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Activation of SIRT1 protects pancreatic β-cells against palmitate-induced dysfunction ☆

Ling Wu [a,b,1], Libin Zhou [a,1], Yan Lu [a], Juan Zhang [a], Fangfang Jian [a], Yun Liu [a], Fengying Li [a], Wenyi Li [a], Xiao Wang [a,*], Guo Li [a,*]

[a] Shanghai Institute of Endocrine and Metabolic Diseases, Department of Endocrine and Metabolic Diseases, Shanghai Clinical Center for Endocrine and Metabolic Diseases, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200025, China

[b] Reproductive Medical Center of Ruijin Hospital, Shanghai Jiaotong University School of Medicine, Shanghai 200025, China

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ABSTRACT

Sirtuin 1 (SIRT1), a nicotinamide adenosine dinucleotide-dependent histone deacetylase, is an important regulator of energy homeostasis in response to nutrient availability. In pancreatic β-cells, SIRT1 has been shown to up-regulate insulin secretion in response to glucose stimulation. However, the potential roles of SIRT1 in islet β-cells against lipotoxicity remain poorly understood. Here, we demonstrated that SIRT1 mRNA and protein expressions were markedly reduced in the islets isolated from rats infused with 20% Intralipid for 24 h. Long-term exposure to 0.4 mmol/L palmitate also decreased SIRT1 expression in cultured INS-1 cells and isolated rat islets, which was prevented by 10 μmol/L resveratrol, a SIRT1 agonist. In addition, resveratrol improved glucose-stimulated insulin secretion decreased by palmitate, which was abrogated by EX527, a specific SIRT1 inhibitor. Furthermore, inhibition of SIRT1 activity by EX527 or a knockdown of SIRT1 suppressed insulin promoter activity, along with decreased insulin, v-maf musculoaponeurotic fibrosarcoma oncogene homolog A (MafA), and NK6 homeodomain 1 (NKX6.1) mRNA expressions. Activation of SIRT1 by resveratrol or overexpression of SIRT1 antagonized palmitate-inhibited insulin transcriptional activity. SIRT1 overexpression exerted an additive effect on pancreatic and duodenal homeobox 1 (PDX1)-stimulated insulin promoter activity and abolished forkhead box O1 protein (FOXO1)-decreased insulin transcriptional activity. Resveratrol reversed FOXO1 nuclear translocation induced by palmitate. Our findings indicate that SIRT1 protects against palmitate-induced β-cell dysfunction.

1. Introduction

Type 2 diabetes mellitus is characterized by insufficient insulin secretion in response to elevations of plasma glucose, and excessive circulating lipid levels have been suggested to contribute, in conjunction with chronic hyperglycemia, to the progressive deterioration of β cell function in this disease [1,2]. Acute exposure of the β cell to free fatty acid (FFA) results in an increase of insulin release, whereas chronic exposure leads to desensitization and suppression of secretion [3,4]. Evidence also indicates that elevated FFA inhibits insulin biosynthesis and decreases cell viability [5–7]. However, the mechanisms underlying the detrimental impact of FFAs on the β cell are still incompletely understood.

The NAD^+-dependent deacetylase Sir2 extends the life span of a lower eukaryote [8]. Its mammalian ortholog sirtuin 1 (SIRT1) plays pivotal roles in various physiological processes including cell cycle regulation, gene silencing, metabolism, and inflammation [9–11]. In skeletal muscle, SIRT1 is suggested to interact with and deacetylate peroxisome proliferator-activated receptor gamma coactivator 1 (PGC-1) to enhance mitochondrial biogenesis [12]. It has been shown that SIRT1 regulates glucose and lipid homeostasis through deacetylating signal transducer and activator of transcription 3 (STAT3) and liver X receptor (LXR) in liver [13,14]. Moreover, glucose-stimulated insulin secretion (GSIS) in islets of SIRT1-knockout mice is blunted [15], whereas GSIS is enhanced in β-cell-specific SIRT1-overexpressing mice [16], suggesting that SIRT1 functions as a positive regulator of insulin secretion and maintenance of β cell function. Sun et al. [17] found that palmitate decreased SIRT1 protein expression in C2C12 myotubes and overexpression of SIRT1

Abbreviations: FFA, free fatty acid; PDX1, pancreatic and duodenal homeobox 1; GSIS, glucose-stimulated insulin secretion; JNK, Jun N-terminal kinase; SIRT1, sirtuin 1; PGC-1, peroxisome proliferator-activated receptor gamma coactivator 1; NeuroD, neurogenic differentiation; NKX6.1, NK6 homeodomain 1; MafA, v-maf musculoaponeurotic fibrosarcoma oncogene homolog A; AMPK, AMP-activated protein kinase

☆ Disclosure summary: the authors have nothing to declare. There are no other conflicts of interest in regard to this study.

* Corresponding authors at: Shanghai Institute of Endocrine and Metabolic Diseases, Department of Endocrine and Metabolic Diseases, Shanghai Clinical Center for Endocrine and Metabolic Diseases, Ruijin Hospital, Shanghai Jiaotong University School of Medicine, 197 Ruijin Road II, Shanghai 200025, China. Tel.: +86 21 64315587; fax: +86 21 64673639.

E-mail addresses: wangxiao1976@hotmail.com (X. Wang), gli_shnfms@yahoo.cn (G. Li).

[1] Ling Wu and Libin Zhou contributed equally to this work.

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improved palmitate-induced insulin resistance. But whether SIRT1 could protect against lipotoxicity in β cells remains elusive.

In the present study, we showed that fatty acids decreased SIRT1 expression in INS-1 insulinoma cells and isolated rat islets *in vivo* and *in vitro*. Resveratrol, a SIRT1 agonist, ameliorated palmitate-suppressed insulin secretion. Therefore, we further investigated the effect of SIRT1 on insulin transcriptional activity under lipotoxic conditions, demonstrating that SIRT1 is a positive regulator of insulin gene expression.

2. Materials and methods

2.1. Reagents

Palmitate, resveratrol, and fatty acid-free bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO, USA). RPMI 1640 medium, fetal bovine serum (FBS), and other culture reagents were obtained from Gibco Life Technologies (Grand Island, NY). EX527 was from Tocris Bioscience (Bristol, UK). Compound C was purchased from Calbiochem (San Diego, CA). Rat insulin RIA kit and anti-SIRT1 antibody were purchased from Millipore Technologies (Billerica, MA, USA). Anti-GAPDH, anti-acetyl-p65, anti-p65, anti-acetyl-p53, anti-p53, and anti-mouse or rabbit IgG conjugated with horseradish peroxidase were from Cell Signaling Technology (Beverly, MA, USA).

2.2. Cell culture

INS-1 cells (passage 22–35) were cultured in RPMI 1640 medium with 11.1 mmol/L glucose, 10% FBS, 10 mmol/L HEPES, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 100 U/mL penicillin G sodium, 100 μg/mL streptomycin sulfate, and β-mercaptoethanol (5 μl/L). At 24 h before the experiment (24 h after seeding), the medium was replaced with RPMI 1640 containing 5.6 mmol/L glucose or 16.7 mmol/L glucose supplemented with either BSA alone or BSA coupled to palmitate. The fatty acid coupling procedure was performed as described previously [18]. This procedure generated BSA-coupled palmitate in a molar ratio of 5:1 (generally, 0.4 mmol/L to 0.52% BSA, final).

2.3. Rat infusions

The animal protocol was reviewed and approved by the Animal Care Committee of Shanghai Jiaotong University School of Medicine. Male Sprague Dawley rats (Shanghai Laboratory Animal Company, Shanghai, China) weighing 250–300 g were housed under controlled temperature (21 °C) and a 12-h light–dark cycle with unrestricted access to water and standard laboratory diet. The animals were randomized into two groups, receiving 0.9% saline or 20% Intralipid with heparin (40 units/mL). The infusion technique was similar to that described by Hagman et al. [19]. Under general anesthesia, indwelling catheters were inserted into the right jugular vein. The catheters were tunneled subcutaneously and exteriorized at the base of the neck. The animals were recovered for 5 days after surgery. Catheter patency was maintained with 50 units/mL heparin in 0.9% saline. Intralipid and saline were infused at a constant rate of 1 mL/h. After 24 h infusion, the animals were killed for islet isolation.

