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Original Article

Effects of miR-155-5p regulating SIRT1 on diabetic nephropathy in mice

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Abstract: To investigate the effects of miR-155-5p regulating silent information regulator 1 (SIRT1) on diabetic nephropathy (DN) in mice. Forty mice were assigned into the DN group (N=30, high-fat diets) and control group (n=10, ordinary diets), and human glomerular mesangial cells (HMCs) were purchased. Changes in miR-155-5p and SIRT1 expression in HMCs and renal tissues of DN mice were determined using quantitative real-time RT-PCR (qRT-PCR). Levels of proliferating cell nuclear antigen (PCNA), cyclin D1, recombinant p21 protein (p21) and apoptosis-related proteins Caspase-3, Caspase-9, Bax and Bcl-2 were quantified. The DN group presented with increased miR-155-5p and decreased SIRT1 expression in renal tissues. Up-regulating miR-155-5p or down-regulating SIRT1 remarkably accelerated abnormal cell proliferation, elevated PCNA and Cyclin D1, suppressed p21, inhibited cell apoptosis in DN mice, as well as down-regulated Bax, Caspase-3, Caspase-9 and up-regulated Bcl-2. There was a regulatory relationship between miR-155-5p and SIRT1 confirmed by dual-luciferase reporter (DLR) assay. MiR-155-5p improves the survival of DN mice through targeted regulation of SIRT1 and may be a new target for diagnosis and gene therapy for DN.

Keywords: miR-155-5p, SIRT1, diabetic nephropathy, renal tissue, cell, expression, effect

Introduction

Diabetes, a common chronic metabolic disease has an increasing prevalence, and is associated with diabetic nephropathy (DN), cardiovascular diseases and other complications [1, 2]. DN is the major cause of endstage renal disease, manifested by glomerular hypertrophy and fibrosis and increased formation of the extracellular matrix (ECM) [3, 4]. Multifactorial pathogenesis of DN leads to a lack of satisfactory treatment options for this latent disease in clinical practice [5]. Therefore, it is necessary to explore and clarify the pathogenesis of DN and seek new potential therapeutic targets to improve the prognosis of patients.

MicroRNAs (miRNAs, miRs) are a set of non-coding protein products that modulate other messenger RNAs [6] and gene expression at epigenetic and post-translational levels, thus affecting multiple cellular processes [7]. Besides, they promote or slow down DN progression by targeting fibrosis, oxidative stress, signaling transduction and inflammation related genes. Some of these miRs function in amplifying circuits, while others exert autonomous effects and cell-specific effects [8]. Hence, it is necessary to explore the molecular mechanism of miR disorders associated with DN. The suppression of miR-21 inhibits inflammation and apoptosis of high glucose-treated podocytes in streptozotocin (STZ)-induced DN rats via targeting tissue inhibitors of metalloproteinase-3 (TIMP3) [9]. MiR-135a is significantly up-regulated in serum and renal tissues of both patients and db/db mice with DN, which is related to microalbuminuria and renal fibrosis. Therefore, inhibition of miR-135a may contribute to the treatment of DN [10]. MiR-155-5p participates in the pathogenesis of DN and shows abnormal up-regulation [11]. Silent information regulator 1 (SIRT1) is a mammalian sirtuin/Sir2 gene in NAD^+-dependent deacetylase family that regulates insulin secretion,

mitochondrial biogenesis and glucose and lipid metabolism in multiple tissues [12]. Moreover, SIRT1 is reduced in the glomeruli of mice and patients with DN, so increasing the activity of SIRT1 slows down DN progression [13].

We noticed that miR-155-5p and SIRT1 shared targeted sites, and there may be a potential regulatory relationship between the two. Therefore, we suspect that miR-155-5p has potential protective effect on renal tissues and cells of DN mice by regulating SIRT1.

Materials and methods

Laboratory animals, materials and reagents

Forty 16-week-old C57BL/6 male mice (Junke Bioengineering Co., Ltd., Nanjing, China, J006) with an average body weight of 27±3 g were exposed to 12-h light/dark cycle, 22±2°C room temperature, 50-65% humidity, and free access to food for 15 days. This study strictly followed the principles of protection and use of laboratory animals [14]. Human glomerular mesangial cells (HMCs) (ATCC, bio-107461) were grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) at 37°C/5% CO_2.

Modeling

Forty mice were allocated into the control group, DN group, DN+miR-155-5p-mimics group, and DN+si-SIRT1 group. Modeling steps [15]: Before the experiment, all mice were fasted for 12 hours and were allowed to drink water. The control mice received only intraperitoneal injection of citrate buffer, and others were intraperitoneally injected with 55 mg/kg STZ (SHR Biotechnology Co., Ltd., Nanjing, China, S817944-1 g). Afterwards, the food and water intake and 24-h urine were monitored. After 72 hours, blood was sampled at the tail tip, and urine glucose test papers were used for measurement continuously for 3 days. Values of random blood glucose > 22.2 mmol/L, fasting blood glucose > 16.7 mmol/L, 24-h urine volume > 150% of the controls, and strongly positive Clinitest indicated the successful establishment of a diabetic model. After 4 weeks of continuous feeding, urine microalbumin > 15 μg/mL indicated the successful modeling of DN.

