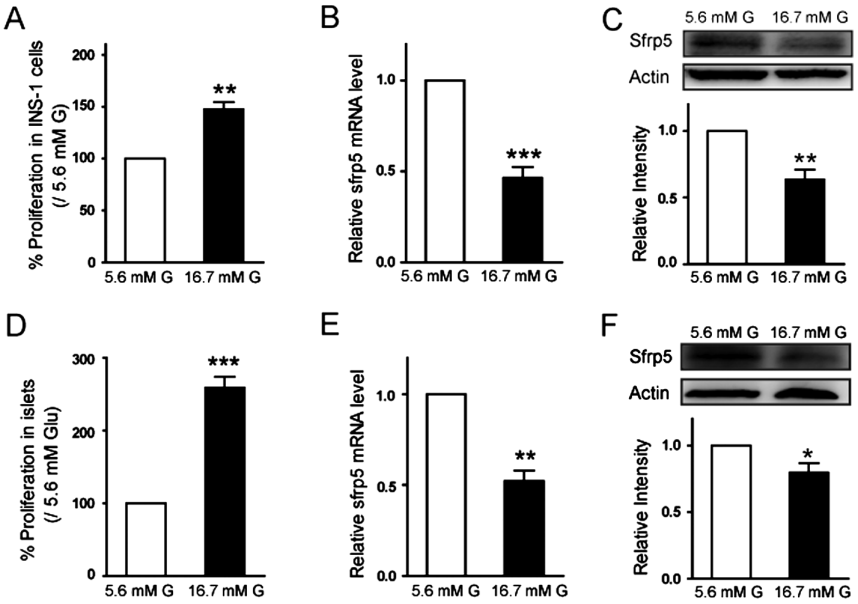

Figure 2
Glucose induces β-cell proliferation and inhibits Sfrp5 expression in vitro *A, D*: INS-1 cells (A) and primary isolated islets (D) were incubated for 24 h with RPMI 1640 medium containing 0.2% BSA and 5.6 or 16.7 mM glucose and then assessed for proliferation by EdU incorporation. *B, E*: Quantitative PCR assay of *Sfrp5* mRNA levels in INS-1 cells (B) and primary isolated islets (E) incubated with 5.6 or 16.7 mM glucose for 24 h *C, F*: Western blot detection of SFPR5 levels in INS-1 cells (C) and primary isolated islets (F) incubated with 5.6 or 16.7 mM glucose for 24 h. Representative immunoblot pictures are shown, and band intensities of SFRP5 are normalized based on the corresponding actin intensity. Data are presented as means ± S.E.M. from at least triplicate independent experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ for differences between the two groups.

Results

Glucose infusion increases β-cell proliferation and reduces *Sfrp5* expression in islets from glucose-infused rats

We and others have reported that short-term continuous glucose infusion via the jugular vein promotes β-cell replication in rodents (Bonner-Weir *et al*. 1989, Alonso *et al*. 2007, Zhang *et al*. 2012). To clarify the underlying mechanisms of glucose-induced β-cell proliferation, we previously performed a clustering analysis of Affymetrix arrays on islets isolated from rats infused with 50% glucose (2 mL/h) or saline. The results revealed that the *Sfrp5* mRNA level decreased by 64% in the islets following 24 and 48 h of glucose infusion compared with saline (Supplementary Fig. 1). To confirm the correlation between *Sfpr5* and glucose-induced β-cell proliferation *in vivo*, we performed the experiments in a 24 h glucose-infusion model. Infusion with 50% glucose for 24 h caused a significant increase in plasma glucose and insulin levels in rats (Supplementary Fig. 2A and B). The pancreatic sections from these rats exhibited more BrdU-positive proliferative cells in the islets compared with the saline-infused rats (Fig. 1A). A quantification of BrdU/insulin-positive cells revealed a robust and significant increase in β-cell replication in rats that received 50% glucose (2.8%) compared with rats that received saline (0.13%) (Fig. 1A). Consistent with our microarray results, islets isolated from 24 h glucose-infused rats showed significant decreases in *Sfrp5* mRNA expression and protein abundance compared with the saline-infused controls (Fig. 1B and C). The downregulation of SFRP5 in the glucose-infusion rat islets was confirmed by immunohistochemistry staining (Fig. 1D).