2.4. Islet isolation and treatment

Islets of Langerhans were isolated by *in situ* pancreas collagenase infusion and separated by density gradient centrifugation [20] from male Sprague Dawley rats. The concentration of collagenase type XI was 0.5 mg/mL. Freshly isolated rat islets were transferred to 24-well plates (10 islets per well) or 6-well (150 islets per well) plates and cultured overnight in RPMI 1640 containing 5.6 mmol/L glucose, 10 mmol/L HEPES, 100 U/mL penicillin G sodium, and 100 μg/mL streptomycin sulfate supplemented with either BSA alone or BSA coupled to palmitate at 37 °C and 5% CO_2.

2.5. Insulin secretion

Islets were washed once in Krebs-Ringer bicarbonate (KRB) buffer [128.8 mmol/L NaCl, 4.8 mmol/L KCl, 1.2 mmol/L KH_2PO_4, 1.2 mmol/L $MgSO_4$, 2.5 mmol/L $CaCl_2$, 5 mmol/L $NaHCO_3$, and 10 mmol/L HEPES (pH 7.4)] with 0.1% BSA containing 3.3 mmol/L glucose, and then were preincubated for 30 min in 1 mL of the same medium at 37 °C. This buffer was then replaced with 1 mL of prewarmed KRB containing other additions as indicated for a further 60 min at 37 °C. An aliquot was then removed for analysis of insulin secretion by RIA. Islets were extracted with 0.18 N HCl in 70% ethanol for determination of insulin content.

2.6. Western blotting

INS-1 cells or isolated islets in 6-well plates were washed twice with ice-cold PBS and placed immediately in lysis buffer containing 25 mmol/L HEPES (pH 7.4), 1% Nonidet P-40, 100 mmol/L NaCl, 2% glycerol, 5 mmol/L NaF, 1 mmol/L EDTA, 1 mmol/L Na_3VO_4, 1 mmol/L NaPPi, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 5 μg/mL leupeptin, and 5 μg/mL pepstatin. Lysates were gently mixed for 10 min at 4 °C and then centrifuged at 13,000 *g* for 15 min at 4 °C. The protein concentration of the extracts was determined according to the method of Bradford, using BSA as the standard. Samples were separated by SDS-PAGE on 8% polyacrylamide gels and transferred to PVDF-Plus membranes (Bio-Rad, Hercules, CA). Primary antibodies were detected with donkey anti-rabbit at 1:2000 for 1 h at room temperature. Blotted membrane was developed with ECL Advance (Cell Signaling Technology, Boston, MA) and imaged with a LAS-4000 Super CCD Remote Control Science Imaging System (Fuji, JAP).

2.7. RNA isolation and real-time PCR

Total RNA was extracted from isolated islets or INS-1 cells using Trizol (Invitrogen) according to the manufacturer's instructions. In order to quantify the transcript abundance of genes of interest, real-time PCR was performed using a SYBR Green Premix Ex Taq (Takara, Shiga, Japan) with an Applied Biosystems 7300 Real-Time PCR machine (Applied Biosystems, Foster City, CA). The results of relative expression were normalized to β-actin mRNA levels in each sample. The specific primers were as follows: SIRT1, 5′-AGG GAACCTCTGCCTCATCTAC-3′ (forward), 5′-GGCATACTCGCCACCTAA CCT-3′ (reverse); Insulin 1 (Ins1), 5′-CCTGCTCGTCCTCTGGGAGCCC AAG-3′ (forward), 5′-CTCCAGTGCCAAGGTCTGAAGATCC-3′ (reverse); Insulin 2 (Ins2), 5′-CCTGCTCATCCTCTGGGAGCCCCGC-3′ (forward), 5′-CTCCAGTGCCAAGGTCTGAAGGTCA-3′ (reverse); neurogenic differentiation (NeuroD), 5′-GGCTCCAGGGTTATGAGATC-3′ (forward), 5′-GCATT CATGGCTTCAAGC-3′ (reverse); pancreatic and duodenal homeobox 1 (PDX1), 5′-CTTTCCCGAATGGAACCGAG-3′ (forward), 5′-GAATTCCTTCT CCAGCTCC-3′ (reverse); NK6 homeodomain 1 (NKX6.1), 5′-CTATTCTCT GGGGATGACGG-3′ (forward), 5′-TCTCGTCGTCAGAGTTCGGGTC-3′ (reverse); v-maf musculoaponeurotic fibrosarcoma oncogene homolog A (MafA), 5′-AGCAAGGAGGAGGTCATC-3′ (forward), 5′-CGTATTTCTCCTT GTACAGG-3′ (reverse); and β-actin, 5′-AGGCCCCTCTGAACCCTAAG-3′ (forward), 5′-GGAGCGCGTAACCCTCATAG-3′ (reverse).

2.8. Plasmid transfection and siRNA interference

Transfection was conducted using Lipofectamine 2000 (Invitrogen) according to the manufacturer's manual. Briefly, INS-1 cells were plated in 24-well plates for 1 day before transfection. At 70–80% confluency, each well of cells was transfected with pECE-SIRT1, pcDNA3.1-PDX1, pcDNA3.1-forkhead box O1 protein (FOXO1) or a control empty vector. After 24 h of transfection, the medium was changed for a further 24 h at 37 °C. SIRT1-specific small interfering RNA (siRNA) and negative control siRNA were designed and synthesized by Shanghai GenePharma

