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Original Article

miR-30a-5p targets Becn1 to ameliorate high-glucose-induced glomerular podocyte injury in immortalized rat podocyte cell line

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Abstract: Objective: Diabetic nephropathy (DN) is a serious kidney-based complication of diabetes, wherein podocyte injury is deemed crucial in the development of early stage. Various miRNAs, as report goes, is involved in the pathogenesis of varieties of kidney diseases including DN. In this study, we found a target relationship between miR-30a-5p and Becn1, of which there are few studies about the role in podocyte injury. We therefore used immortalized rat podocyte cell line to explore the role and molecular mechanism of miR-30a-5p targeting Becn1 gene in high-glucose-induced glomerular podocyte injury. Methods: The mRNA and protein expressions of miR-30a-5p and Becn1 were detected respectively by quantitative reverse transcriptase PCR and western blotting. The proliferation, apoptosis, and the levels of interleukin (IL)-6 and tumor necrosis factor (TNF)-α were detected by MTT assay, flow cytometry, and enzyme-linked immuno sorbent assay, respectively. Intracellular reactive oxygen species (ROS), superoxide dismutase (SOD) and malondialdehyde (MDA) levels were also determined. Results: Compared with normal group, miR-30a-5p in model groups were down-regulated, while Becn1 expression was significantly up-regulated, with slower proliferation, higher apoptosis rate, lower SOD level, and significantly higher ROS, MDA, IL-6, and TNF-α levels (all $P<0.05$). Overexpression of miR-30a-5p or Becn1 knock-out could lower Becn1 expression, apoptosis rate, promote proliferation, with relatively higher SOD level and lower ROS, MDA, Il-6, and TNF-α levels of model cells (all $P<0.05$). Conclusion: Up-regulation of miR-30a-5p can suppress the expression of Becn1 to increase the growth and inhibit the apoptosis of immortalized rat podocyte cell line, therefore ameliorating podocyte injury induced by high glucose in vitro.

Keywords: Podocyte, miR-30a-5p, Becn1, proliferation, apoptosis

Introduction

Diabetic nephropathy (DN), characterized by alteration in the renal tissue and loss of kidney function, is one of the most common microvascular complications in diabetic patients, which is also a significant cause of end-stage kidney disease globally and a major source of increasing morbidity and mortality worldwide [1]. Proteinuria and hematuria are the main clinical manifestations of DN, along with which DN can lead to chronic kidney disease [2]. Proteinuria in DN is closely related to transdifferentiation of mesenchymal cells and damage of glomerular filtration barrier [3]. Generally, DN presents with glomerular dysfunction, glomerular sclerosis, and reduction of the glomerular filtration barrier [4].

Podocyte injury plays a critical role in the development of DN in the early stage [4]. Podocytes are a group of highly differentiated visceral epithelial cells lining the outermost layer of the glomerular basement membrane, which serve as a major component of the ultrafiltration apparatus [5]. There is a link between podocyte injury and the development and progression of albuminuria. DN may due to proteinuria worsen and

eventually develop into end-stage renal disease [6]. Therefore, DN is considered to be podocyte disease [7]. It has been confirmed that high glucose contributes to podocyte injury and thus causes persistent proteinuria and decrease in glomerular filtration rate in DN patients [8]. Their presence is related to apoptosis, inflammation concerning interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), and mitochondrial stress involving superoxide dismutase (SOD), malondialdehyde (MDA) and reactive oxygen species (ROS) [9]. Therefore, there is an urgent need to find effective and novel podocyte-based therapeutic strategies for DN.

MicroRNAs (miRNAs) can bind to 3' untranslated region of a certain messenger RNA, to suppress the gene expression of target [10]. Various miRNAs have been reported to be involved in the pathogenesis of varieties of kidney diseases including DN [11]. miR-370 promotes high-glucose-induced podocyte injury via inhibition on angiotensin II type 1 receptor-associated proteins [12]. Down-regulation of miR-217 suppresses hyperglycemia-induced podocyte injury [13]. miR-30 plays various and critical role in tumor initiating, adipogenesis and other many biological processes [14-16]. miR-30a-5p is a member of miR-30 family and was related with the development of many diseases [17, 18]. For example, miR-30a-5p targets protein kinase B to promote the apoptosis of chondrocytes in patients with osteoarthritis [19]. miR-30a-5p inhibits Neurod 1 via MAPK/ERK signaling to improve inflammatory response and oxidative stress induced by spinal cord injury [20]. However, there are few ones concerning cell damage. Therefore, it is imperative to further elucidate the role of miR-30a-5p in the pathogenesis of podocyte injury in the downstream.