Glucose induces islet β-cell proliferation and inhibits *Sfrp5* expression *in vitro*

In line with our *in vivo* observations, exposure of INS-1 cells and isolated islets to 16.7 mM glucose for 24 h significantly increased the cell proliferation rates, which were approximately 1.5- and 2.5-fold higher than those of cells exposed to basal glucose concentrations (5.6 mM), respectively (Fig. 2A and D), as determined by EdU incorporation. Furthermore, there were significant decreases in *Sfrp5* mRNA and protein abundance in INS-1 cells incubated with 16.7 mM glucose compared with 5.6 mM glucose (Fig. 2B and C). Similar results demonstrating the glucose-inhibited *Sfrp5* mRNA (Fig. 2E) and protein expression (Fig. 2F) were also observed in isolated rat islets, indicating that this phenomenon occurred in primary β-cells. These *in vitro* results demonstrated that glucose-induced β-cell proliferation was accompanied by a reduction in *Sfrp5* expression.

Overexpression of SFRP5 attenuates glucose-induced proliferation in INS-1 cells and primary islets

Our present study demonstrated that high glucose stimulated β-cell proliferation, which was correlated with decreased *Sfrp*5 expression. *Sfrp5* downregulation has been

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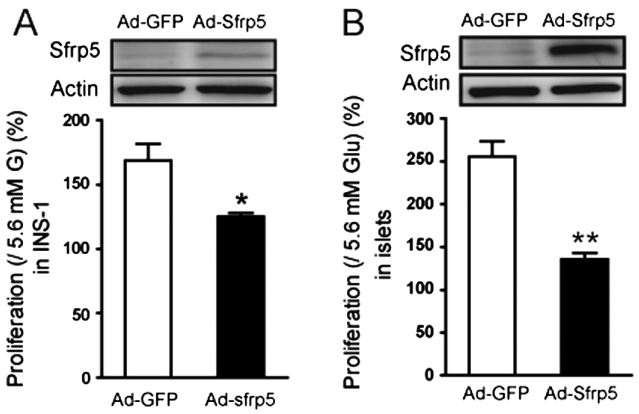

Figure 3
Overexpression of SFRP5 attenuates glucose-induced proliferation in INS1 cells and primary islets *A, B*: Cell proliferation rates due to exposure to 5.6 or 16.7 mM glucose were measured by the rate of EdU incorporation in INS-1 cells (A) and primary islets (B) after infection with Ad-GFP or Ad-*Sfrp5*. The upper portion of A, B shows representative immunoblot pictures of SFRP5 and actin in INS-1 cells and islets infected with the indicated adenoviruses for 48 h. The results are presented as the means ± S.E.M. from at least triplicate independent experiments. $*P < 0.05$, $**P < 0.01$ for differences between the two groups.

shown to enhance the proliferation of pancreatic β-cells (Rebuffat *et al*. 2013). To elucidate the function of *Sfrp5* in glucose-induced β-cell proliferation, we overexpressed SFRP5 in INS-1 cells and primary islets using an adenoviral vector. After 48 h of infection, the INS-1 cells and primary islets were subjected to the glucose-induced proliferation assay. As shown in Fig. 3A, the infection of INS-1 cells with Ad-*Sfrp5* increased the abundance of SFRP5 as determined by immunoblotting. Overexpression of SFRP5 abolished glucose-induced β-cell proliferation as determined by EdU incorporation compared with INS-1 cells treated with Ad-GFP (Fig. 3A) Similar observations were obtained in dispersed rat islets with SFRP5 overexpression (Fig. 3B). Meanwhile, overexpression of SFRP5 did not affect the EdU incorporation in INS-1 cells and dispersed rat islets at basal glucose concentration (5.6 mM) (Supplementary Fig. 3A and B). These findings indicated that *Sfrp5* may play a negative role in glucose-induced β-cell proliferation.