Cell culture and transfection

HMCs were incubated in DMEM (Chreagen Biotechnology Co., Ltd., Beijing, China, 120-002) with 10% FBS at 5% CO_2/37°C. After passaging, the cells were transfected with: inhibitor (miR-155-5p inhibition sequence), mimics (miR-155-5p over-expression sequence), miR-negative control (miR-NC), targeted over-expression of SIRT1 RNA (sh-SIRT1), targeted inhibition of SIRT1 RNA (si-SIRT1). DN models were established in all groups except the control group. After the cells reaching confluence, the culture solution was replaced with DMEM with 25 mmol/L glucose.

Quantitative real-time RT-PCR (qRT-PCR)

After extracting total RNA with Trizol (Chreagen Biotechnology Co., Ltd., Beijing, China, 121-238), the purity and concentration were measured. Total RNA (5 μg) was reverse-transcribed into cDNA with a reverse transcription kit (Qiming Biotech Co., Ltd., Shanghai, China, OX02700). Reaction parameters: 37°C for 15 min, 42°C for 15 min, 70°C for 5 min. Amplification system (20 μL): 1 μL of cDNA, 0.4 μL each of upstream and downstream primers, 10 μL of 2 × TransTaq® Tip Green qPCR SuperMix, 0.4 μL of Passive Reference Dye (50X), 0.8 μL of H_2O. Amplification conditions: PCR conditions: 94°C for 30 sec, followed by 40 cycles of 94°C for 5 sec, 60°C for 30 sec. Each sample was measured 3 times in 3 repeated wells. U6 and β-actin were employed as internal reference for miR and mRNA, respectively. Primers were all designed by GenePharma (Shanghai, China), and the data were processed using $2^{-\Delta\Delta CT}$.

Western blotting (WB)

Renal tissue (50 mg) was lysed in 500 μL lysis buffer (Hengfei Biotech, Shanghai, China, S0015). After homogenization in ice bath, centrifugation (12,000 × g) was carried out at 4°C for 20 min. The protein concentration in supernatant was measured by BCA kit (Rongbai Biotech, Shanghai, China, LCB004). Following 12% sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) separation (Xinyu Biotech, Shanghai, China, XY-0672), the proteins were moved to a polyvinylidene difluoride (PVDF) membrane, which were then placed

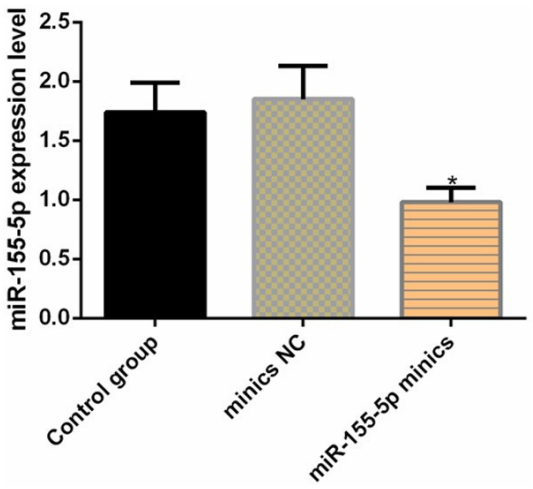

Figure 1. Transfection efficacy of miR-155-5p. The level of miR-155-5p in miR-155-5p mimics group is remarkably lower than that in control group and mimics NC group. Note: *P < 0.05.

in 5% skim milk powder (Yiyan Biotech, Beijing, China, ISEQ00011) for immune reaction. After incubating overnight with the membrane at 4°C, primary antibody (1:1000) was removed, then goat anti-rabbit secondary antibody (horseradish peroxidase coagulated, 1:1000) was added and incubated for 1 h at 37°C. The membrane was rinsed 3 times with phosphate buffer saline (PBS) for 5 min each. Development was carried out with enhanced chemiluminescence (ECL), and Quantity One infrared imaging system was adopted to capture images. Relative expression of target protein = gray value of target band/gray value of internal reference band.

Proliferation test (CCK-8)

Cells harvested 24 h after transfection were inoculated in 96-well plates (4×10^6 cells/well) and cultured for 24 h, 48 h, 72 h, and 96 h. CCK solution (10 μL) and basic medium (DMEM, 90 μL) were applied to each well, followed by another culture at 37°C for 2 h. The optical density (OD) value was read at 570 nm under an Elisa reader.