Becn1, also known as phosphatidylinositol-3-kinase (PI3KCIII) complex, serves as a component of the BECN1/VPS34/VPS15 complex. Becn1 is further assembled into multiprotein complexes in response to complicated extracellular signals [21]. There are few reports about Becn1 concerning podocyte injury, and it has been reported alone that catalase ameliorates diabetes-induced cardiac damage by reducing p65/RelA-mediated Becn1 transcription [22].

In this study, a target relationship between miR-30a-5p and Becn1 was found through bioinformatics prediction. Following above knowledges and findings, we speculated that miR-30a-5p may down-regulate Becn1 expression, thereby ameliorating high-glucose-induced glomerular podocyte injury.

Material and methods

Cell culture and processing

In this in vitro study, the conditionally immortalized rat podocyte cell line was purchased from the Cell Resource Center of Peking Union Medical College for this study. The cells were cultured in RPMI 1640 medium (Hyclone, Utah, USA) containing 10% fetal bovine serum (FBS; Gibco, MD, USA), 10 U/mL of interferon-γ (IFN-γ; Invitrogen, CA, USA), and 1% penicillin-streptomycin (Sigma-Aldrich, MO, USA) in a 5% CO_2-humidified atmosphere at 37°C. Subsequently, the cells were treated with high glucose (30 mM D-glucose) and normal glucose (5 mM D-glucose) for 48 h [23, 24], followed by cell transfection.

Cell transfection

In this experiential study, the rat podocytes in logarithmic growth phase were seeded into 6-well culture plates ($1*10^5$ cells per well). Cells were cultured by serum-free RPMI 1640 medium (Gibco, USA) a day before transfection. The cells were divided into seven groups. All the groups except Normal group were treated with high glucose, including the Model group (without treatment), the NC mimic group (transfected with NC mimic), miR-30a-5p mimic group (transfected with miR-30a-5p mimic), si-NC group (transfected with si-NC), si-Becn1 group (transfected with si-Becn1), and miR-30a-5p mimic+oe-Becn1 group (transfected with miR-30a-5p mimic+oe-Becn1). All the miRNAs and siRNA were synthesized by Shanghai GenePharma Co., Ltd. The transfection procedure was carried out using Lipofectamine™ 2000 transfection reagents according to the instructions of the kit. The serum-free medium was replaced with Gibco™ RPMI 1640 medium containing 10% fetal bovine serum after 6 h culturing. The cells were harvested 48 h after transfection and stored in a -80°C refrigerator for subsequent experiments.

Table 1. Primer sequences

	Forward primers	Reverse primers
miR-30a-5p	5'-ACACTCCAGCTGGGTGTAAACATCCTCGAC-3'	5'-CAGTGCGTGTCGTGGAGT-3'
Becn1	5'-GTGCTCCTGTGGAATGGAAT-3'	5'-GCTGCACACAGTCCAGAAAA-3'
U6	5'-CTCGCTTCGGCAGCACA-3'	5'-AACGCTTCACGAATTTGCGT-3'
GAPDH	5'-AAGCCCATCACCATCTTCCAGGAG-3'	5'-AGCCCTTCCACAATGCCAAAG-3'

Dual-luciferase reporter system assay

The bioinformatics prediction website, TargetScan software, was used to predict the target of miR-30a-5p and Becn1 was screened out. The target relationship between miR-30a-5p and Becn1 was then verified by dual-luciferase reporter system assay. The reporter vectors, wildtype vector (PGL3-Becn1 wt), and mutant (on binding site) vector (PGL3-Becn1 mut), were constructed and co-transfected into human embryonic kidney 293T cells with renilla plasmid+miR-30a-5p plasmid or renilla plasmid+NC plasmid, respectively. After 24 h of transfection, luciferase activity was detected by the kit purchased from Promega (Boston, MA, US) [8].