Sfpr5 impairs glucose-induced Wnt signaling pathway activation and decreases cyclin D2 expression in INS-1 cells

Sfrp5 is a negative regulator of Wnt signaling. A previous study demonstrated that the Wnt signaling pathway regulated β-cell proliferation (Rulifson *et al*. 2007), while glucose enhanced Wnt signaling to promote cancer cell proliferation (Chocarro-Calvo *et al*. 2013, Mao *et al*. 2013). We examined whether *Sfrp5* was involved in glucose-induced β-cell expansion by inhibiting the Wnt signaling pathway. As shown in Fig. 4A, 16.7 mM glucose increased the accumulation of β-catenin, the characteristic protein of the Wnt pathway, in the nucleus of INS-1 cells (Fig. 4A). Overexpression of SFRP5 in INS-1 cells blocked this translocation under the high glucose condition (Fig. 4A). These data indicated that high glucose may activate Wnt signaling and increase subsequent cell proliferation via *Sfrp5* inhibition. To further verify this possibility, we used a Wnt signaling reporter assay (TCF responsive luciferase reporter vector) to measure Wnt signaling in INS-1 cells stimulated by high glucose. Indeed, high glucose promoted TCF luciferase gene reporter activity, and overexpression of SFRP5 impaired the glucose-mediated activity (Fig. 4B). Additionally, we analyzed the expression of cyclin D2, a cell cycle regulator regulated by Wnt signaling in β-cells (Kushner *et al*. 2005, Rulifson *et al*. 2007, Chen *et al*. 2012). We found that overexpression of SFRP5 muted glucose-dependent cyclin D2 expression (Fig. 4C). These findings suggested that *Sfrp5* diminished glucose-stimulated proliferation in β-cells through the inhibition of the Wnt signaling pathway and the subsequent cyclin D2 expression.

Sfrp5 expression is modulated by glucose through the PI3K/AKT pathway

The PI3K/AKT pathway is known to be an important intracellular signaling cascade for glucose-stimulated pancreatic β-cell proliferation (Weir & Bonner-Weir 2007, Assmann *et al*. 2009). We postulated that the regulation of *Sfrp5* by glucose in β-cells may also be mediated by the PI3K/AKT pathway. We used a PI3K inhibitor (Ly294002) to inhibit endogenous PI3K activity in INS-1 cells. As expected, LY294002 reduced AKT phosphorylation and reversed glucose-induced *Sfrp5* repression at both the mRNA and protein levels (Fig. 5A and B). To further confirm our hypothesis, we transfected INS-1 cells with constitutively active AKT (caAKT) and found that these ca*Akt*-transfected cells showed significantly decreased *Sfrp5* expression levels compared with cells transfected with the control vectors (Fig. 5C and D). We concluded that glucose decreased *Sfrp5* expression in a PI3K/AKT-dependent manner in β-cells.

Discussion

The proliferation of β-cells is an important mechanism in the maintenance of the functional β-cell mass in response

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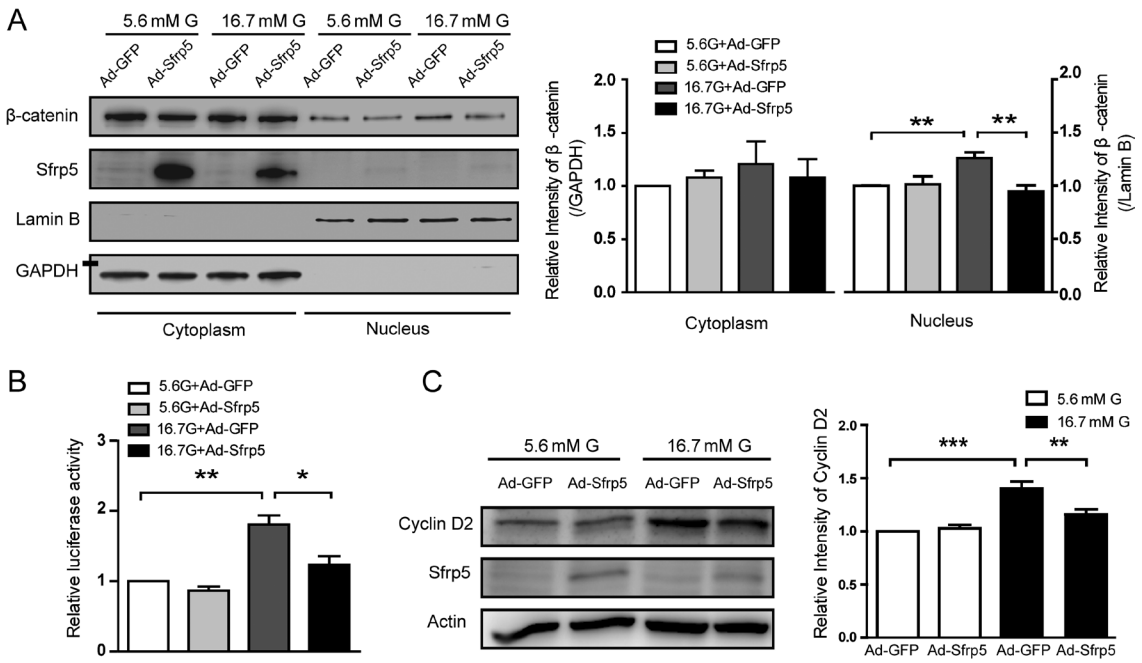

