

# SIRT4 overexpression protects against diabetic nephropathy by inhibiting podocyte apoptosis

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**Abstract.** Diabetic nephropathy is a diabetic complication associated with capillary damage and increased mortality. Sirtuin 4 (SIRT4) plays an important role in mitochondrial function and the pathogenesis of metabolic diseases, including aging kidneys. The aim of the present study was to investigate the association between SIRT4 and diabetic nephropathy in a glucose-induced mouse podocyte model. A CCK-8 assay showed that glucose simulation significantly inhibited podocyte proliferation in a time- and concentration-dependent manner. Reverse transcription-quantitative polymerase chain reaction and western blot analysis showed that the mRNA and protein levels of SIRT4 were notably decreased in a concentration-dependent manner in glucose-simulated podocytes. However, SIRT4 overexpression increased proliferation and suppressed apoptosis, which was accompanied by increases in mitochondrial membrane potential and reduced production of reactive oxygen species (ROS). Notably, SIRT4 overexpression downregulated the expression of apoptosis-related proteins NOX1, Bax and phosphorylated p38 and upregulated the expression of Bcl-2 in glucose-simulated podocytes. In addition, SIRT4 overexpression significantly attenuated the inflammatory response, indicated by reductions in the levels of TNF- $\alpha$ , IL-1 $\beta$  and IL-6. These results demonstrate for the first time that the overexpression of SIRT4 prevents glucose-induced podocyte apoptosis and ROS production and suggest that podocyte apoptosis represents an early pathological mechanism leading to diabetic nephropathy.

## Introduction

Diabetic nephropathy is a serious and common microvascular complication of diabetes and a major cause of end-stage renal disease worldwide (1). Several factors have been shown to

contribute to the progression of diabetic nephropathy; hyperglycemia, hypertension, obesity and advancing age have been extensively characterized (2,3). However, the precise mechanism for this condition remains unclear. Diabetic nephropathy is characterized by changes in kidney morphology and ultrastructure, resulting in an increased glomerular filtration rate, increased glucose level and blockade of the renin-angiotensin system (4-6).

Although maintaining control of the glycemic index is challenging, it lightens the symptoms of diabetic complications, suggesting that hyperglycemia is the critical induction factor in the development and progression of diabetic complications, including diabetic nephropathy (7,8). Treatment strategies for diabetic nephropathy, such as glycemic and blood pressure control, target various pathways contributing to the development of diabetic nephropathy (9,10). However, numerous patients continue to experience progressive renal injury. Thus, investigations of additional pathogenic pathways and relevant therapeutic strategies involving candidate targets with a potential impact on diabetic nephropathy are worthwhile. Importantly, reactive oxygen species (ROS), apoptosis and inflammatory response in the kidney are associated with the development and progression of diabetic nephropathy (11,12).

The sirtuin family has seven members, SIRT1-SIRT7, which have functions in lifespan regulation. In mammals, SIRT1, SIRT6 and SIRT7 are located in the nucleus, SIRT3, SIRT4 and SIRT5 are located in the mitochondria and SIRT2 is located in the cytoplasm (13). SIRT1 has been shown to be associated with the regulation of apoptosis, inflammation, metabolism and mitochondrial biogenesis, and play a pivotal role in neural development and age-related diseases, including type 2 diabetes (14). SIRT3 enhances lipid catabolism, regulates the tricarboxylic acid cycle and reduces the levels of ROS (15). The SIRT4 protein, which is localized to the mitochondrial cellular compartment, uses nicotinamide adenine dinucleotide to adenosine diphosphate (ADP)-ribosylate glutamate dehydrogenase (GDH) and thereby repress GDH activity and limit the generation of adenosine triphosphate (16,17). The expression of sirtuins has been observed in the kidneys, and shown to be modulated by calorie restriction to protect against the development and progression of damage in the aging kidney (18), suggesting that sirtuins may be involved in evoking susceptibility to diabetic nephropathy.

In the present study, the first comprehensive characterization of SIRT4 as a candidate gene for diabetic nephropathy is

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provided, and the association between SIRT4 overexpression and diabetic nephropathy investigated in an experimental glucose-induced mouse podocyte model.

## Materials and methods

**Cell culture and glucose treatment.** Mouse podocytes were obtained from Shanghai Cell Bank, Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI-1640 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Invitrogen; Thermo Fisher Scientific, Inc.), 100X penicillin-streptomycin solution and 10 U/ml interferon (IFN)- $\gamma$  (ProSpec-Tany TechnoGene Ltd., East Brunswick, NJ, USA), and incubated in a humidified atmosphere at 33°C with 5% CO<sub>2</sub>. After proliferation to 70%-80% confluence, podocytes were cultured in the same medium without 10 U/ml IFN- $\gamma$  and incubated in a humidified atmosphere at 37°C with 5% CO<sub>2</sub> for 10-14 days. Podocytes were exposed to normal glucose (5.5 mM) and high glucose (10, 20, 30 and 40 mM), respectively. The normal glucose (5.5 mM) treatment was used as control.