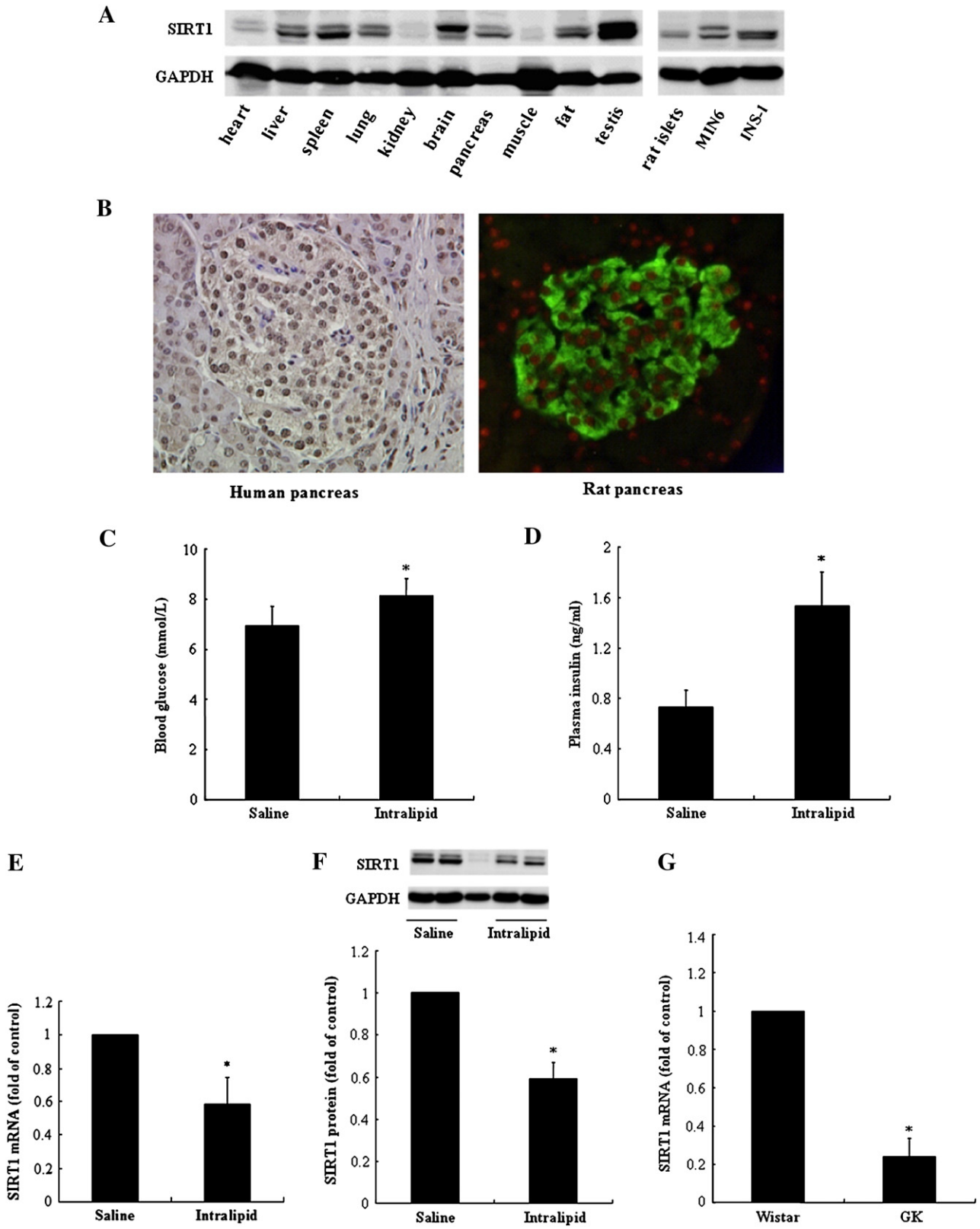

Fig. 1. Expression of SIRT1 in various mouse tissues and effect of fatty acids on its expression in rat islets *in vivo*. (A) Western blot analysis of SIRT1 expression in various mouse tissues, isolated rat islets, MIN6 and INS-1 pancreatic β cell lines. (B) SIRT1 immunostaining of human and rat pancreatic sections. Immunoperoxidase staining labeled SIRT1 in human pancreatic section. Immunofluorescent staining labeled insulin (green) and SIRT1 (red) in rat pancreatic section. Rats were infused with 20% Intralipid for 24 h ($n=5$). Plasma glucose (C) and insulin (D) levels were detected. Islets were isolated for SIRT1 mRNA (E) and protein (F) level assay by real-time PCR and Western blot. (G) After GK rats were fed with high-fat diet for 10 weeks, islets were isolated for SIRT1 mRNA level assay by real-time PCR. Data are expressed as mean ± SEM. *$P<0.05$ *vs.* control group.

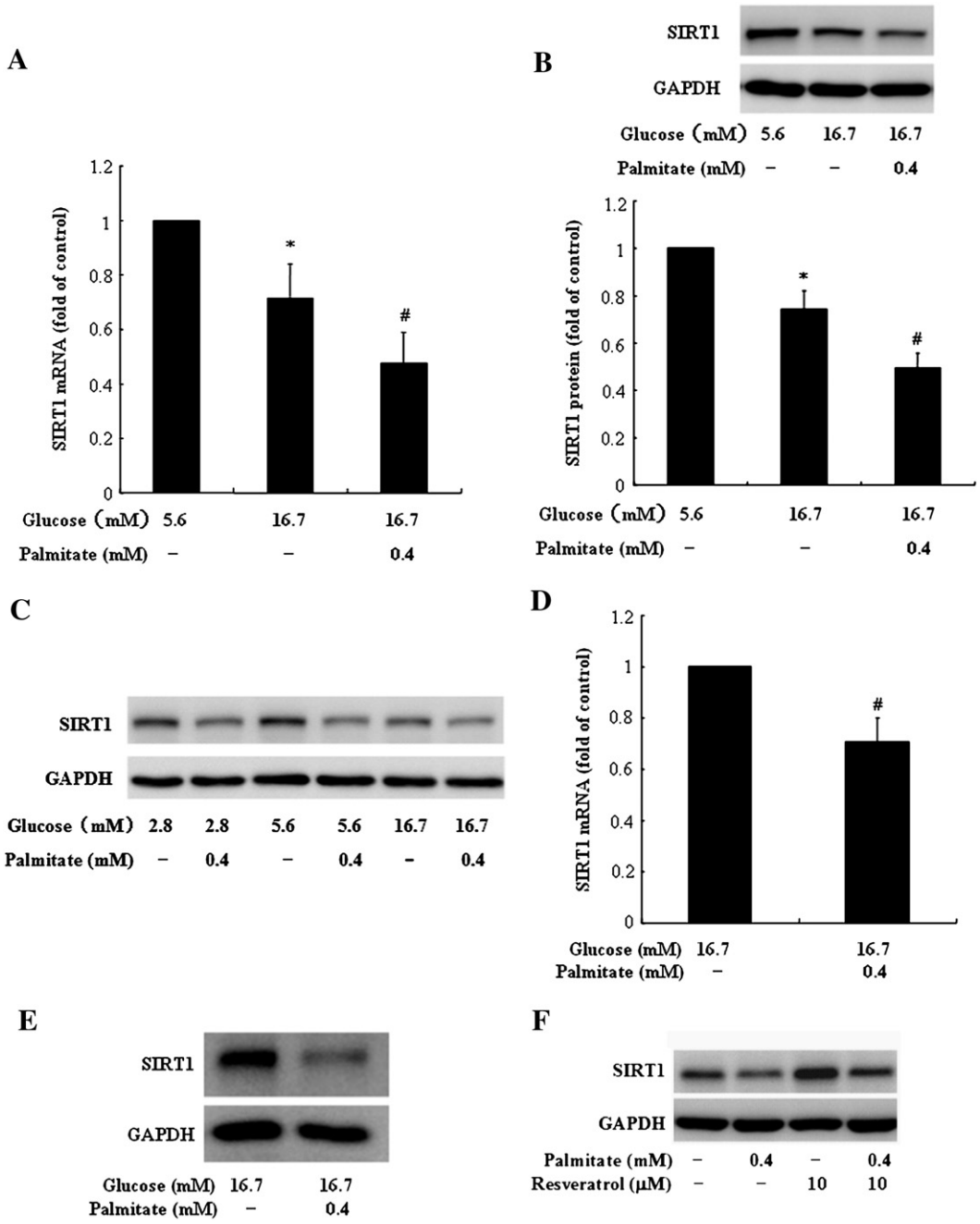

Fig. 2. Effects of fatty acids on SIRT1 expressions in INS-1 cells and rat islets *in vitro*. INS-1 cells and islets were incubated with the indicated concentrations of glucose in the presence or absence of 0.4 mmol/L palmitate for 24 h. The levels of SIRT1 mRNA (A) and protein (B and C) were detected by real-time PCR and Western blot. (D and F) SIRT1 mRNA and protein expressions in rat islets. (G) INS-1 cells were treated with 0.4 mmol/L palmitate in the presence or absence of 10 μmol/L resveratrol for 24 h. SIRT1 expression was detected by Western blot. Data are expressed as mean ± SEM for three separate experiments. $^{*}P<0.05$ *vs.* 5.6 mmol/L glucose; $^{\#}P<0.05$ *vs.* 16.7 mmol/L glucose alone. A representative blot from three independent experiments is shown. All three experiments showed similar results.

company and transiently transfected with siRNA using Lipofectamine 2000 as described above. The sequences of three SIRT1 siRNAs were as follows: si-1, sense—CACCUGAGUUGGAUGAUAUTT, antisense—AUAU CAUCCAACUCAGGUGTT; si-2, sense—CUGUGGCAGAUUGUUAUUATT, antisense—UAAUAACAAUCUGCCACAGTT; and si-3, sense—GAUCAAG AGAUGGUAUUUATT, antisense—UAAAUACCAUCUCUUGAUCTT.

2.9. Luciferase reporter assay

After INS-1 cells were plated in 24 well plates for 24 h, each well of cells was transfected with 0.2 μg of rat insulin promoter (RIP) luciferase plasmid. pRL-SV40 expressing *Renilla* luciferase (Promega) was used to normalize the luciferase activity. Cells were harvested and luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega).