Figure 4

SFPR5 impairs glucose-induced activation of the canonical Wnt signaling pathway and decreases cyclin D2 expression in INS1 cells. (A) Western blot detection of β-catenin level in the cytoplasm and nucleus of INS-1 cells treated with 5.6 or 16.7 mM glucose after infection with Ad-*Sfrp5* or Ad-GFP. Representative immunoblot pictures are shown, and band intensities of SFRP5 were normalized based on the corresponding GAPDH intensity. (B) Overexpression of SFRP5 decreased TCF luciferase gene reporter activity activated by glucose in INS-1 cells. (C) Western blot detection of cyclin D2 levels in cells treated with 5.6 or 16.7 mM glucose after infection with Ad-*Sfrp5* or Ad-GFP. Representative immunoblot pictures are shown, and band intensities of cyclin D2 were normalized based on the corresponding actin intensity. Data are presented as the means ± S.E.M. from at least triplicate independent experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ for differences between the two groups.

to various metabolic demands (Dor *et al.* 2004, Ogino *et al.* 2006, Gupta *et al.* 2007, Okada *et al.* 2007, Teta *et al.* 2007). The failure to increase β-cell proliferation may result in glucose intolerance and eventually lead to diabetes mellitus (Butler *et al.* 2003, Prentki & Nolan 2006). Thus, understanding how β-cells proliferate and elucidating the molecular mechanisms that natural mitogens stimulate the expansion of functional β-cells is beneficial for diabetes therapy. Glucose is a well-known potent β-cell mitogen in mice, rats, and humans, and plays a dominant role in the β-cell compensation of insulin demands (Chick 1973, Bonner-Weir *et al.* 1989, Alonso *et al.* 2007, Terauchi *et al.* 2007, Weir & Bonner-Weir 2007, Levitt *et al.* 2011). However, the mechanisms underlying its mitogenic activity remain unclear. Our study demonstrated that glucose-stimulated β-cell proliferation may at least in part be mediated by *Sfrp5* regulation. We found that glucose-induced islet β-cell proliferation correlated with reduced *Sfrp5* expression both *in vitro* and *in vivo*. Overexpression of SFRP5 inhibited glucose-induced proliferation through the inactivation of the Wnt pathway.

SFRP5 is a member of the SFRP protein family and contains a cysteine-rich domain homologous to the putative Wnt-binding site of the frizzled proteins, which act as negative modulators of Wnt signaling (Kawano & Kypta 2003). Previous studies on *Sfrp5* mainly focused on adipogenesis and development of obesity but produced contradictory results. Ouchi *et al.* (2010) reported that SFRP5 secretion by adipocytes exerted salutary effects on metabolic dysfunction by controlling inflammatory cells within the adipose tissue. *Sfrp5*-deficient mice exhibited severe glucose intolerance upon consumption of a high fat–high sugar diet. However, in a different study, *Sfrp5*-deficient mice were reported to exhibit mild improvements in glucose tolerance upon consumption of a high-fat diet (Mori *et al.* 2012). Studies investigating the function of *Sfrp5* in β-cells are limited. Recently, *Sfrp5* was demonstrated to regulate β-cell proliferation in obese rats (Rebuffat *et al.* 2013) and may also play a role in the regulation of pancreatic β-cell function (Rulifson *et al.* 2014). Rebuffat *et al.* (2013) found the *Sfrp5* was downregulated in the islets of cafeteria diet-fed rats. In turn, this downregulation promoted β-cell proliferation by activating the Wnt signaling pathway. In our study, we found that glucose decreased *Sfrp5* expression in high glucose-treated INS-1 cells, primary