Quantitative reverse transcriptase PCR

Total RNA of the harvested cells in each group was extracted using Invitrogen™ TRIzol® reagent (Thermo Fisher, Harbin, China). TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher, USA) was used to synthesize cDNA through reverse transcription. Fluorescence quantitative PCR was performed using SYBR® Premix EX Taq™ II (Tli RNaseH Plus) system (Takara, Guangzhou, China). ABI Prism® 7300 Real-Time PCR instrument (Applied Biosystems, Shanghai, China) was used for performing PCR. The PCR program was 95°C for 10 min, 95°C for 15 s, 60°C for 30 s, 72°C for 1 min, 32 cycles. The fold change of relative expression level of a target gene was calculated by the $2^{-\Delta\Delta Ct}$ method. U6 and GAPDH were used as the internal controls for miR-30a-5p and for other genes, respectively. **Table 1** presents the sequences of primers.

Western blotting

The total protein in the cells was extracted using radioimmunoprecipitation assay lysis buffer containing phenylmethanesulfonyl fluoride (R0010; Beijing Solarbio Science & Technology Co., Ltd., Shanghai, China). The protein concentration was determined by QuantiPro™ BCA Assay Kit (Sigma, USA). Then 50 μg protein sample was processed by polyacrylamide gel electrophoresis at a constant voltage of 70 V for 3 h and transferred to polyvinylidene fluoride membrane (ISEQ00010; Millipore, Billerica, MA, USA) under constant current of 150 mA. The membranes were immersed and blocked with 5% skimmed milk for 2 h at 4°C. The membranes were washed using TBST buffer after discarding the blocking buffer. The membranes were then incubated with primary rabbit anti-mouse antibodies against Becn1 (1:1,000; ab210498; Abcam, UK) and GAPDH (1:2,000; ab22555; Abcam, UK) overnight at 4°C, followed by washing with TBST buffer 3 times (6 min each time). The membranes were subsequently incubated for 2 h at room temperature after adding sheep anti-rabbit IgG antibody (1:5,000; Beijing Zhongshan Biotechnology Co., Ltd., China) conjugated with horseradish peroxidase, and were then washed with TBST 3 times (6 min each time), and immersed in Tris-buffered saline. The membranes were then immersed in chemiluminescence reaction solution (BB-3501, Ameshame, UK) to develop colors/images. The protein bands were obtained through Bio-Rad® Image Analysis System, and their gray values were analyzed and calculated using Image J software. The relative protein content was shown by the ratio of target band gray value to GAPDH band gray value.

MTT assay

The harvested cells were collected and counted using routine enzymatic digestion. The cells were seeded into 96-well plates at density of $3\sim6*10^3$ cells per well (100 μL/per well), and 6 wells were repeated for each group. After adding 20 μL of 5 mg/mL MTT (Gibco, USA) to each well at 24 h, 48 h and 72 h, the cells were incubated for 4 h in the dark. Then 100 μL of dimethyl sulfoxide (DMSO; Sigma-Aldrich, St Louis, MO, USA) was added to each well for dis-

solving the formazan crystals. Optical value (OD) of each well was measured at 495 nm using the enzyme-linked immunosorbent detector (NYW-96M; Beijing Nyaw Instruments Co., Ltd., China). The cell activity curve chart was draw with OD value as the ordinate and time point as the abscissa.

Flow cytometry assay

The harvested cells were processed by centrifugation at 2,000 r/min for 30 min. The supernatant was discarded and were washed with pre-cooled PBS 3 times. Annexin V-FITC Apoptosis Detection Kit (Sigma-Aldrich, St Louis, MO, USA) was used for detection of apoptotic cells. The fluorescence of the cells was detected using flow cytometer with a 488 nm excitation source for evaluating apoptosis.

Determination on ROS, SOD, and MDA levels

ROS production in cells was determined by the cell-permeant 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA; Sigma-Aldrich, St Louis, MO, USA). The treated podocytes were incubated with 5 μg of DCFH-DA at 37°C for 30 min. After collection and rinsing, fluorescence intensity of the cells was detected by using FASCalibur (BD Biosciences, NJ, USA) at an emission wavelength of 530 nm and an excitation wavelength of 485 nm.