Apoptosis test (flow cytometry)

Transfected cells were trypsinized (0.25%, Yuanye Biotech Co., Ltd., Shanghai, China, R20109) and washed twice with PBS, then prepared into suspension (1×10^6 cells/mL) with 100 μL of binding buffer. AnnexinV-FITC and propidium iodide (PI) were added in sequence, the cells were grown in the dark at room temperature for 5 min to detect the apoptosis with a flow cytometer (Beamdiag Biotech Co., Ltd., Suzhou, China, 1026). The experiment was performed 3 times and the average was taken.

Dual-luciferase reporter (DLR) assay

SIRT1-3' untranslated regions (3'UTR) wild type (Wt) and SIRT1-3'UTR Mutant (mut) were transferred into HMCs using Lipofectamine™ 2000. After 2 days, a DLR assay kit was used to determine the luciferase activity (Baiao Laibo Technology, Beijing, China, KFS303-LBV).

Statistical analysis

Data processing was performed with SPSS 19.0, and figure illustration was done with GraphPad 7. Normal distribution data were represented by mean ± SD, and between-group comparison was conducted by independent samples t test. Non-normal distribution data expressed by quartiles [M (P25-P75)] were tested by nonparametric test (denoted by Z). Multi-group comparison was performed with one-way analysis of variance (ANOVA), and difference analysis was conducted with Fisher's least significant difference-t (LSD-t) test. The differences were indicated as statistically significant at P < 0.05.

Results

Transfection efficacy of miR-155-5p

qRT-PCR revealed that mice in the miR-155-5p mimics group showed suppressed miR-155-5p expression compared to those in the control group and mimics NC group (P < 0.05) (**Figure 1**).

Effects of miR-155-5p on HMC proliferation and cyclins

No differences were shown in HMC proliferation between the control group and DN group, while HMC proliferation clearly decreased in the miR-155-5p-inhibitor group and clearly increased in the miR-155-5p mimics group (P < 0.05). Proliferating cell nuclear antigen (PCNA) and Cyclin D1 increased and p21 decreased in HMCs treated with miR-155-5p-mimics (both P