2.10. Immunostaining

An adult human paraffin-embedded, formaldehyde-fixed pancreatic section from the Department of Pathology, Shanghai Ruijin Hospital, and a rat frozen pancreatic section were blocked with 5% (wt/vol.) BSA in PBS for 1 h. The human pancreatic section was incubated overnight at 4 °C with rabbit anti-SIRT1 (1:100) and detected with biotinylated anti-rabbit IgG in combination with streptavidin horseradish peroxidase.

The rat pancreatic section was incubated overnight at 4 °C with rabbit anti-SIRT1 (1:100) and guinea pig anti-insulin (1:400) primary antibodies, and then detected with rhodamine-coupled anti-rabbit IgG and FITC-labeled antiguinea pig IgG secondary antibodies.

HEK293 cells grown on glass coverslips were transfected with HA-tagged FOXO1 plasmid for 24 h and treated as indicated. The cells were fixed in 4% (vol./vol.) paraformaldehyde for 15 min, permeabilized with 2% (vol./vol.) Triton-X-100 for 10 min, and blocked in 5% (wt/vol.) BSA for 1 h. Cells were then incubated overnight at 4 °C with monoclonal anti-HA antibody (1:200) and stained with rhodamine-coupled anti-rabbit IgG antibody. DAPI (Sigma-Aldrich) was used for nuclear staining. The cellular localization of HA-tagged FOXO1 was photographed and analyzed using a fluorescence microscope (Olympus BX51; Olympus America, Inc., Melville, NY).

2.11. Statistics

Data were presented as mean ± SEM. Comparisons were performed using ANOVA for multiple groups or the Student's *t* test for two groups. Significance was established at $P<0.05$.

3. Results

3.1. SIRT1 is expressed in pancreatic β cells

To examine the connection between SIRT1 and β cell function, we first analyzed the tissue distribution of SIRT1 protein in C57BL/6 mice tissue. As shown in Fig. 1A, a moderate level of SIRT1 was expressed in the pancreas compared to other tissues. SIRT1 was highly expressed in the spleen, brain, and testis. Liver and fat tissues showed comparable SIRT1 expression levels to that of the pancreas, but a very low level of SIRT1 was detectable in skeletal muscle. In addition, SIRT1 expressions were also detected in primary islets isolated from rats, MIN6 and INS-1 pancreatic β cell lines. We further immunostained SIRT1 on human and rat pancreatic sections with an antibody against SIRT1. As in other cells reported previously [21], SIRT1 was expressed in the nucleus of pancreatic β cells, not in the cytoplasm (Fig. 1B).

3.2. Fatty acids decrease SIRT1 expression in INS-1 cells and isolated rat islets in vivo and in vitro

To investigate whether SIRT1 is involved in pancreatic β cell lipotoxicity, we first examined its expression in isolated islets from rats infused with Intralipid for 24 h. Plasma glucose and insulin levels were increased in the Intralipid infusion group compared with the saline infusion group ($P<0.05$, Fig. 1C and D). SIRT1 mRNA level was decreased by 42% in islets of Intralipid-infused rats ($P<0.05$, Fig. 1E). SIRT1 protein expression showed a similar decrease (Fig. 1F). In isolated islets of Goto–Kakizaki (GK) rats fed with a high-fat diet, SIRT1 mRNA level was also decreased ($P<0.05$, Fig. 1G). To detect the effect of fatty acids on SIRT1 expression *in vitro*, INS-1 cells were exposed to 0.4 mmol/L palmitate for 24 h at a concentration of 16.7 mmol/L glucose. SIRT1 mRNA and protein levels were significantly reduced by palmitate treatment (Fig. 2A and B). At the concentrations of 2.8, 5.6, and 16.7 mmol/L glucose, palmitate all decreased SIRT1 protein expression (Fig. 2C). We further examined SIRT1 expression in isolated rat islets, and found a similar decline in palmitate-treated primary islets (Fig. 2D and E). However, 10 μmol/L resveratrol antagonized palmitate-suppressed SIRT1 protein expression in INS-1 cells (Fig. 2F).

3.3. Resveratrol ameliorates palmitate-inhibited GSIS

The pattern of insulin secretion directly reflected the physiological function of pancreatic islets. Here, insulin secretion was observed in islets isolated from Sprague Dawley rats. As expected, GSIS was markedly decreased after isolated rat islets were pretreated with 0.4 mmol/L palmitate at a concentration of 16.7 mmol/L glucose, consistent with previous studies [6,22]. 10 μmol/L resveratrol had no effect on insulin secretion in the presence of low glucose and palmitate while improved GSIS suppressed by the combination of high glucose and palmitate (Fig. 3A, $P<0.05$). As it has been reported, resveratrol stimulates both AMP-activated protein kinase (AMPK) and SIRT1 activities [23]. We further investigated which of AMPK and SIRT1 mediated resveratrol-improved insulin secretion. Isolated rat islets were pre-incubated with 10 μmol/L compound C (an AMPK inhibitor) or 10 μmol/L EX527 (a SIRT1 inhibitor) for 1 h, and then co-incubated with 10 μmol/L resveratrol for 24 h. Resveratrol-improved insulin secretion in the presence of 0.4 mmol/L palmitate was abolished by EX527, not by compound C. In addition, both EX527 and compound C had no significant effects on insulin secretion in the absence of resveratrol compared with corresponding controls (Fig. 3B).

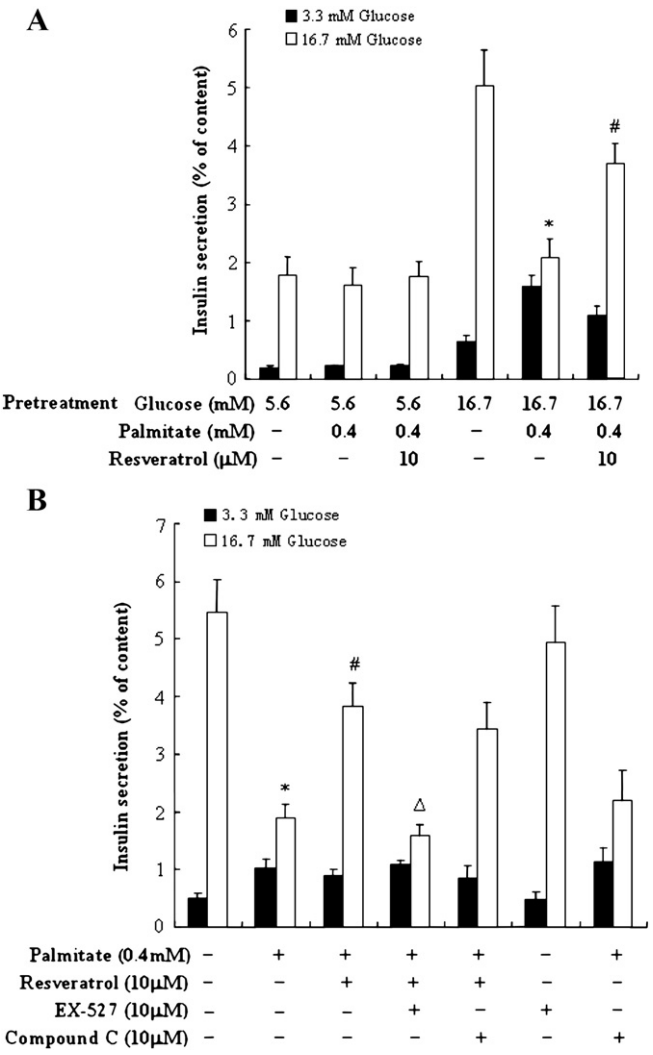

Fig. 3. Resveratrol ameliorated palmitate-induced inhibition of glucose-stimulated insulin secretion. (A) Isolated rat islets were pretreated with 0.4 mmol/L palmitate in the presence or absence of 10 μmol/L resveratrol at the indicated concentrations of glucose for 24 h, and then islets from each group were stimulated for 1 h in KRB buffer at 3.3 and 16.7 mmol/L glucose for insulin secretion assay. (B) Isolated rat islets were pretreated with 10 μmol/L EX527 or 10 μmol/L compound C in the presence of 0.4 mmol/L palmitate and 16.7 mmol/L glucose for 1 h, and then incubated with 10 μmol/L resveratrol for 24 h. The islets from each group were stimulated for 1 h in KRB buffer at 3.3 and 16.7 mmol/L glucose for insulin secretion assay. Data are expressed as mean ± SEM for three separate experiments. $^{*}P<0.05$ *vs.* 16.7 mmol/L glucose alone; $^{\#}P<0.05$ *vs.* 16.7 mmol/L glucose plus 0.4 mmol/L palmitate; $\Delta P<0.05$ *vs.* 16.7 mmol/L glucose plus 0.4 mmol/L palmitate and 10 μmol/L resveratrol.