The levels of MDA and SOD were measured by WST-1 method and thiobarbituric acid (TBA) method respectively. The harvested cells were digested with pancreatin at 37°C for 30 min, followed by a PBS rinse for digestion termination. The digested mixture was then filtered through a 300-mesh nylon mesh, followed by centrifugation at 1,000 rpm for 10 min. The supernatant was discarded, and the cells were resuspended in the PBS buffer for detection. The operations were carried out strictly as per the instructions of the kits for SOD (A001-3-2; Nanjing Jiangcheng Bioengineering Institute, China) and MDA (A003-1-2; Nanjing Jiangcheng Bioengineering Institute, China).

Enzyme-linked immuno sorbent assay

The pro-inflammatory cytokines, TNF-α and IL-6, were measured using ELISA assay kits (69-40133, 69-25328, respectively; Beyotime, Wuhan, China) according to the instructions.

Statistical analysis

All the data were analyzed using the SPSS 21.0 software package (SPSS Inc., Chicago, II, USA). The measurement data were expressed as the mean ± standard deviation. The comparisons between multiple groups were performed using one-way analysis of variance, and pairwise comparisons of average value between groups were compared by Tukey post-hoc test. For all analyses, $P<0.05$ was considered statistically significant.

Results

Expression of miR-30a-5p and Becn1 in injured rat podocytes in each group

To investigate how miR-30a-5p regulates the effect of Becn1 on rat podocyte injury induced by high glucose, we detected the mRNA expressions of miR-30a-5p and Becn1 by qRT-PCR, and determined the protein expression level of Becn1 by western blot. The results were shown in **Figure 1**. Compared with Normal group, other groups showed down-regulation on miR-30a-5p expression and up-regulation on Becn1 expression (all $P<0.05$). There were no statistical differences in Becn1 expression between Model group, NC mimic group, si-NC group, and miR-30a-5p mimic+oe-Becn1 group (all $P>0.05$). While compared with Model group, the expression levels of Becn1 gene in miR-30a-5p mimic group and si-Becn1 group were significantly reduced (all $P<0.05$). Compared with miR-30a-5p mimic group, Becn1 expression level in miR-30a-5p mimic+oe-Becn1 group was significantly increased ($P<0.05$).

miR-30a-5p negatively regulates Becn1 expression

The bioinformatics prediction website found that there was a specific binding site between miR-30a-5p and Becn1 gene, as shown in **Figure 2A**. The results of dual-luciferase reporter system assay were shown in **Figure 2B**. Compared with NC mimic group, luciferase activity intensity in co-transfection group of miR-30a-5p and Wt-Becn1 decreased significantly ($P<0.05$), while co-transfection group of miR-30a-5p and Mut-Becn1 had no obvious change in the activity intensity ($P>0.05$), indicating that miR-30a-5p targets the 3'UTR of Becn1 gene, and can negatively regulate Becn1 gene expression.