**Lentiviral production and transduction.** The SIRT4 coding sequence was cloned into a pLVX-AcGFP-C1 lentiviral vector (Sangon Biotech, Shanghai, China). A blank vector was used as negative control. The constructs were then transduced into HEK293T cells (Shanghai Cell Bank, Chinese Academy of Sciences, Shanghai, China) with psPAX2 and pMD2G lentiviral packaging vectors (Addgene, Cambridge, MA, USA) using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. After 48 h of transduction, the lentivirus was collected and used to infect mouse podocytes. Mouse podocytes were infected with the lentivirus at a multiplicity of infection of 20 in the presence of 8  $\mu$ g/ml Polybrene (Sigma-Aldrich).

**Cell proliferation assay.** Podocytes were washed, trypsinized and adjusted to 3x10<sup>3</sup> cells/well in 96-well plates, and cultured for 0, 12, 24, 36, 48, 60 and 72 h after transduction. Cell proliferation was then determined using a Cell Counting kit (CCK)-8 assay (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's protocol. Briefly, CCK-8 solution (10  $\mu$ l in 100  $\mu$ l RPMI-160 medium) was added to each well and incubated for 1 h at 37°C with 5% CO<sub>2</sub>. After incubating, the absorbance at 450 nm was measured using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

**Cell apoptosis assay.** Cell apoptosis analysis was performed using flow cytometry and an Annexin V apoptosis detection kit (eBioscience, Inc., San Diego, CA, USA). Briefly, podocytes were plated in 6-well plates at a density of 1x10<sup>5</sup> cells/well and incubated with 195  $\mu$ l Annexin V and 5  $\mu$ l propidium iodide for 15 min in the dark at 4°C. The early apoptotic cells were represented in the lower right quadrant of the fluorescence-activated cell sorting histogram.

**Mitochondrial membrane potential (MMP) measurement.** Tetrachloro-tetraethylbenzimidazolyl carbocyanine iodide (JC-1) fluorescent probe was used to detect the changes of MMP. Podocytes were resuspended in phosphate-buffered

saline (PBS) and the density was adjusted to 1x10<sup>5</sup> cells/ml. The podocytes were then incubated with 0.5 ml JC-1 in an incubator (37°C, 100% humidity and 5% CO<sub>2</sub>) for 20 min and subsequently subjected to flow cytometric analysis.

**ROS detection.** A dichlorodihydrofluorescein diacetate (DCFH-DA) fluorescent probe combined with flow cytometric analysis was used to detect the changes of ROS levels, as described previously (19). Briefly, podocytes were resuspended in PBS and the density was adjusted to 5x10<sup>5</sup> cells/ml. The podocytes were then incubated with 10  $\mu$ M DCFH-DA for 20 min in the dark at 37°C and subsequently subjected to flow cytometric analysis.

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was extracted from the podocytes using TRIzol reagent (Gibco-BRL; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Briefly, 1  $\mu$ g RNA was reverse transcribed to cDNA using a cDNA synthesis kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. qPCR was then performed to measure the mRNA levels of SIRT4 using an ABI-7300 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). Maxima SYBR Green/ROX qPCR Master Mix (K0223; Finnzymes; Thermo Fisher Scientific, Inc.) was used, according to the manufacturer's protocol. The PCR cycling conditions were as follows: 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 45 sec, and a final extension step of 95°C for 15 sec, 60°C for 1 min, 95°C for 15 sec and 60°C for 15 sec. Gene expression was calculated using the  $\Delta\Delta$ Cq method (20). The primers used were as follows: SIRT4, 5'-TTGTGGGCTGGCCTCAATTC-3' and 5'-AGTGCAAAGCGTCCACGTTTC-3'; and GAPDH, 5'-ATC ACTGCCACCCAGAAG-3' and 5'-TCCACGACGGACACA TTG-3'. The experiment was repeated three times.