3.4. Inhibition or knockdown of SIRT1 suppresses insulin transcription

Palmitate inhibits insulin gene transcription in the presence of high glucose [3]. A reduction of SIRT1 expression under lipotoxic conditions lead to a postulation that the inhibition of SIRT1 activity might be responsible for the defect of insulin gene transcription. To address this question, we treated the INS-1 cells with EX527, which has shown potential and specific inhibition of SIRT1 [13,24]. Although EX527 did not suppress SIRT1 mRNA expression (Fig. 4A), it dramatically enhanced p65 and p53 acetylation, which are two well-known substrates for SIRT1 [11,24]. On the contrary, resveratrol decreased p65 and p53 acetylation (Fig. 4B and C), suggesting that EX527 suppressed and resveratrol stimulated SIRT1 activity. At the concentrations of 0.2, 0.4, and 0.8 mmol/L, palmitate increased p53 acetylation (Fig. 4D, $P<0.05$).

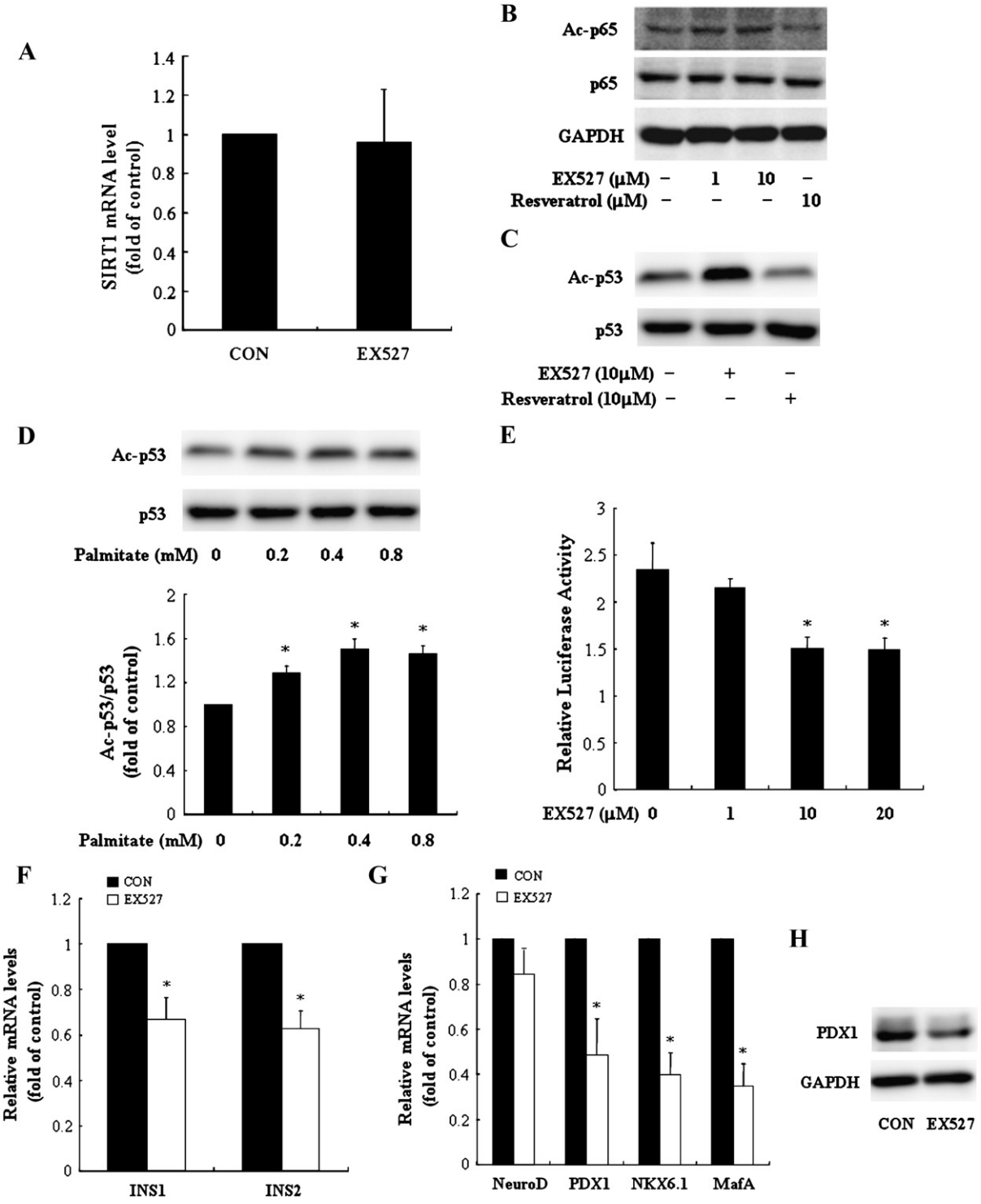

Fig. 4. Inhibition of SIRT1 activity decreased insulin transcription. INS-1 cells were incubated with EX527, resveratrol or palmitate at the indicated concentrations for 24 h. (A) 10 μmol/L EX527 did not change SIRT1 mRNA expression analyzed by real-time PCR. (B) The acetylated p65 (Ac-p65) level was assayed by Western blot. (C and D) The acetylated p53 (Ac-p53) was assayed by Western blot. (E) Insulin promoter activity was detected by the Dual-Luciferase Reporter Assay System after SIRT1 activity was inhibited by EX527. (F and G) After INS-1 cells were incubated with 10 μmol/L EX527 for 24 h, insulin 1 (INS1), insulin 2 (INS2), NeuroD, PDX-1, NKX6.1, and MafA mRNA expressions were detected by real-time PCR. (H) PDX1 protein expression was detected by Western blot. Data are expressed as mean ± SEM for three independent experiments. *$P<0.05$ *vs.* control (CON).

As expected, EX527 significantly decreased RIP activity at the concentrations of 10 and 20 μmol/L (Fig. 4E, both $P<0.05$). Furthermore, mRNA expressions of Ins1, Ins2, and several transcription factors including MafA, NKX6.1, and PDX1 were suppressed by 10 μmol/L EX527 (Fig. 4F and G, all $P<0.05$). Western blot also showed that EX527 decreased PDX1 protein expression (Fig. 4H).

To further characterize the roles of SIRT1, INS-1 cells were transfected with three siRNAs against SIRT1. As shown in Fig. 5A, SIRT1 protein levels were reduced by about 50%. The knockdown of SIRT1 expression significantly inhibited RIP activity (Fig. 5B, $P<0.05$). In addition, mRNA expressions of Ins2, MafA, and NKX6.1 were also down-regulated. A tendency toward decreased expressions, though not reaching significance, was also observed for Ins1 and PDX1 (Fig. 5C and D).