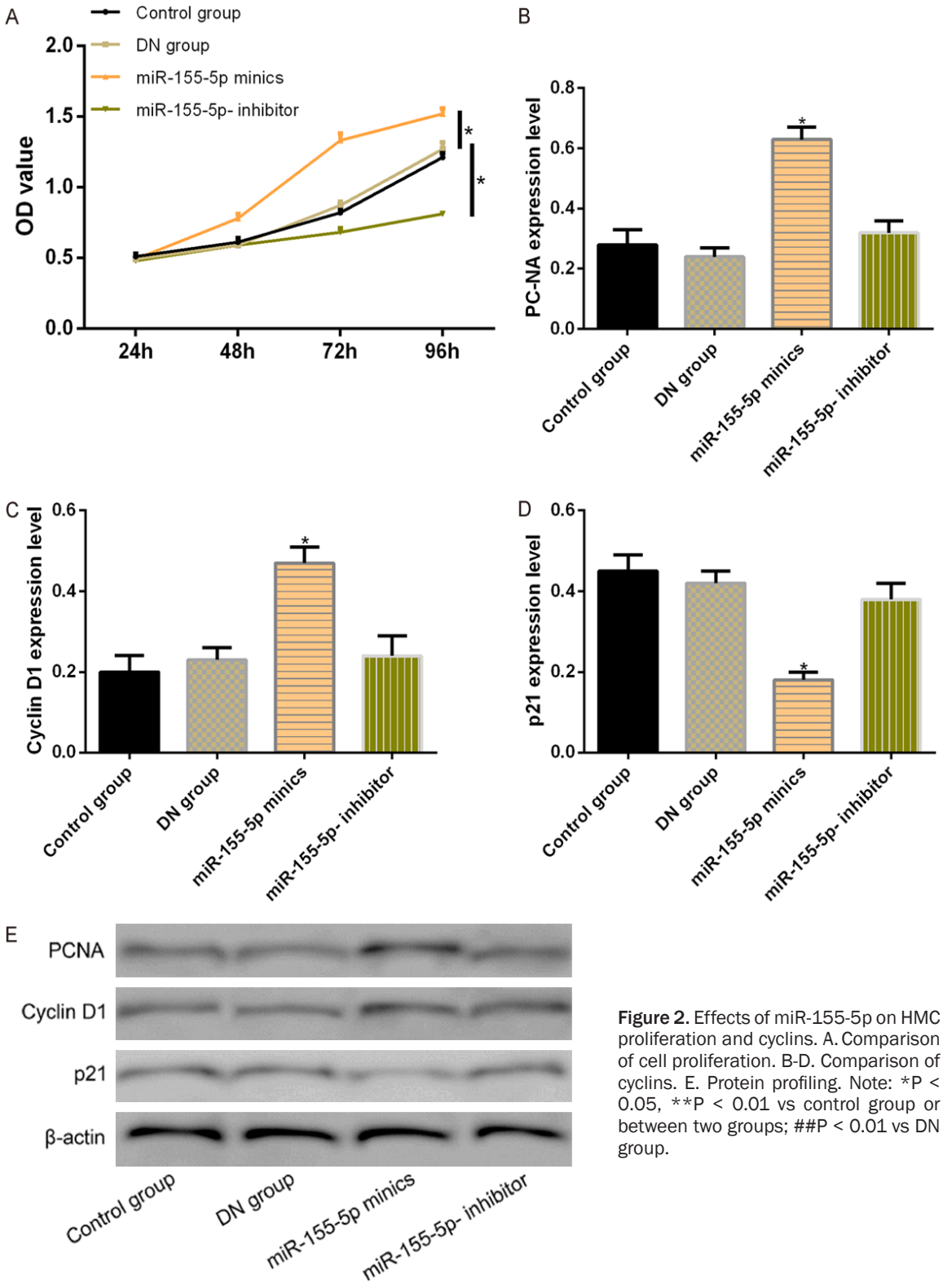

Figure 2. Effects of miR-155-5p on HMC proliferation and cyclins. A. Comparison of cell proliferation. B-D. Comparison of cyclins. E. Protein profiling. Note: *P < 0.05, **P < 0.01 vs control group or between two groups; ##P < 0.01 vs DN group.

< 0.05), and miR-155-5p-inhibitor showed no such effects on them (P > 0.05). Therefore, miR-155-5p accelerated the proliferation of HMCs (**Figure 2**).

Effects of down-regulation of miR-155-5p on HMC apoptosis and related proteins

Compared with the control group, HMC apoptosis in the DN group was remarkably enhanced ($P < 0.05$). Bax, Caspase-3, and Caspase-9 were elevated ($P < 0.05$) and Bcl-2 was suppressed ($P < 0.05$). MiR-155-5p-inhibitor reversed these results ($P < 0.05$) (**Figure 3**).

Effects of SIRT1 on HMC proliferation and cyclins

Compared with the control group, HMC proliferation in the DN group decreased ($P < 0.05$). After transfection of sh-SIRT1, PCNA and Cyclin D1 increased ($P < 0.05$) and p21 decreased ($P < 0.05$) in HMCs, but si-SIRT1 had no effect on them ($P > 0.05$) (**Figure 4**).

Effects of up-regulation of SIRT1 on HMC apoptosis and related proteins

HMC apoptosis in the DN group increased remarkably compared to that in control group ($P < 0.05$), and Bax, Caspase-3, and Caspase-9 were elevated ($P < 0.05$), while Bcl-2 was suppressed ($P < 0.05$). However, sh-SIRT1 reversed these results ($P < 0.05$) (**Figure 5**).

SIRT1 is regulated by miR-155-5p

Targetscan7.2 demonstrated SIRT1 shared targeted binding sites with miR-155-5p. It was revealed by DLR assay that after inhibiting miR155-5p in HMC cells, luciferase activity of SIRT1 3'UTR-Wt increased ($P < 0.05$), but SIRT1 3'UTR-Mut revealed no changes ($P > 0.05$). In WB, SIRT1 was clearly elevated in cells treated with miR-155-5p-inhibitor ($P < 0.05$) (**Figure 6**).

Discussion

DN is a prevalent chronic microvascular complication of diabetes mellitus, and approximately 40% of diabetic patients suffer from DN. Abnormal accumulation of glomerular basement membrane thickening and tubulointerstitial dilatation are the main pathological features [16], the control of blood lipids and blood sugar, and anti-inflammatory therapy are preferred treatments for DN, but the effectiveness of these treatments are disappointing. Metabolic and hemodynamic alterations are considered to be part of the pathophysiologic processes of DN [17]. Therefore, identifying the mechanisms and novel targets is necessary to improve the prognosis of DN patients. MiRs participate in the progress of diseases by influencing cell biological functions [18]. A study shows us the intermediary role of miRs in the pathogenesis of DN [19]. Besides, suppressed miR-155-5p is found in DN [20], but its role in this disease is not fully understood.

Our findings demonstrated that miR-155-5p was highly expressed in renal tissues and HMCs of DN mice, suggesting its participation in DN progression. Subsequently, we transfected HMCs with miR-155-5p mimics and miR155-5p-inhibitors and observed the changes of PCNA, Cyclin D1 and p21. PCNA is a nucleoprotein and a marker of cell proliferation that evaluates proliferation disorder, and increase of PCNA-immunoreactivity increases cell proliferation [21]. Cyclin D1, a regulatory protein in cell cycle, is over-expressed in many tumors [22]. p21 is a cyclin-dependent kinase (CDK) inhibitor regulating cell cycle through inactivated CDK modulators [23]. The proliferation of HMCs in miR-155-5p mimics group was enhanced, while that in miR-155-5p-inhibitor group was weakened, indicating that miR-155-5p is associated with the proliferation of HMCs. In miR155-5p mimic-treated cells, PCNA and Cyclin D1 increased and p21 decreased. However, miR-155-5p-inhibitor had no effect on them, indicating that overexpression of miR-155-5p effectively improves the expression of cyclins in DN and promotes the proliferation of HMCs.