**Protein extraction and western blotting.** Podocytes were harvested and lysed on ice for 30 min in radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Haimen, China) containing 1 mM phenylmethylsulfonyl fluoride. The protein concentration was assessed using a bicinchoninic acid protein assay kit (cat. no. PICPI23223; Thermo Fisher Scientific, Inc.). Equal amounts of cell lysates (35  $\mu$ g) were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the blots were incubated with primary antibodies against SIRT4 (1:1,000; ab124508; Abcam, Cambridge, MA, USA), NADPH oxidase 1 (NOX1; 1:500; ab131088; Abcam), B-cell lymphoma 2 (Bcl-2; 1:200; Sc-492; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), Bcl-2-associated X protein (Bax; 1:200; Sc-493; Santa Cruz Biotechnology, Inc.), p38 (1:1,000; cat. no. 9212; Cell Signaling Technology, Inc.), phospho (p)-p38 (1:1,000; cat. no. 9211, Cell Signaling Technology, Inc.) and GAPDH (1:1,500; cat. no. 5174; Cell Signaling Technology, Inc.) at 4°C overnight. The membranes were subsequently washed three times with Tris-buffered saline with Tween 20 (Amresco, LLC, Solon, OH, USA). The membranes were then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1,000; cat. no. A0208; Beyotime Institute of Biotechnology) and goat anti-mouse IgG (1:1,000; A0216; Beyotime Institute of Biotechnology) secondary antibodies for 1 h at 37°C, and

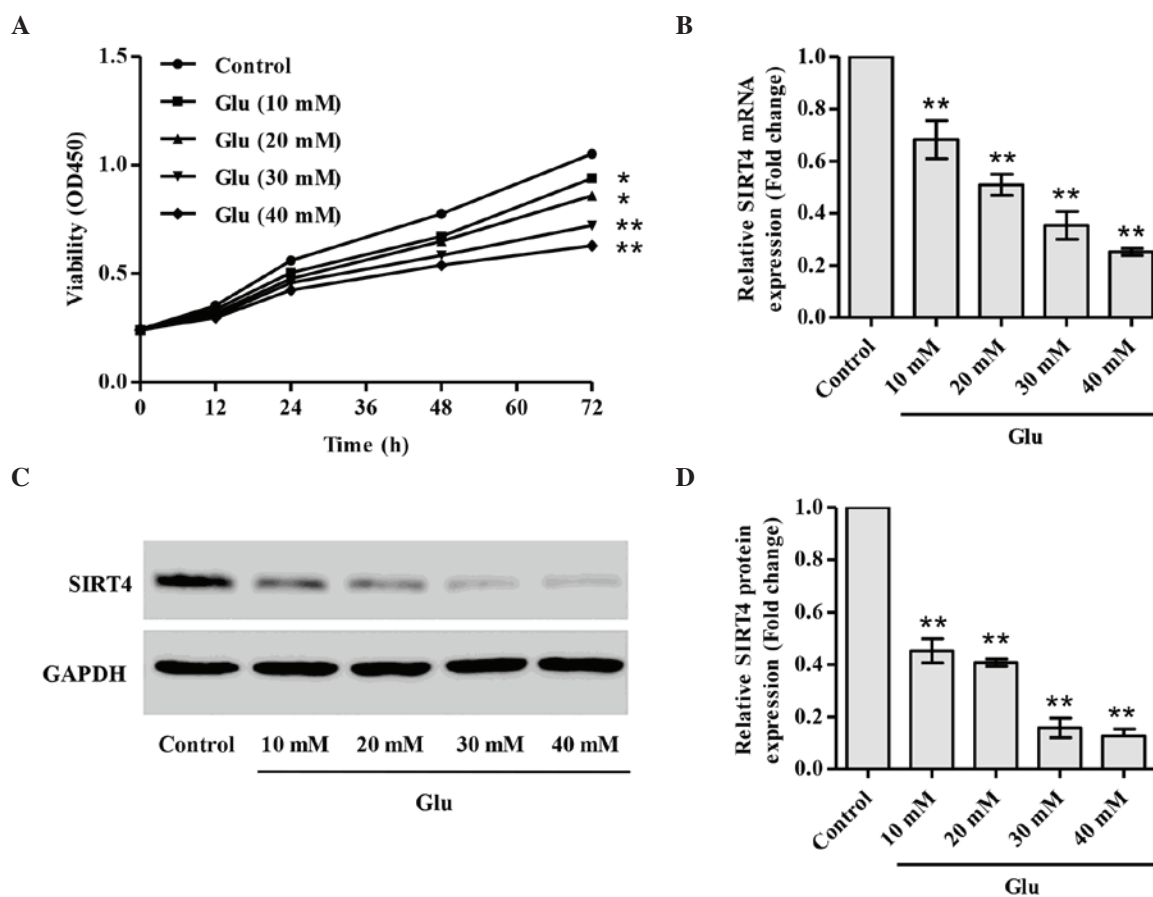


Figure 1. Glucose inhibits podocyte proliferation and downregulates SIRT4 expression. Podocytes were stimulated with glucose at different concentrations. (A) Proliferation was analyzed by Cell Counting kit-8 assay after 0, 12, 24, 36, 48, 60 and 72 h glucose stimulation. (B) mRNA expression levels of SIRT4 were examined using quantitative polymerase chain reaction, and protein expression levels of SIRT4 were examined using western blot analysis; (C) representative blots and (D) relative SIRT4 expression levels are shown. \* $P < 0.05$ , \*\* $P < 0.01$  vs. control. SIRT4, sirtuin 4; Glu, glucose; OD, optical density.

washed three times with Tris-buffered saline with Tween 20 (Amresco, LLC). The blots were visualized using enhanced chemiluminescence (Millipore, Billerica, MA, USA) and signal intensity was determined using ImageJ software version 1.46 (National Institutes of Health, Bethesda, MD, USA).