3.5. Overexpression or activation of SIRT1 ameliorates palmitate-inhibited insulin transcriptional activity

A previous study showed that SIRT1 could prevent interleukin-1β and interferon-γ induced cytokine toxicity and maintain normal insulin-secreting responses to glucose in isolated rat islets [25]. Resveratrol has been receiving increasing attention as a potent activator of SIRT1 [26]. At the concentration of 16.7 mmol/L glucose, 10 μmol/L resveratrol dramatically increased RIP activity (Fig. 6A, $P<0.05$). Hence, we further investigated whether overexpression of SIRT1 could attenuate the decline of insulin transcriptional activity under lipotoxicity. As shown in Fig. 6B, 16.7 mmol/L glucose increased RIP activity compared with 2.8 mmol/L, which was markedly decreased by 0.4 mmol/L palmitate, consistent with previous studies [6,7]. Palmitate-suppressed insulin promoter activity was completely reversed by overexpression of SIRT1 and abrogated by resveratrol (both $P<0.05$). Moreover, resveratrol exerted a synergistic effect with SIRT1 overexpression on RIP activity in the presence or absence of palmitate (Fig. 6C).

3.6. SIRT1 enhances PDX1-stimulated and antagonizes FOXO1-inhibited insulin promoter activity

PDX1 is a crucial factor required for the early development of pancreas and interacts synergistically with NeuroD and MafA to activate the insulin promoter [27]. Thus, we examined the effect of SIRT1 on the activity of PDX1 in the insulin promoter. SIRT1 exerted an additive effect on PDX1 up-regulated RIP activity (Fig. 7A, $P<0.05$). It has been previously demonstrated that expression of a constitutively active mutant of FOXO1 results in a complete lack of PDX-1 expression [28]. Fig. 7B showed that overexpression of FOXO1 inhibited insulin promoter activity, which was antagonized by SIRT1 overexpression ($P<0.05$).

3.7. Resveratrol abrogates palmitate-stimulated FOXO1 nuclear translocation

It has been reported that palmitate stimulates FOXO1 nuclear translocation in MIN6 cells [29]. To detect whether resveratrol antagonize this action, HEK293 cells were transfected with HA-tagged FOXO1 plasmid for 24 h, and incubated with 0.4 mmol/L palmitate and 10 μmol/L resveratrol for 4 h. The results showed that resveratrol significantly suppressed palmitate-stimulated HA-tagged FOXO1 protein nuclear translocation (Fig. 8A and B, $P<0.05$).

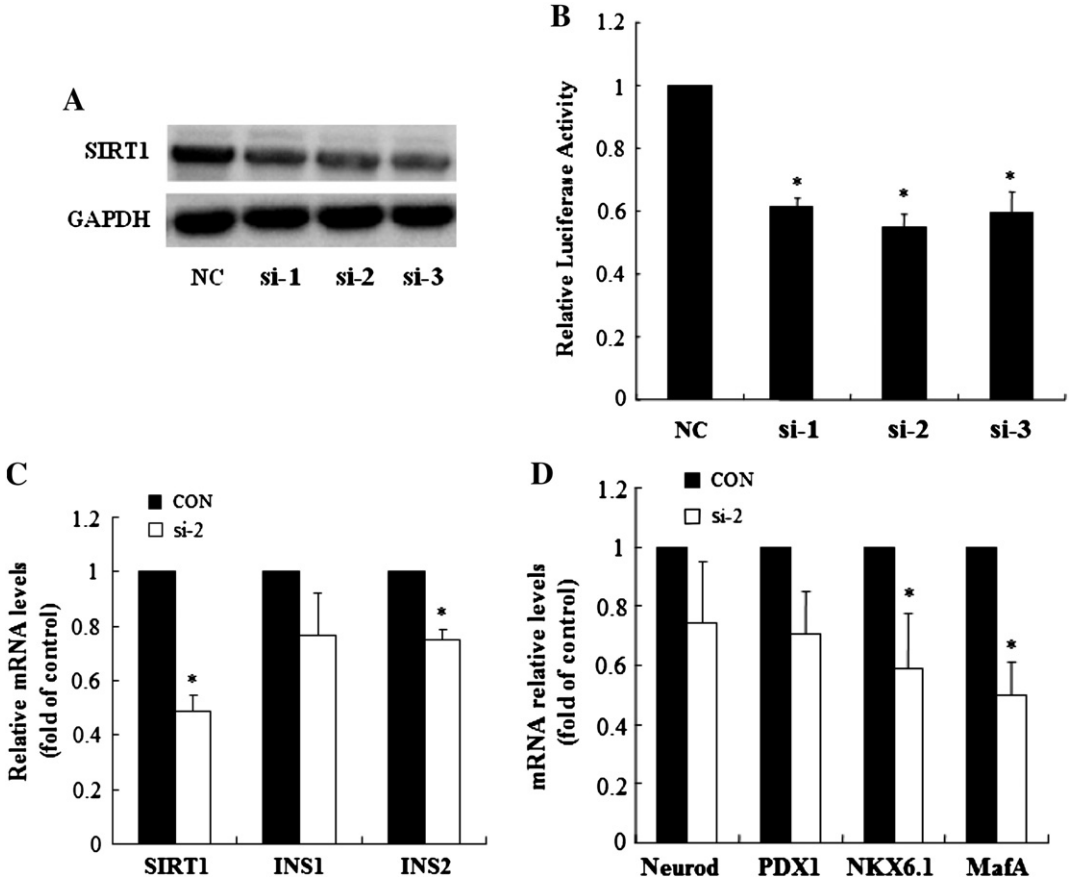

Fig. 5. Knockdown of SIRT1 suppressed insulin transcription. (A) INS-1 cells were transfected with three SIRT1 siRNAs (si-1, si-2, and si-3) or control scrambled siRNA (CON) for 48 h. SIRT1 protein expression was determined by Western blot. (B) INS-1 cells were cotransfected with rat insulin promoter plasmid and three SIRT1 siRNAs for 48 h. The luciferase activity was measured using the Dual-Luciferase Reporter Assay System. (C and D) After INS-1 cells were transfected with SIRT1 siRNA or control scrambled siRNA for 48 h, SIRT1, insulin 1 (INS1), insulin 2 (INS2), NeuroD, PDX-1, NKX6.1, and MafA mRNA expressions were detected by real-time PCR. Data are expressed as mean ± SEM for three independent experiments. *$P<0.05$ *vs.* control (CON).