MiRs affect cellular biological functions by regulating the target genes. Increasing attention has been paid to the regulatory role of miR-155-5p in DN. For example, Guo et al pointed out that miR-155-5p is elevated in DN rats and cell models, while dihydromyricetin inhibits high sugar-induced fibrosis and promotes autophagy by inhibiting miR-155-5p in NRK-52E cells [24]. In renal tubular injury of diabetic kidney disease, miR-155-5p increases with the elevation of high glucose concentration in HK-2 cells, and the increase in miR-155-5p may activate P53 and inhibit SIRT1, as well as form a positive feedback pathway [25]. These results suggest the involvement of miR-155-5p and SIRT1 in the pathological mechanism of DN

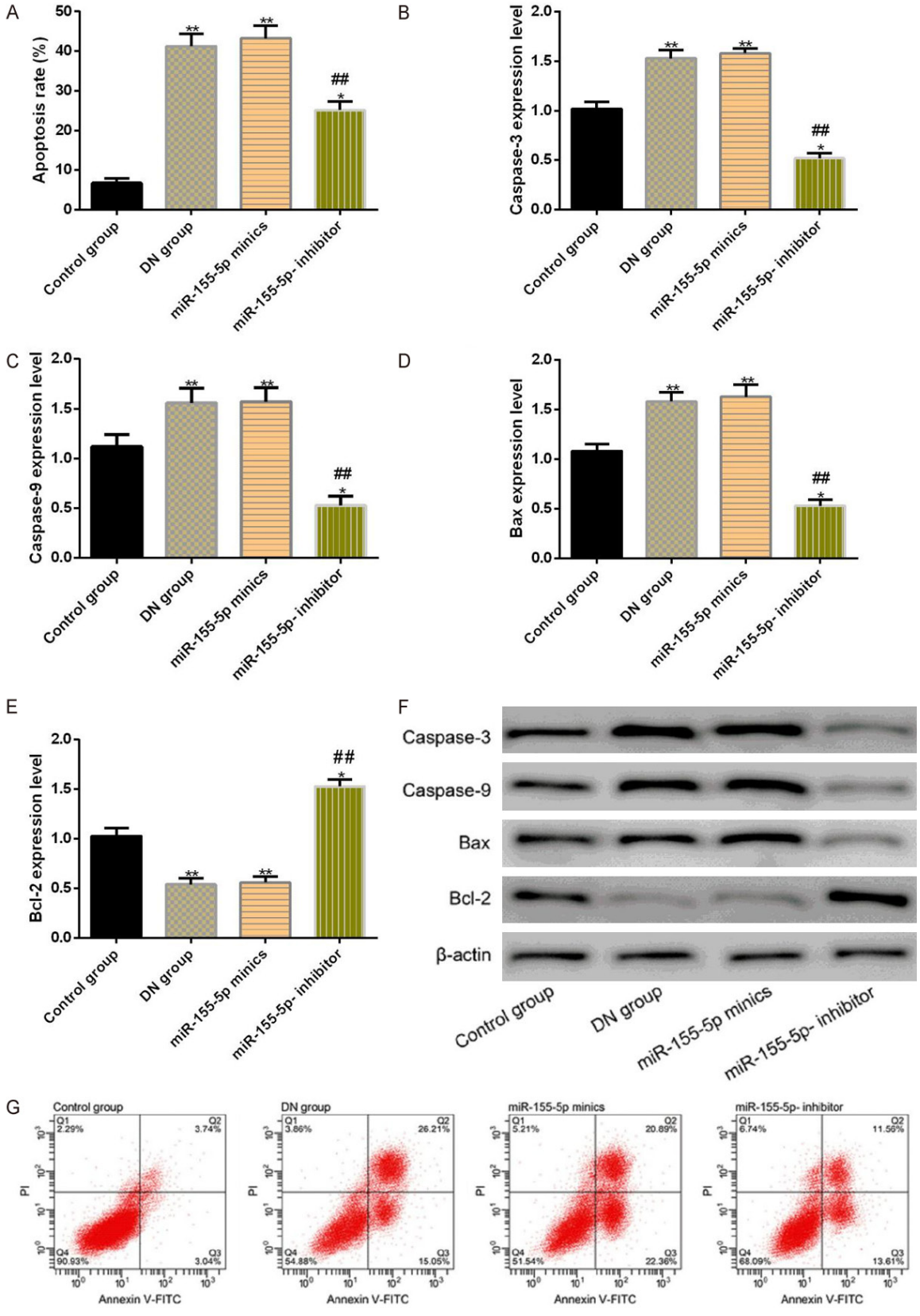

Figure 3. Effects of down-regulation of miR-155-5p on HMC apoptosis and related proteins. A. Down-regulation of miR-155-5p enhances HMC apoptosis. B-E. Down-regulation of miR-155-5p improves the levels of apoptosis-related proteins. F. Protein profiling of apoptosis-related proteins. G. Cell cytometry. Note: *P < 0.05, **P < 0.01 vs control group or between two groups; ##P < 0.01 vs DN group.