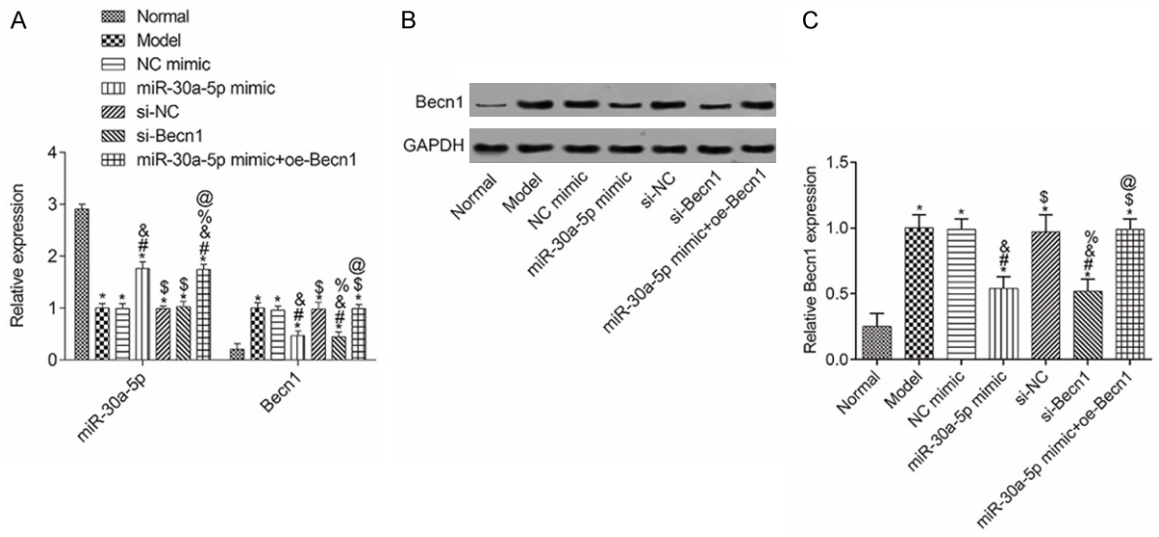

Figure 1. Expression of miR-30a-5p and Becn1 in each group of cells. A. Histogram of the relative expression levels of miR-30a-5p and Becn1 mRNA in podocytes. B. Diagram of protein band of Becn1. C. Histogram of expression level of Becn1 protein in podocytes. *P<0.05 vs. Normal group; #P<0.05 vs. Model group; &P<0.05 vs. NC mimic group; $P<0.05 vs. miR-30a-5p mimic group; %P<0.05 vs. si-NC group; @P<0.05 vs. si-Becn1 group.

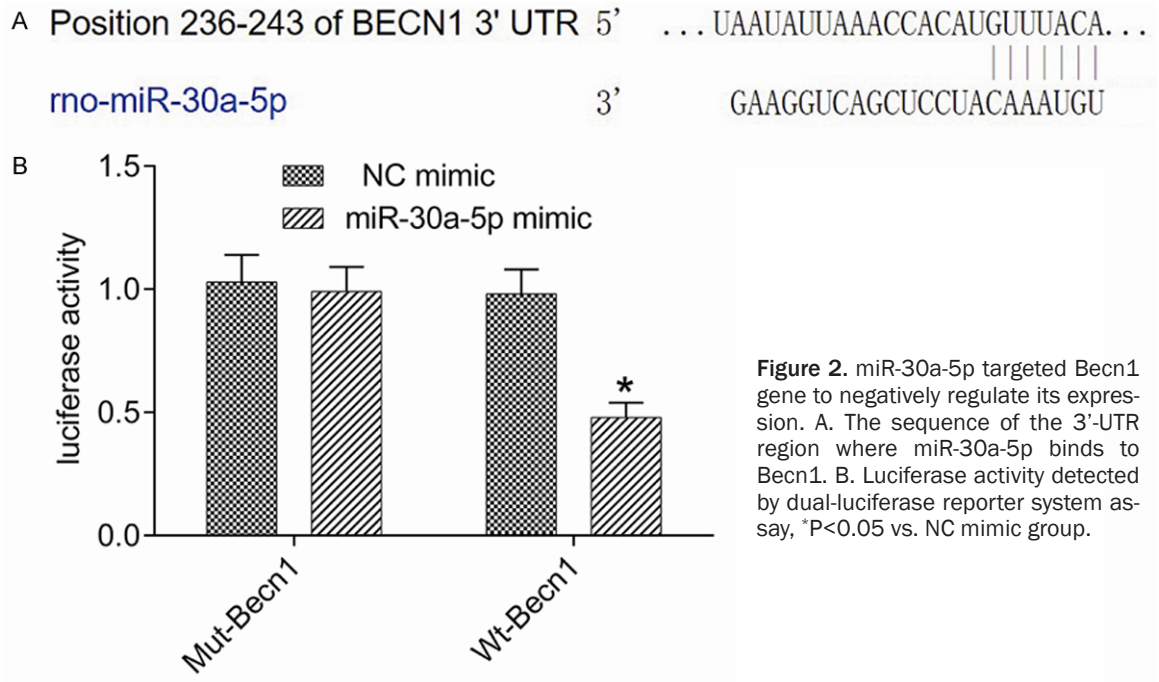

Figure 2. miR-30a-5p targeted Becn1 gene to negatively regulate its expression. A. The sequence of the 3'-UTR region where miR-30a-5p binds to Becn1. B. Luciferase activity detected by dual-luciferase reporter system assay, *P<0.05 vs. NC mimic group.

miR-30a-5p suppresses Becn1 expression to promote the cell proliferation of injured rat podocytes