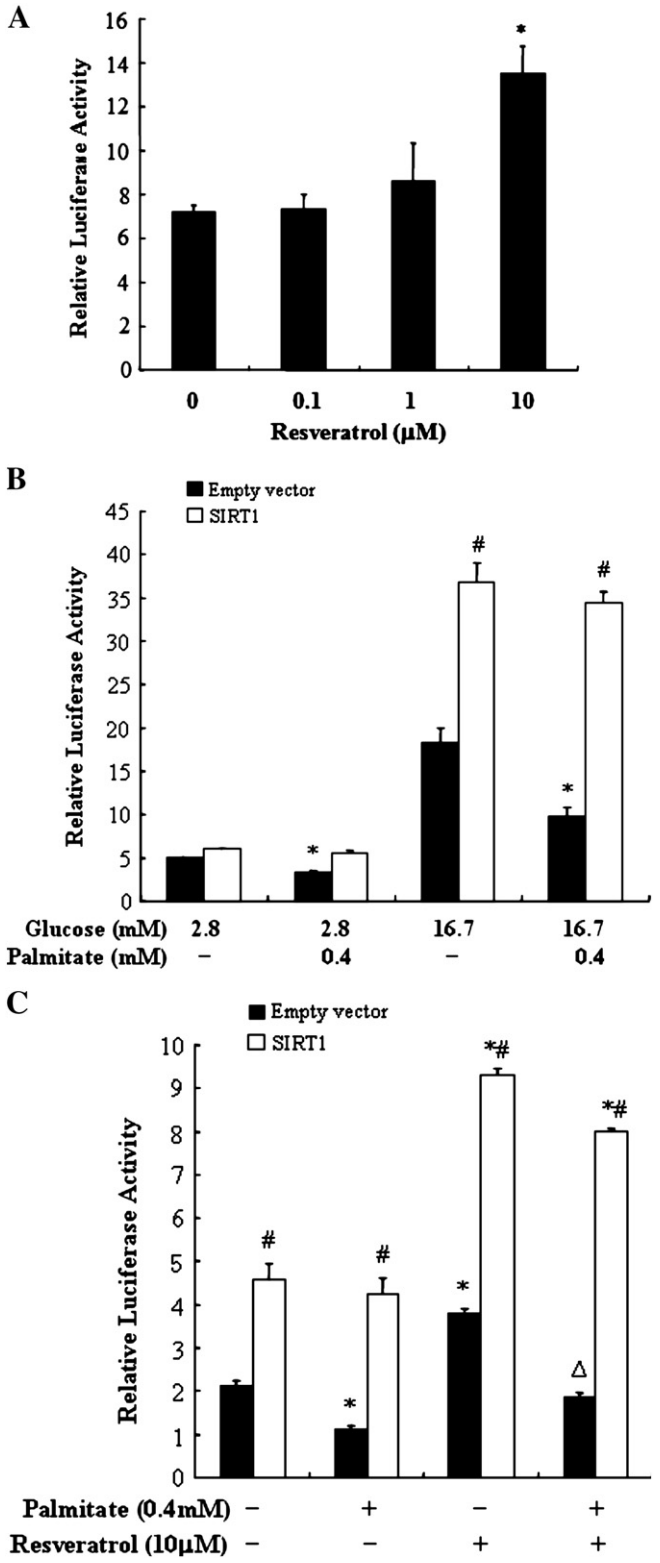

Fig. 6. SIRT1 antagonized palmitate-suppressed insulin promoter activity. (A) INS-1 cells were transfected with the rat insulin promoter for 24 h, and then incubated with various concentrations of resveratrol for 24 h. Dual-luciferase assay was performed. (B) INS-1 cells were cotransfected with the rat insulin promoter and pECE-SIRT1 or empty vector for 24 h, and then media were changed with the indicated glucose concentration in the presence or absence of 0.4 mmol/L palmitate. Dual-luciferase assay was performed. (C) INS-1 cells were cotransfected with the rat insulin promoter and pECE-SIRT1 or empty vector for 24 h, and then incubated with 0.4 mmol/L palmitate or 10 μmol/L resveratrol at the concentration of 16.7 mmol/L glucose. The dual-luciferase assay was performed. Data are expressed as mean ± SEM of three independent experiments. $^{*}P<0.05$ *vs.* control (basal or without palmitate); $^{\#}P<0.05$ *vs.* empty vector; $\Delta P<0.05$ *vs.* palmitate alone.

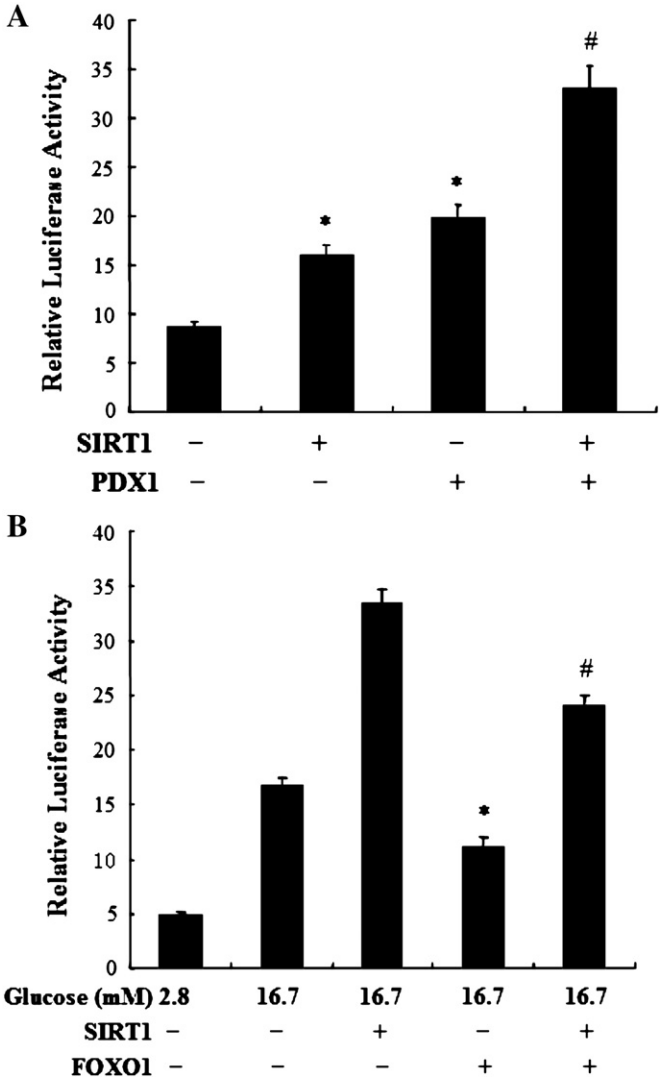

Fig. 7. SIRT1 enhanced PDX1-stimulated and antagonized FOXO1-suppressed insulin promoter activity. (A) INS-1 cells were cotransfected with the rat insulin promoter and the expression vectors pcDNA3.1-PDX1, pECE-SIRT1 or both at the concentration of 16.7 mmol/L glucose for 24 h. The dual-luciferase assay was performed. (B) INS-1 cells were cotransfected with the rat insulin promoter and the expression vectors pcDNA3.1-FOXO1, pECE-SIRT1 or both for 24 h. The dual-luciferase assay was performed. $^{*}P<0.05$ *vs.* empty vector. $^{\#}P<0.05$ *vs.* pcDNA3.1-PDX1 or pcDNA3.1-FOXO1 alone. Data are expressed as mean ± SEM of three independent experiments.

4. Discussion

SIRT1 has been demonstrated to improve GSIS as evidenced by β cell-specific SIRT1 overexpression transgenic mice [16]. A recent report also revealed that overexpression of SIRT1 protected β cell against cytokine toxicity [25]. Here we show that SIRT1 can protect β cell against lipotoxicity induced by palmitate for the following reasons. First, SIRT1 expression was significantly decreased in isolated islets from rats infused with Intralipid. Chronic exposure to palmitate yielded a similar result in INS-1 cells and isolated islets *in vitro*. Second, resveratrol improved palmitate-suppressed GSIS. Third, knockdown or inhibition of SIRT1 suppressed insulin transcription while overexpression or activation of SIRT1 ameliorated palmitate-inhibited RIP activity.

Circulating FFA levels are elevated in most patients of type 2 diabetes, and it may be present for years and even decades [5]. Clearly, long-term exposure to FFA induced insulin resistance [30]. In the light of increased demands for insulin biosynthesis during insulin resistance conditions, any inhibitory effect on insulin biosynthesis seems inappropriate and potentially deleterious. Therefore, protecting β cell against