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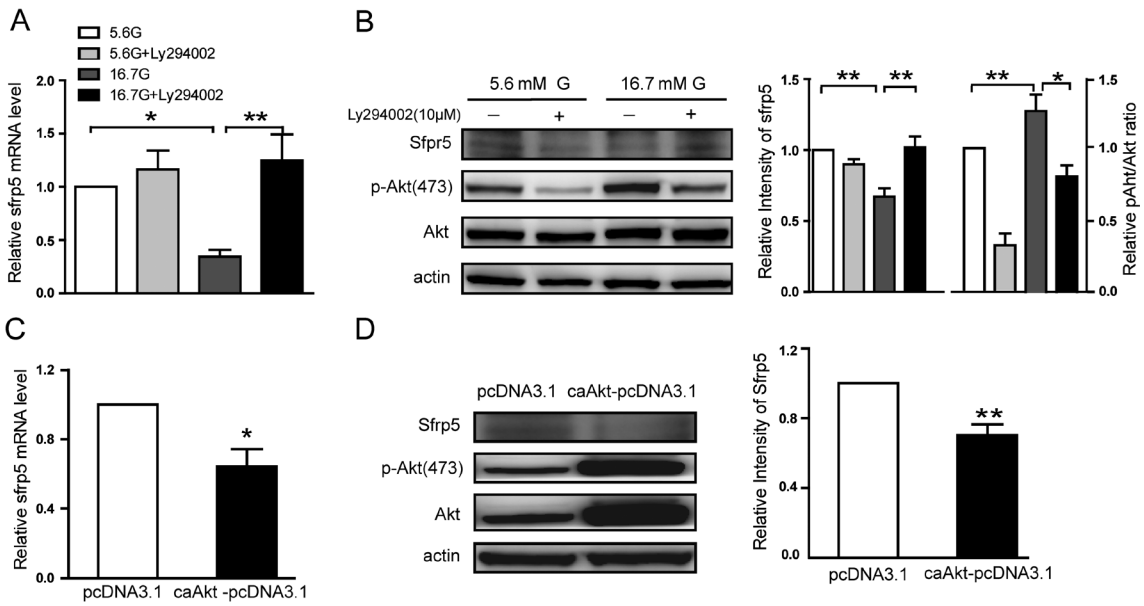

Figure 5

Sfrp5 expression is modulated by glucose through the PI3K/Akt pathway (A) Quantitative PCR analysis of *Sfrp5* mRNA levels in INS-1 cells exposed to 10 μM Ly294002. (B) Western blot detection of SFRP5, pAkt(473) protein levels in INS-1 cells exposed to 10 μM Ly294002. Band intensities of SFRP5 were normalized based on the corresponding actin intensity, pAkt(473) were normalized to total Akt. (C) Quantitative PCR analysis of *Sfrp5* mRNA levels in INS-1 cells transfected with caAkt-pcDNA3.1 or the control vector. (D) Western blot detection of SFRP5, pAKT(473), and AKT protein levels in INS-1 cells transfected with caAkt-pcDNA3.1 or the control vector. Band intensities of SFRP5 were normalized based on the corresponding actin intensity. Data are presented as the means ± S.E.M. from at least triplicate independent experiments. $*P < 0.05$, $**P < 0.01$ for differences between the two groups.

isolated islets, and islets isolated from glucose-infused rats. Our observation may also have clinical relevance because circulating SFRP5 levels were found to be decreased in humans with hyperglycemia (Hu *et al.* 2013). Moreover, overexpression of SFRP5 diminished glucose-induced proliferation due to the inactivation of the Wnt pathway. These results demonstrated that *Sfrp5* was also involved in glucose-induced β-cell proliferation. Obesity and hyperglycemia are common physiological conditions with increased insulin demand that result in compensatory β-cell proliferation. Based on the findings from Rebuffat *et al.* (2013) and this study, we suggest that *Sfrp5* is a critical component involved in compensatory β-cell proliferation. Whether *Sfrp5* is involved in pancreatic β-cell proliferation in response to other physiological replication stimuli deserves further investigation.