The results from MTT assay in **Figure 3** showed that all groups had progressive increase in the cell growth with time. But compared with Normal group, other groups had lower elevation in the podocyte proliferation rate (all P<0.05). There were no statistical differences in the podocyte proliferation rate between Model group, NC mimic group, si-NC group, and miR-30a-5p mimic+oe-Becn1 group (all P>0.05). While compared with Model group, miR-30a-5p mimic group and si-Becn1 group showed significant increase in the podocyte proliferation rate (both P<0.05). Compared with miR-30a-5p mimic group, the cell proliferation rate of miR-

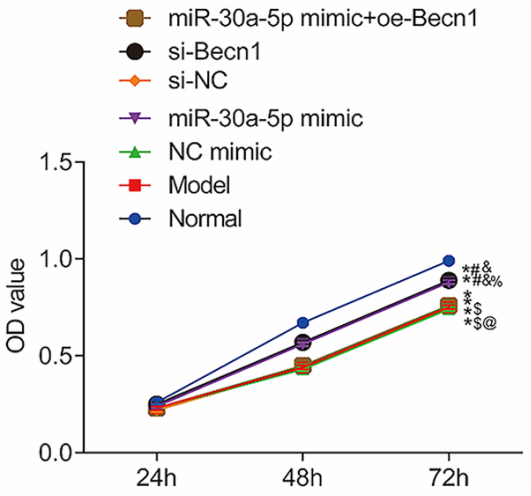

Figure 3. Cell proliferation in each group detected by MTT assay. *P<0.05 vs. Normal group; #P<0.05 vs. Model group; &P<0.05 vs. NC mimic group; $P<0.05 vs. miR-30a-5p mimic group; %P<0.05 vs. si-NC group; @P<0.05 vs. si-Becn1 group. OD, optical value.

30a-5p mimic+oe-Becn1 group was decreased (P<0.05).

miR-30a-5p suppresses Becn1 expression to inhibit the cell apoptosis of injured rat podocytes

Flow cytometry was used to detect cell apoptosis, and the results were showed in **Figure 4**. Compared with Normal group, other groups showed substantial increase in apoptosis rate (all P<0.05). There were no statistical differences in apoptosis rate between Model group, NC mimic group, si-NC group, and miR-30a-5p mimic+oe-Becn1 group (all P>0.05). While compared with Model group, miR-30a-5p mimic group and si-Becn1 group presented with significantly lower apoptosis rate (both P<0.05). In addition, apoptosis rate in miR-30a-5p mimic+oe-Becn1 group was significantly higher than that in miR-30a-5p mimic group (P<0.05).

miR-30a-5p suppresses Becn1 expression to inhibit oxidative stress reaction of injured rat podocytes

Figure 5A-C showed the ROS, SOD and MDA levels in the podocytes of rats in each group. Compared with Normal group, other groups presented with lower SOD level, but with higher ROS and MDA levels in the podocytes of the rats (all P<0.05). No statistical differences were shown in ROS, SOD and MDA levels between Model group, NC mimic group, si-NC group, and miR-30a-5p mimic+oe-Becn1 group (all P>0.05). Compared with Model group, miR-30a-5p mimic group and si-Becn1 group showed significantly higher SOD level and lower ROS and MDA levels in the podocytes (both P<0.05). While compared with miR-30a-5p mimic group, miR-30a-5p mimic+oe-Becn1 group had lower SOD level, and higher ROS and MDA levels (P<0.05).

miR-30a-5p suppresses Becn1 expression to inhibit inflammatory response of injured rat podocytes

In terms of inflammatory factor, **Figure 5D**, **5E** showed the IL-6 and TNF-α levels in each group. Compared with Normal group, the levels of IL-6 and TNF-α in the podocytes of rats in other groups were significantly higher (all P< 0.05). No statistical differences were shown in IL-6 and TNF-α levels between Model group, NC mimic group, si-NC group, and miR-30a-5p mimic+oe-Becn1 group (all P>0.05). Compared with Model group, miR-30a-5p mimic group and si-Becn1 group presented with lower IL-6 and TNF-α levels (both P<0.05). While compared with miR-30a-5p mimic group, miR-30a-5p mimic+oe-Becn1 group had higher IL-6 and TNF-α levels (P<0.05).