**Enzyme-linked immunosorbent assay (ELISA).** The levels of tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1 $\beta$  and IL-6 present in the podocytes were determined using commercially available murine-specific sandwich ELISA kits (cat. nos. RTA00, RLB00 and R6000B, respectively; R&D Systems, Inc., Minneapolis, MN, USA) following the manufacturer's protocol.

**Statistical analysis.** Data are presented as the mean  $\pm$  standard deviation. Paired, two-tailed Student's t-tests were used to analyze the significance of difference between groups.  $P < 0.05$  was considered to indicate a statistically significant result.

## Results

**Glucose inhibits podocyte proliferation and downregulates SIRT4 expression.** The dose-dependent effect of glucose on podocyte proliferation was investigated. The treatment of podocytes with different doses of glucose from 10 to 40 mM for 72 h significantly inhibited the proliferation of podocytes

in a dose-dependent manner (Fig. 1A). To investigate the mechanisms underlying the inhibition of proliferation and other biological behaviors induced by glucose in podocytes, the present study focused on SIRT4, an ADP-ribosylating mitochondrial enzyme that downregulates GDH activity. The mRNA and protein levels of SIRT4 were measured using RT-qPCR and western blot analysis. Notably, the mRNA and protein levels of SIRT4 were significantly decreased in the glucose-stimulated podocytes in a dose-dependent manner (Fig. 1B-D).

**SIRT4 overexpression promotes the proliferation of glucose-stimulated podocytes.** To investigate the functions of SIRT4, a SIRT4-overexpressing vector was constructed and its effect on proliferation in the presence of high glucose was examined. In glucose-treated podocytes, the mRNA and protein levels of SIRT4 were increased 0.92- and 2.19-fold, respectively, with SIRT4 transfection (Fig. 2A-C). Under glucose stimulation, SIRT4-overexpressing podocytes exhibited increased proliferation compared with the podocytes transfected with blank vector (Fig. 2D), suggesting that SIRT4 attenuates the reduction in proliferation induced by high glucose in podocytes.

**SIRT4 overexpression decreases cell apoptosis.** Treatment of podocytes with 30 mM glucose markedly increased the cell apoptosis of podocytes compared with normal glucose treatment (Fig. 2E). The effects of SIRT4 on the apoptosis of