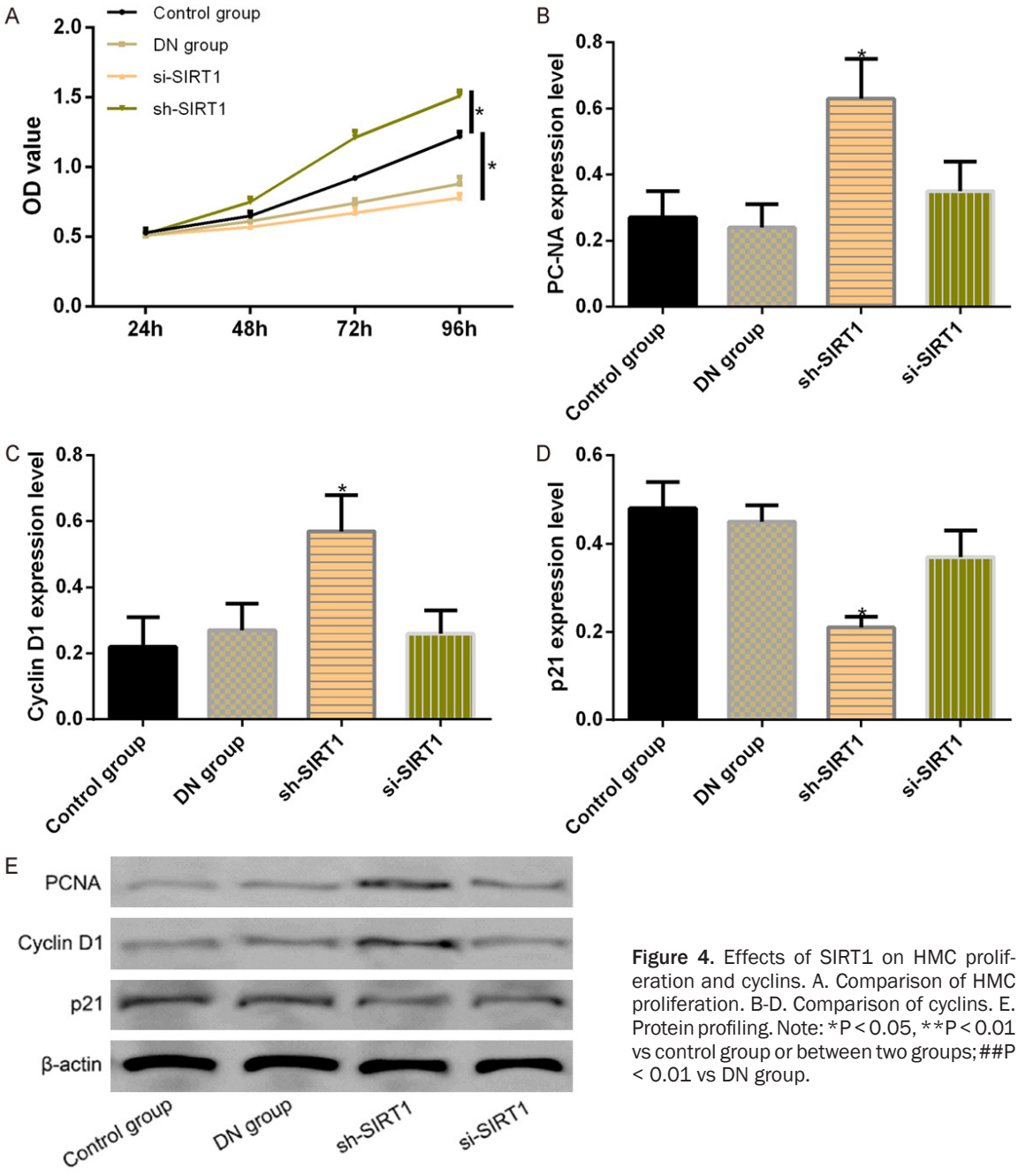

Figure 4. Effects of SIRT1 on HMC proliferation and cyclins. A. Comparison of HMC proliferation. B-D. Comparison of cyclins. E. Protein profiling. Note: *P < 0.05, **P < 0.01 vs control group or between two groups; ##P < 0.01 vs DN group.

mice, which is similar to our results. HMCs were treated with high glucose and miR-155-5p transfection in this study to analyze the apoptosis and related proteins. It turned out that the DN group had higher apoptosis and higher levels of Caspase-3, Caspase-9 and Bax. Caspase-3, Caspase-9 and Bax are pro-apoptotic proteins that have a higher level in the high glucose-induced group, while Bcl-2, an anti-apoptotic protein, shows the opposite results, which are reversed after effective intervention [26, 27]. This is similar to our findings, that is, suppressing miR-155-5p is able to improve expression of apoptosis-related proteins and effectively accelerate apoptosis in DN. The levels of PCNA and Cyclin D1 were elevated and p21 was decreased in HMCs transfected with sh-SIRT1, but si-SIRT1 had no effect on PCNA, Cyclin D1 and p21, which suggested that SIRT1 also participates in the proliferation of HMCs. After SIRT1 transfection, HMC apoptosis in the DN group was enhanced, Caspase-3,