Previous studies revealed that Wnt signaling controlled islet β-cell proliferation and that glucose could enhance Wnt signaling to promote cancer cell proliferation (Rulifson *et al.* 2007, Liu & Habener 2008, Figeac *et al.* 2010, Chocarro-Calvo *et al.* 2013, Mao *et al.* 2013). However, whether glucose could activate Wnt signaling to promote cell growth in β-cells is unknown. In our study, we found that glucose allowed β-catenin translocation into the nucleus and promoted TCF luciferase reporter gene activity, indicating that the Wnt pathway was activated. Overexpression of SFRP5 not only inhibited the glucose-induced activation of the Wnt pathway but also impaired the glucose-stimulated proliferation of β-cells. These results demonstrated that glucose may promote β-cell proliferation, at least in part, through the activation of the Wnt pathway. Cyclin D2 is an essential cell cycle regulator that is controlled by Wnt signaling in pancreatic β-cells (Rulifson *et al.* 2007). Indeed, cyclin D2 was found to be upregulated in insulinoma cell lines treated with high glucose and islets from glucose-infused rats (Alonso *et al.* 2007, Rulifson *et al.* 2007, Salpeter *et al.* 2011). Our results demonstrated that cyclin D2 upregulation initiated by glucose could be attenuated by overexpression of the Wnt signaling inhibitor SFRP5. Our finding suggests a new mechanism for glucose-stimulated β-cell proliferation in which glucose inhibits *Sfrp5* and thereby activates Wnt signaling to increase the level of cyclin D2.

In this study, we identified a novel mechanism through which glucose boosts β-cell proliferation by repressing *Sfrp5* expression and subsequently leads to activation of the Wnt signaling pathway. However, the mechanism by which glucose regulates

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Sfrp5 expression is not understood. Glucose has been demonstrated to upregulate IRS2 expression, leading to the downstream activation of PI3K/AKT signaling (Lingohr *et al*. 2006), which plays a critical role in maintaining β-cell proliferation and survival (Kasuga 2006). Our findings showed that the inhibition of PI3K reversed glucose-induced *Sfrp5* repression, whereas the sustained activation of AKT significantly decreased *Sfrp5* expression. Our data indicated that glucose decreased *Sfrp5* expression in a PI3K/AKT-dependent manner in β-cells. In line with our findings, Rebuffat and coworkers recently demonstrated that *Sfrp5* expression in β-cells was promoted by IGFBP3 (Rebuffat *et al*. 2013), which exerted anti-proliferative effects in other cell types by blocking IGF1-mediated PI3K/AKT (Buckbinder *et al*. 1995). However, further studies are required to establish the precise mechanisms by which transcriptional factors may be involved in the regulation. Furthermore, Wnt signaling could be modulated by the PI3K/AKT signaling pathway through the regulation of GSK3β phosphorylation and the direct inhibition of β-catenin nuclear localization (Fukumoto *et al*. 2001, Lee *et al*. 2010). Our results indicate another mechanism: in addition to phosphorylated GSK3β, the PI3K/AKT pathway can activate the Wnt pathway by downregulating the expression of the Wnt pathway inhibitor SFRP5. Thus, it is plausible that *Sfrp5* is another molecule that mediates the cross talk between the Wnt and PI3K/AKT signaling pathways.

In summary, our results demonstrated that the SFRP5/Wnt pathway was required for glucose-induced β-cell proliferation and was regulated by glucose via the PI3K/AKT signaling pathway. Our findings provide novel insights into the mechanism of compensatory β-cell proliferation in response to increased insulin demand. Therefore, *Sfrp5* may serve as a target to expand functional pancreatic islets in diabetic patients.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/JOE-15-0535.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contributions

B G and W L researched data, wrote the manuscript, and reviewed/edited the manuscript; F L, Y X, Q N and Y G researched data and contributed to discussion; X L and Q W contributed to discussion, edited the manuscript; H Z conceived the project and designed the experiments, contributed to discussion, wrote the manuscript, and reviewed/edited the manuscript; N G contributed to discussion, and reviewed the manuscript.

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