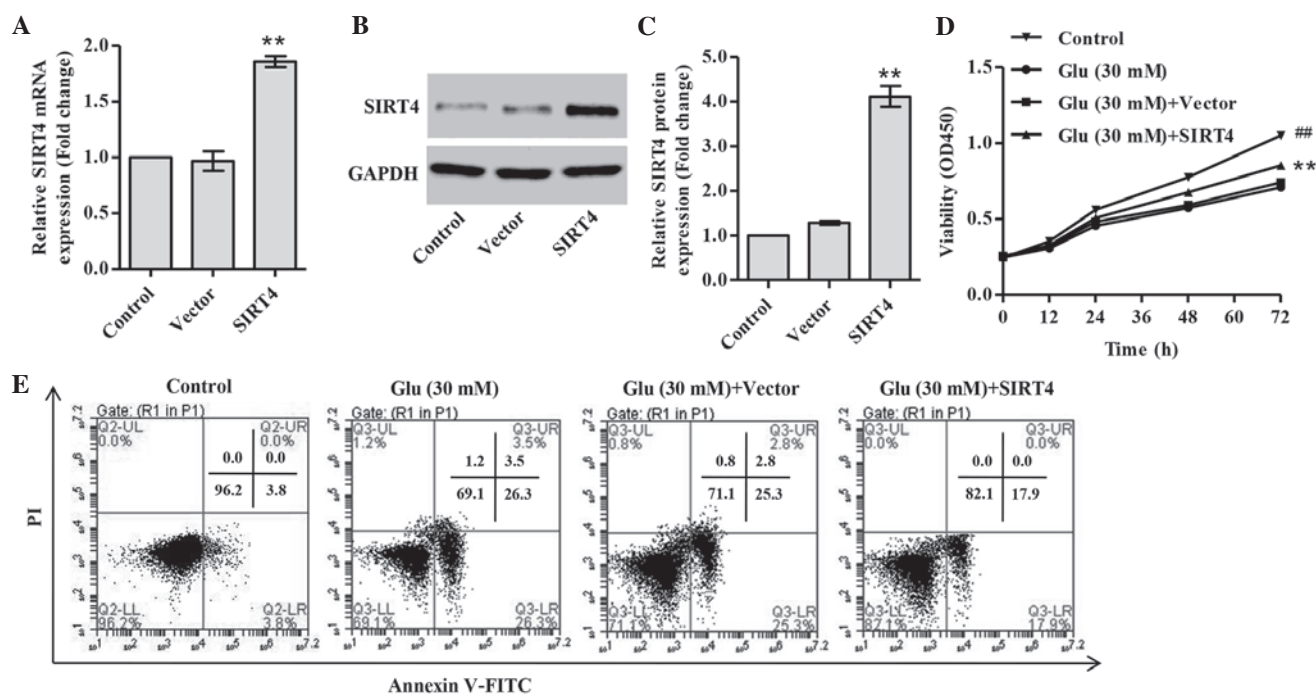


Figure 2. SIRT4 overexpression induces podocyte proliferation and inhibits apoptosis. Podocytes were stimulated with 30 mM glucose following transduction with SIRT4-overexpressing vector. (A) mRNA expression of SIRT4 was examined using reverse transcription-quantitative polymerase chain reaction, and protein levels were evaluated using western blot analysis; (B) representative blots and (C) relative SIRT4 expression levels are shown. (D) Proliferation was analyzed by Cell Counting kit-8 assay. (E) Apoptosis was analyzed by flow cytometry. \*\* $P < 0.01$  vs. control; ## $P < 0.01$  vs. Glu (30 mM). SIRT4, sirtuin 4; Glu, glucose; OD, optical density.

podocytes under glucose stimulation were then investigated. It was observed that the proportion of apoptotic cells was significantly decreased in SIRT4-overexpressing podocytes compared with podocytes transduced with blank vector (Fig. 2E). This indicates that SIRT4 reduces cell apoptosis in glucose-stimulated podocytes.

**SIRT4 overexpression inhibits apoptosis via the mitochondrial pathway.** Loss of MMP is correlated with the mitochondrial apoptotic pathway (21). Thus, the effect of SIRT4 overexpression on the MMPs of podocytes under glucose stimulation was next assessed. As shown in Fig. 3A and B, treatment of podocytes with 30 mM glucose significantly decreased the MMP levels of podocytes compared with normal glucose treatment ( $P < 0.01$ ). SIRT4 overexpression significantly increased the MMPs of podocytes compared with those of podocytes transduced with blank vector ( $P < 0.01$ ), suggesting that SIRT4 causes the polarization of mitochondrial membranes (Fig. 3A and B). However, ROS generation is also associated with mitochondria (22). Fluorescence probe DCFH-DA was used to determine the levels of ROS production in glucose-stimulated podocytes. The data indicated that treatment of podocytes with 30 mM glucose significantly increased the ROS accumulation of podocytes compared with normal glucose treatment ( $P < 0.01$ ; Fig. 3C and D). SIRT4 overexpression significantly decreased ROS accumulation compared with that in the podocytes transduced with blank vector ( $P < 0.01$ ; Fig. 3C and D). These results suggest that SIRT4 overexpression inhibits apoptosis via the mitochondrial pathway in glucose-stimulated podocytes.

**Expression of apoptosis-related proteins.** To clarify the mechanism by which glucose induces podocyte apoptosis, the expression levels of apoptosis-related proteins were determined by western blotting. As shown in Fig. 4, treatment of podocytes with 30 mM glucose significantly increased expression levels of NOX1 and Bax, as well as the phosphorylation of p38 (p-p38), but decreased the expression of Bcl-2 in glucose-stimulated podocytes compared with the normal glucose treatment ( $P < 0.01$ ). However, SIRT4 overexpression attenuated these changes. These results indicate that the mechanism by which SIRT4 overexpression inhibits the podocyte apoptosis induced by glucose may involve inhibition of p38 activation.

**SIRT4 overexpression inhibits the release of TNF- $\alpha$ , IL-1 $\beta$  and IL-6.** To elucidate the effect of SIRT4 overexpression on inflammatory responses under glucose stimulation, the expression levels of the pro-inflammatory cytokines TNF- $\alpha$ , IL-1 $\beta$  and IL-6 were investigated. As shown in Fig. 5, treatment of podocytes with 30 mM glucose significantly increased the production of TNF- $\alpha$ , IL-1 $\beta$  and IL-6 compared with normal glucose treatment. However, SIRT4 overexpression attenuated these changes. These data suggest that SIRT4 may protect glucose-stimulated podocytes against inflammation.