Discussion

Podocytes play a crucial role in maintaining glomerular structure and thus the filtration barrier function, and they cannot self-renew due to their unlimited capacity to divide [25]. Podocytes are highly differentiated glomerular epithelial cells lacking a key property for regeneration after an injury [26]. In the past few years, a large amount of studies indicate that miRNA plays an important role in controlling various basic cells. Recently, mounting evidence shows the potential role of miRNA in regulation of podocytes in physiology and pathogenesis [27]. For example, studies have found that in aminonucleoside-treated rats, the loss of miR-30s promotes podocyte injury and in the meantime induces proteinuria [28]. However, there was study reporting the role and effect of miR-30a-5p in podocyte injury. In this study, we found that miR-30a-5p was down-regulated in podocytes under high glucose. Hence, we speculated that miR-30a-5p may play roles in the development of podocyte injury.

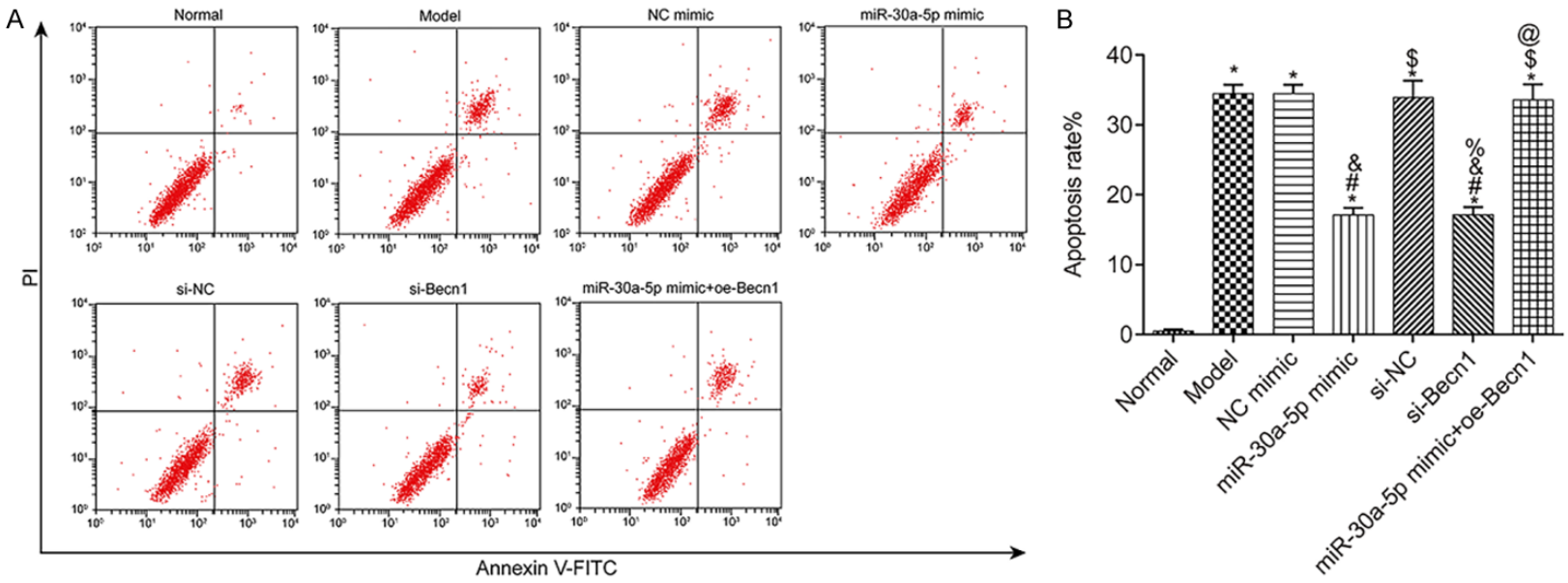

Figure 4. Cell apoptosis rate (A) in each group detected by flow cytometry, and (B) histogram of apoptosis rate in each group. *P<0.05 vs. Normal group; #P<0.05 vs. Model group; &P<0.05 vs. NC mimic group; $P<0.05 vs. miR-30a-5p mimic group; %P<0.05 vs. si-NC group; @P<0.05 vs. si-Becn1 group.