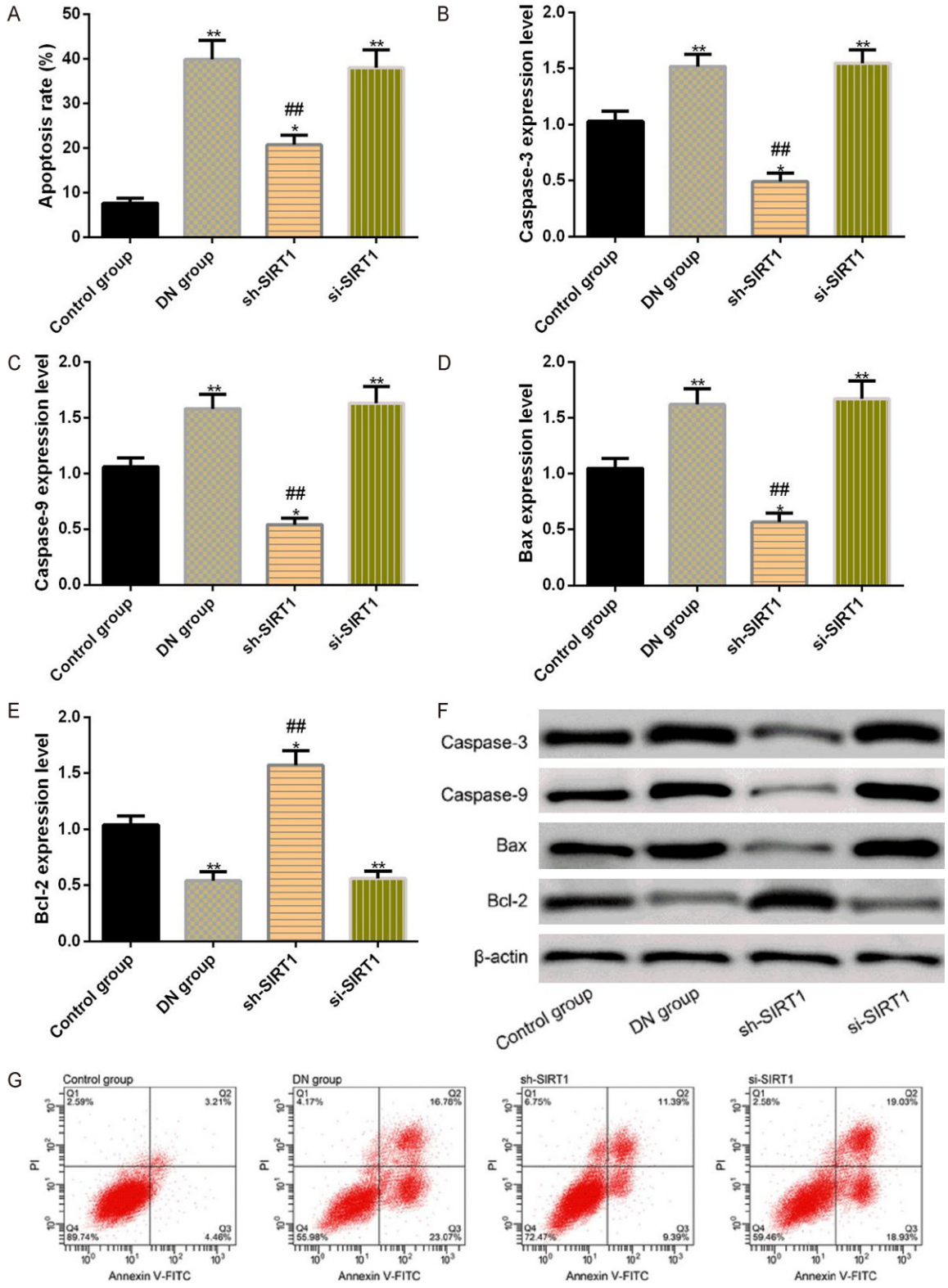

Figure 5. Effects of up-regulation of SIRT1 on HMC apoptosis and related proteins. A. Up-regulation of SIRT1 remarkably accelerates the apoptosis of cells. B-E. Up-regulation of SIRT1 improves the levels of apoptosis-related proteins. F. Protein profiling of apoptosis-related proteins. G. Flow cytometry. Note: *P < 0.05, **P < 0.01 vs control group or between two groups; ##P < 0.01 vs DM group.