## Discussion

The results of the present study indicate that SIRT4 inhibits podocyte apoptosis in a glucose-induced diabetic nephropathy model. SIRT4 is an enzyme that converts glutamate to  $\alpha$ -ketoglutarate in mitochondria and regulates the ability of pancreatic  $\beta$  cells to secrete insulin in response to glucose

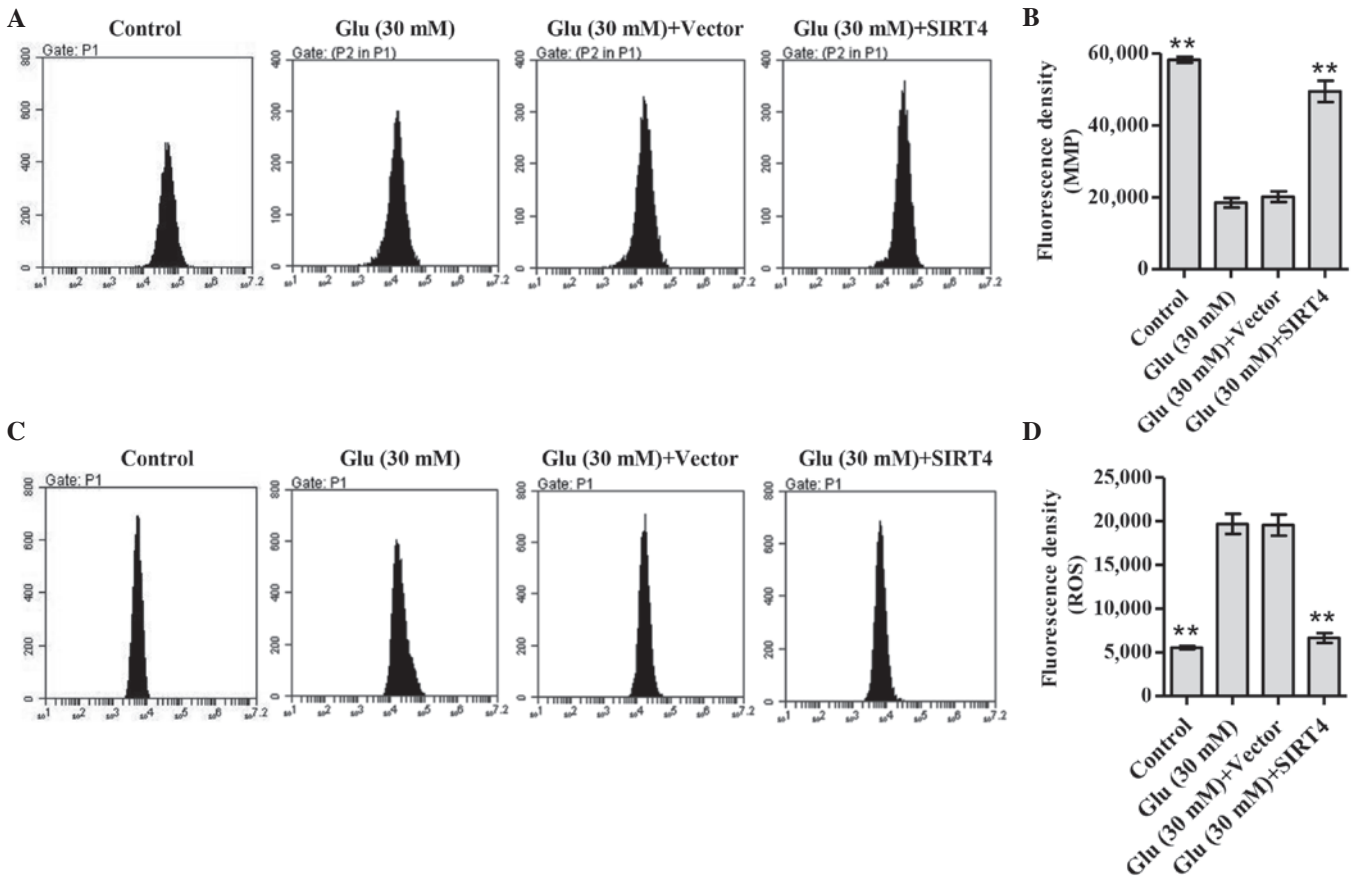


Figure 3. SIRT4 overexpression increases MMP elicitation and decreases ROS production. Podocytes were stimulated with 30 mM glucose after transduction with SIRT4-overexpressing vector. (A) Podocytes incubated with fluorescent probe JC-1 were analyzed by flow cytometry; (B) fluorescent density indicated the MMP. (C) Podocytes were incubated with fluorescent probe dichlorodihydrofluorescein diacetate and analyzed by flow cytometry; (D) fluorescent density indicated ROS levels. \*\*P<0.01 vs. Glu (30 mM). SIRT4, sirtuin 4; MMP, mitochondrial membrane potential; ROS, reactive oxygen species; Glu, glucose.