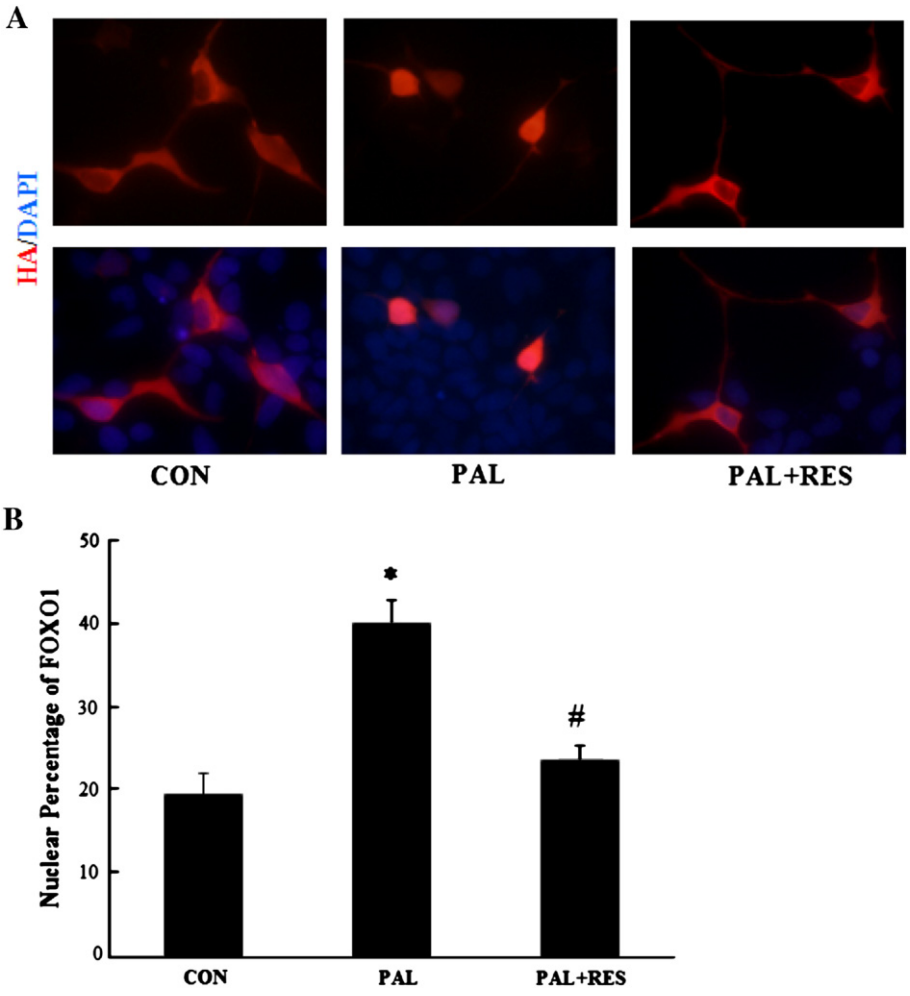

Fig. 8. Resveratrol reversed palmitate-induced FOXO1 nuclear translocation. (A) HEK293 cells were transfected with HA-FOXO1 plasmid for 24 h, pretreated with 10 μmol/L resveratrol (RES) for 30 min, and then incubated with 0.4 mmol/L palmitate (PAL) for 4 h. Cellular localization of HA-tagged FOXO1 was detected by immunofluorescence and DAPI staining. (B) The number of cells with HA-tagged FOXO1 in the nucleus was counted (mean ± SEM, $n = 600–1000$ cells from three separate experiments). $^{*}P<0.05$ *vs.* control (CON), $^{\#}P<0.05$ *vs.* PAL alone.

palmitate-decreased insulin biosynthesis and secretion seems crucial. Skeletal muscle insulin resistance is a key component of the etiology of type 2 diabetes. Many studies have shown that SIRT1 promotes insulin sensitivity of skeletal muscle [17,31]. In this study, SIRT1 protein expression in pancreas was significantly higher than in skeletal muscle, suggesting that SIRT1 may also play an important role in the regulation of islet β cell function. β-cell-specific SIRT1 mice enhanced GSIS and improved glucose tolerance after being fed with a high-fat diet [16]. Our study showed that SIRT1 expression was markedly reduced in islets isolated from rats infused with 20% Intralipid. The results were similar in palmitate-treated INS-1 cells and isolated rat islets, which is consistent with the results in C2C12 myotubes reported previously [17]. Moreover, palmitate increased the acetylation level of p53, a target protein of SIRT1, suggesting that palmitate inhibited SIRT1 activity. Resveratrol antagonized the effect of palmitate on SIRT1 expression, suggesting that SIRT1 may protect pancreatic β cell against lipotoxicity.

Resveratrol, a polyphenol compound, is known for its effects on energy homeostasis dependent or independent of SIRT1 [12,32,33]. Several papers have shown that resveratrol also stimulates AMPK in different tissues [22,34,35]. Our previous studies showed that troglitazone acutely inhibited GSIS *via* activating AMPK in MIN6 cells [36,37]. Chen et al. [38] reported that resveratrol acutely enhanced insulin secretion by blocking K_{ATP} and K_V channels of β cells, while Szkudelski et al. [39] displayed adverse results. Little is known about the long-term effect of resveratrol on islet function. In the present study, we found that resveratrol ameliorated palmitate-decreased insulin secretion, which was abolished by a SIRT1 inhibitor, EX527, not by AMPK inhibitor compound C. The result suggests that chronic resveratrol treatment potentiates GSIS through a SIRT1-dependent pathway.

Transcriptional activation of insulin gene expression is a comprehensive process. Palmitate impairment of insulin gene expression appears to be mediated by direct inhibition of insulin promoter activity [7]. Insulin promoter is mainly controlled in a 340 bp region upstream of the transcription start site. Most of the responsiveness inherent to the insulin promoter are conferred by the A3, E1 and C1 sites, which are bound by the transcription factors PDX-1, NeuroD/Beta2, and MafA respectively. These transcription factors act in a coordinated and synergistic manner to stimulate insulin gene expression in response to increased glucose levels [27]. Several studies showed that palmitate inhibited PDX-1 and MafA expressions, and reduced PDX-1 nuclear localization, which contribute to decreased insulin gene expression [7,40,41]. NKX6.1 is known to be involved in pancreatic differentiation and beta cell function. Overexpression of NKX6.1 has been shown to increase GSIS in rat islets [42]. In this study, the inhibition of SIRT1 activity by EX527 decreased insulin promoter activity and insulin mRNA expression, along with decreased mRNA expressions of transcription factors PDX-1, MafA, and NKX6.1. Knockdown of SIRT1 by siRNA verified the results. Overexpression of SIRT1 or activation of SIRT1 by resveratrol abrogated palmitate-inhibited insulin promoter activity. Moreover, the effect of SIRT1 was additive to PDX1-increased insulin promoter activity. It has

been demonstrated that PDX1 could be acetylated by P300, a well-known acetyltransferase [43]. Hence, whether SIRT1 exerts its effects by modulating the deacetylation of PDX1 needs to be determined.

Accumulating evidence indicates that the forkhead transcription factor FOXO1 serves as a negative regulator in pancreatic β-cell dysfunction. Inhibition of FOXO1 protects against fatty acid and endoplasmic reticulum stress-induced β-cell apoptosis [29]. A gain-of-function FOXO1 mutation targeted to liver and pancreatic β-cells results in diabetes, mainly due to a combination of increased hepatic glucose production and impaired β-cell compensation [44]. Pancreatic β-cells overexpressing FOXO1 also show decreased glucose use and insulin secretion [45]. Knockdown of FOXO1 led to notable increases in the beta cell population as well as insulin mRNA levels in human fetal islet epithelial cell clusters [46]. Our study showed that FOXO1 overexpression inhibited glucose-stimulated insulin promoter activity, which was antagonized by overexpressing SIRT1. Recent studies have revealed that FOXO1 negatively regulated PDX1 by modulating its subcellular localization [27,47]. H_2O_2 and palmitate stimulated FOXO1 nuclear translocation by Jun N-terminal kinase (JNK) pathway [28,29]. It is well-known that FOXO1 is a substrate of SIRT1 [8]. In our study, resveratrol reversed palmitate-stimulated FOXO1 nuclear translocation. Therefore, the inhibition of FOXO1 may be one important mechanism underlying how SIRT1 improves pancreatic β cell dysfunction.

Recently Wu et al. [48] found that sirtinol, an inhibitor of SIRT1, attenuated Wld^S (Wallerian degeneration slow)-induced activation of insulin promoter. SIRT1 was required for the improved insulin transcription and secretion to streptozotocin-induced hyperglycemia caused by Wld^S. The study of Park et al. [49] demonstrated that resveratrol inhibited phosphodiesterases, elevated cAMP level, and activated the cAMP effector protein Epac (the exchange proteins activated by cyclic AMP) in C2C12 myotubes. Epac mediates stimulatory actions of the second messenger cAMP on insulin secretion from pancreatic β cells [50]. Therefore, the role of Epac in the regulation of resveratrol and SIRT1 on pancreatic β cell function deserves further study.

Until now, little is known about the role of SIRT1 in islet function. The suppression of uncoupling protein 2 is the only known explanation for SIRT1-enhanced GSIS [15]. Our data demonstrate that SIRT1 activation ameliorates palmitate-suppressed GSIS and insulin transcriptional activity, which may be attributed to the increased expressions of β cell specific transcription factors.

Acknowledgements

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