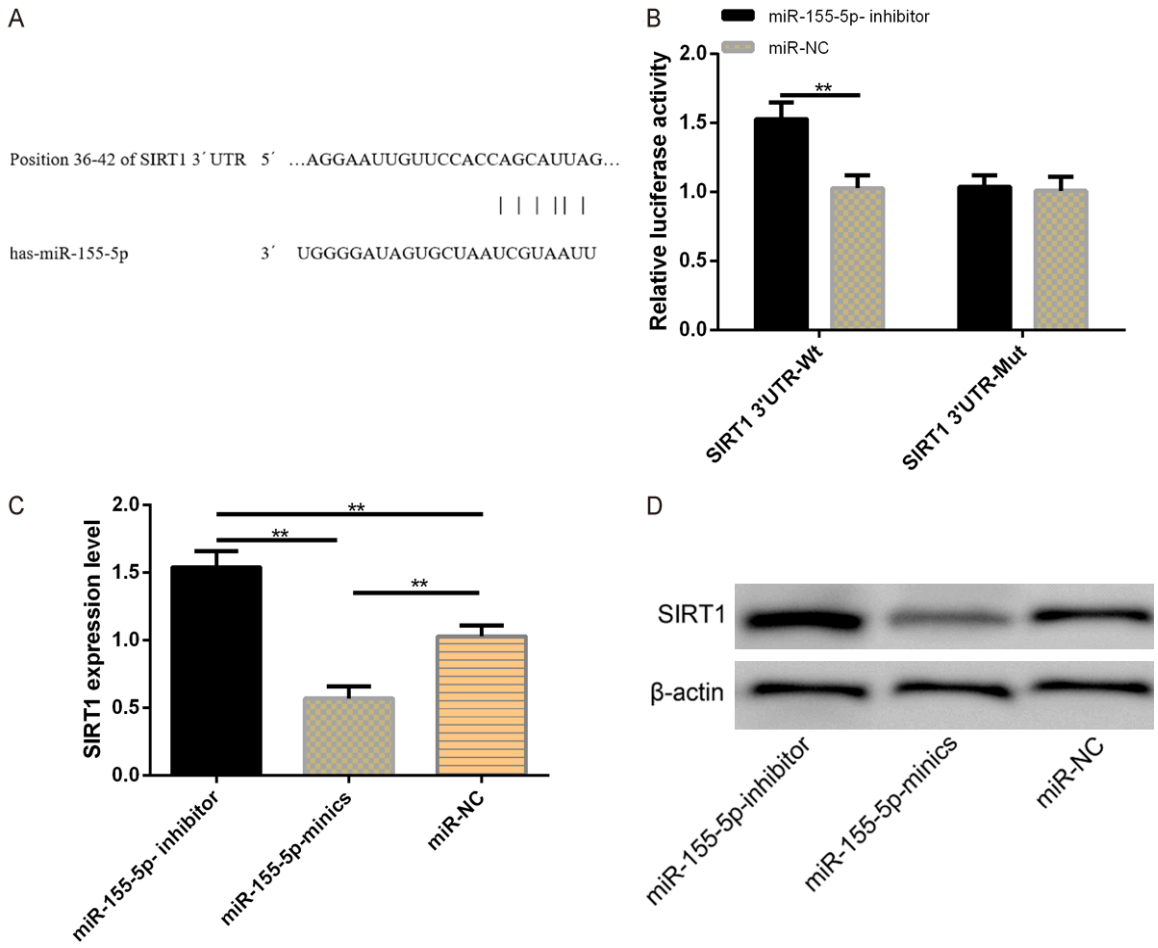

Figure 6. SIRT1 is targetedly regulated by miR-155-5p. A. There are targeted binding sites between miR-155-5p and SIRT1. B. Relative luciferase activity-DLR assay. C. Expression of SIRT1 in transfected HMC cells. D. Protein profiling. Note: **P < 0.01.

Caspase-9 and Bax were elevated while Bcl-2 was suppressed. Sh-SIRT1 reversed the above results. TargetScan database showed us that miR-155-5p and SIRT1 were targetedly related. Inhibiting miR-155-5p in HMCs enhanced luciferase activity of SIRT1 3'UTR-Wt and exerted no effect on SIRT1 3'UTR-Mut. After injection of miR-155-5p-inhibitor, SIRT1 was remarkably up-regulated. These indicate that miR-155-5p regulates cyclins, inhibits the proliferation of HMCs induced by high glucose, as well as promotes apoptosis by regulating SIRT1.

To sum up, miR-155-5p promotes the survival of DN mice through targeted regulation of SIRT1 and may be a new target for diagnosis and gene therapy for DN. However, there is still room for improvement. First of all, the inhibitory effect of miR-155-5p and SIRT1 on inflammatory factors needs further exploration to figure out their potential protective effects on kidney cells in DN. In addition, the regulation on oxidative stress indicators needs to be supplemented to identify their involvement in the protective mechanism of oxidative stress injury. Besides, the relationship between miR-204-5p and other target genes can also be investigated.

Disclosure of conflict of interest

None.

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