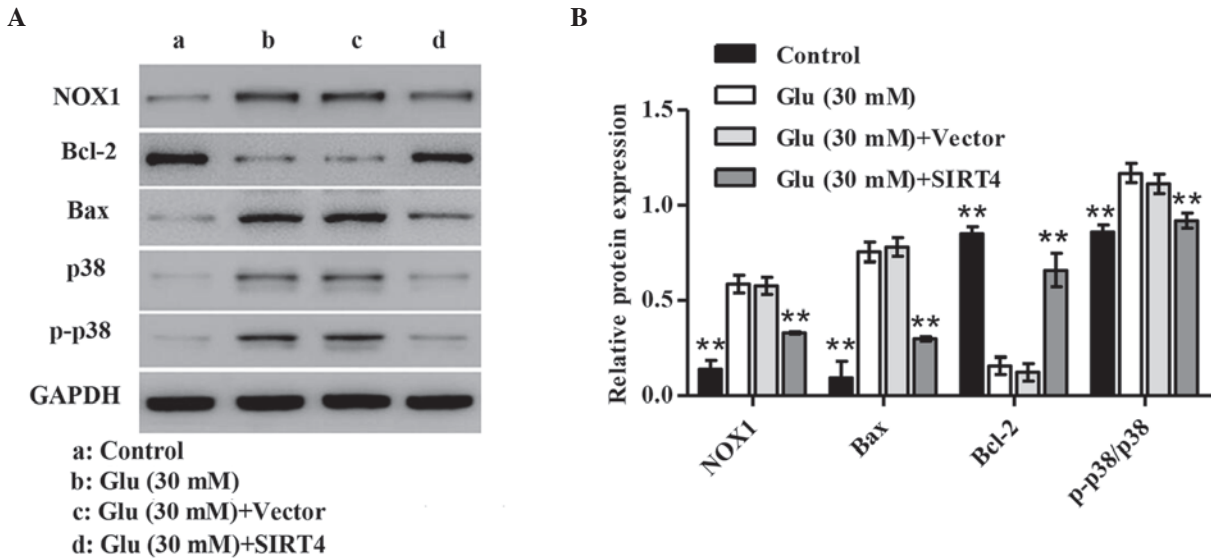


Figure 4. SIRT4 overexpression regulates apoptosis-related protein expression. Podocytes were stimulated with 30 mM glucose after transduction with SIRT4-overexpressing vector. (A) Western blotting and (B) quantification of NOX1, Bax, Bcl-2 and p38 expression levels in different groups. \*\*P<0.01 vs. Glu (30 mM). SIRT4, sirtuin 4; NOX1, NADPH oxidase 1; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; Glu, glucose.

and amino acids (23). SIRT4 knockdown and calorie restriction activate G6PD and upregulate the secretion of insulin by  $\beta$  cells, suggesting that the effects of SIRT4 oppose those of

calorie restriction in pancreatic  $\beta$  cells (23), i.e., the expression of SIRT4 is upregulated under hyperglycemic conditions. SIRT4 is downregulated in insulin-resistant rats (24) and in

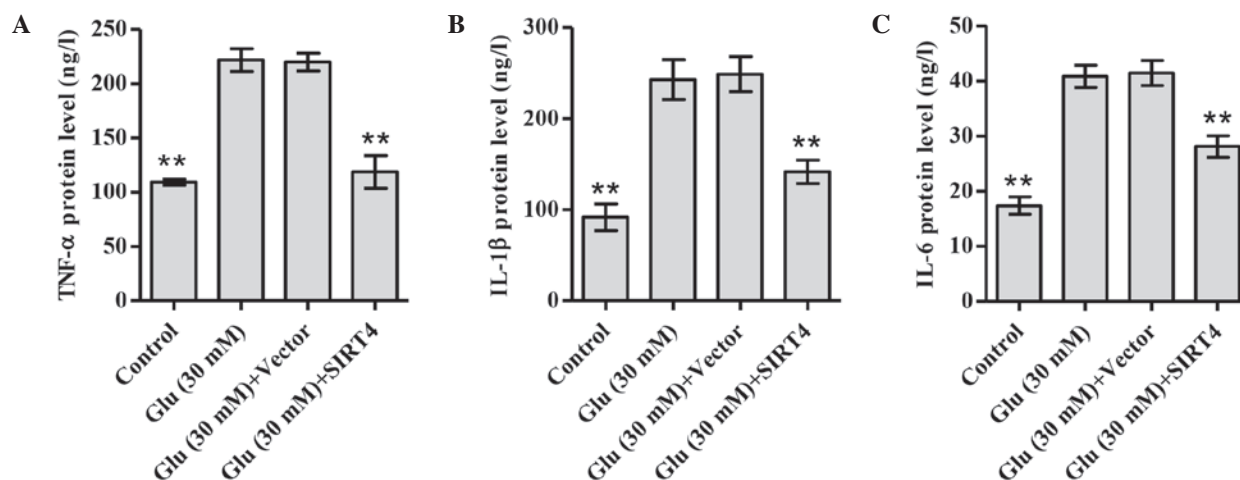


Figure 5. SIRT4 overexpression represses the production of TNF- $\alpha$ , IL-1 $\beta$  and IL-6. Podocytes were stimulated with 30 mM glucose after transduction with SIRT4-overexpressing vector. ELISA analysis of (A) TNF- $\alpha$ , (B) IL-1 $\beta$  and (C) IL-6 protein expression in different groups. \*\* $P < 0.01$  vs. Glu (30 mM). SIRT4, sirtuin 4; TNF, tumor necrosis factor; IL, interleukin; Glu, glucose.

a type 2 diabetes mouse model (25), which is consistent with the results of the present study showing the downregulation of SIRT4 in glucose-induced podocytes.

Although a reduction in the number of podocytes is one of the strongest predictors of progression of diabetic nephropathy (26), the cause, molecular pathways and pathological mechanisms underlying the depletion of podocytes in diabetic nephropathy remain poorly understood. Podocyte apoptosis has been observed in various mouse models of nondiabetic renal disease, including nephritis and glomerulosclerosis with TGF- $\beta$ 1 induction by CD2-associated protein knockdown (27). The observation in the present study of inhibited podocyte proliferation and increased apoptosis with hyperglycemia indicates that the cytotoxicity of glucose contributes to apoptosis. Previous studies have shown that glucose induces apoptosis in several cell types, including glomerular cells, proximal tubular cells and podocytes (28-30). However, the mechanism of glucose-induced podocyte apoptosis has not been previously elucidated. In the present study, it was observed that 30 mM glucose significantly inhibited podocyte proliferation and increased podocyte apoptosis. Notably, SIRT4 overexpression attenuated the effects of glucose on podocyte proliferation. These data suggest that SIRT4 overexpression may inhibit podocyte apoptosis and reduce podocyte injury under hyperglycemic conditions.

In the present study, the MMP and ROS levels were also examined in podocytes, and it was found that glucose stimulation was associated with a decline in the MMP and increased ROS production in podocytes. This is consistent with the findings of Bock *et al*, who found that glucose impaired the MMP and increased ROS levels (31). However, in the present study, SIRT4 overexpression increased the MMP and reduced ROS levels in podocytes under hyperglycemic conditions, indicating that SIRT4 overexpression inhibits apoptosis by reducing the generation of ROS from mitochondrial sources in podocytes. In addition, NOX1 is an NADPH oxidase that was observed to be significantly increased in glucose-induced podocytes, indicating that NADPH oxidase-dependent ROS generation may be involved in glucose-induced podocyte apoptosis *in vitro*,

which is validated in diabetic cardiovascular and renal complications (32). In the present study, the results showed a significant induction of ROS production followed by an increase in the expression of the proapoptotic Bax and phosphorylated p38 and reduction of antiapoptotic Bcl-2 in podocytes following exposure to glucose. A possible molecular association between ROS and p38 activation was demonstrated by the observation that increased oxidative stress induces p38 activation followed by podocyte apoptosis (33). Importantly, SIRT4 overexpression opposed the effects of glucose induction in podocytes in the present study, suggesting that SIRT4 attenuates podocyte apoptosis via the inhibition of p38 pathway activation.

Although diabetic nephropathy has been considered a non-immune disease, the overproduction of leukocytes in the kidneys of diabetic humans and in experimental animal models of diabetes has been found in a previous study (34). Several reviews have examined the role of pro-inflammatory cytokines in diabetic nephropathy; however, the mechanism remains poorly understood (35,36). In the present study, the results showed that the pro-inflammatory cytokines TNF- $\alpha$ , IL-1 $\beta$  and IL-6 were notably decreased in SIRT4-overexpressing podocytes with glucose stimulation. TNF- $\alpha$  plays an important role in the development and progression of diabetic nephropathy supported by the observation of increases in renal TNF- $\alpha$  levels in diabetic animal models and patients (37,38), indicating that increased TNF- $\alpha$  levels result in renal damage. Clinical studies have reported significant increases in the renal production of IL-1 $\beta$  and IL-6 in patients with type 2 diabetic nephropathy compared with that in diabetic patients without nephropathy, suggesting a role of IL-1 $\beta$  and IL-6 in the pathogenesis of diabetic nephropathy (39,40). Similar to the aforementioned opposing effects of SIRT4 in glucose-induced podocytes, SIRT4 overexpression also attenuated the production of TNF- $\alpha$ , IL-1 $\beta$  and IL-6. These data support a role for SIRT4 in the inflammatory response of diabetic nephropathy.

In conclusion, this study found that SIRT4 is associated with diabetic nephropathy and the present data suggest that SIRT4 is a good candidate for diabetic nephropathy; however the hypothesis should be evaluated in further studies.

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