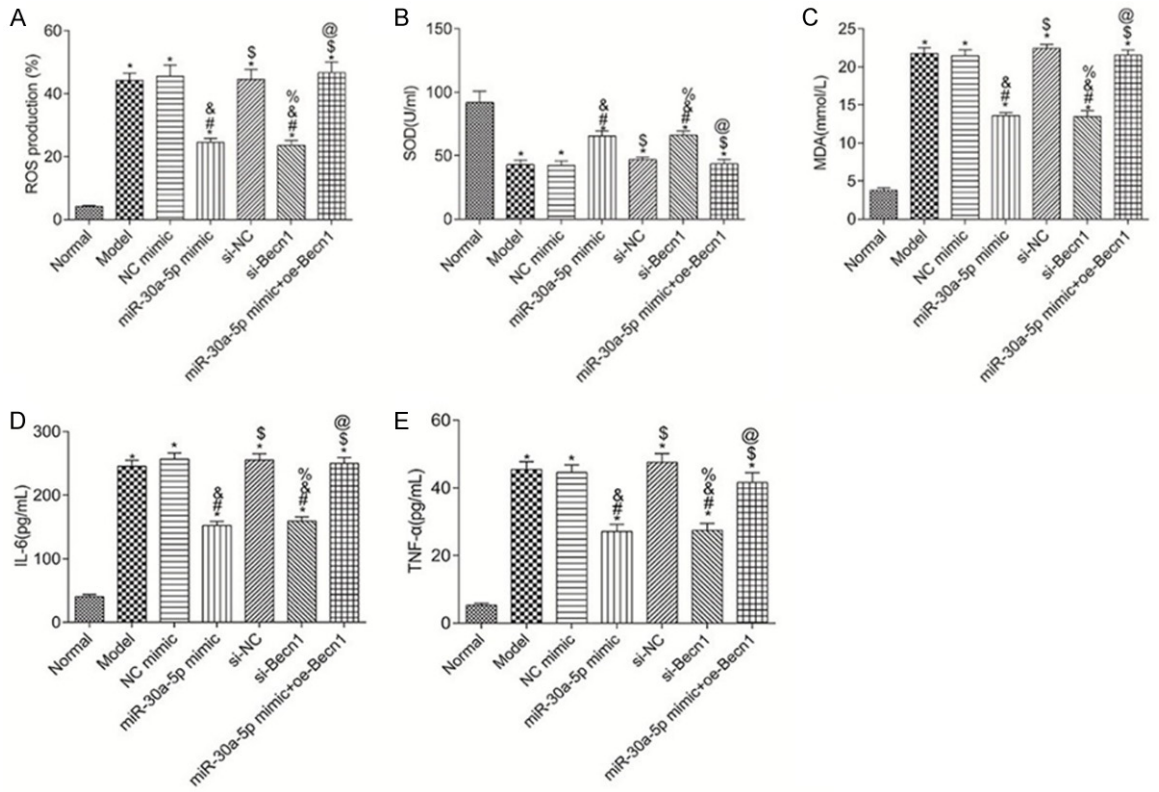

Figure 5. Levels of (A) ROS, (B) SOD and (C) MDA in the podocytes and levels of inflammatory cytokines (D) IL-6 and (E) TNF-α in each group. *P<0.05 vs. Normal group; #P<0.05 vs. Model group; &P<0.05 vs. NC mimic group; $P<0.05 vs. miR-30a-5p mimic group; %P<0.05 vs. si-NC group; @P<0.05 vs. si-Becn1 group. ROS, reactive oxygen species; SOD, superoxide dismutase; MDA, malondialdehyde; IL, interleukin; TNF, tumor necrosis factor.

In order to future explore role of miR-30a-5p in podocyte injury, we used TargetScan software to screen the downstream target and BECN1 attracts our attention. BECN1 is reported to related with autophagy, apoptosis and inflammatory response [29]. Park et al. found that ULK1 phosphorylates Ser30 of BECN1 in association with ATG14 to stimulate autophagy induction [30]. Wu et al. reported that in human triple-negative breast cancer cells BECN1-knockout could impair tumor growth, migration and invasion by suppressing the cell cycle and partially suppressing the epithelial-mesenchymal transition [31]. In this study, we found that Becn1 was overexpressed in podocytes under high glucose, meanwhile dual-luciferase reporter system assay confirmed that miR-30a-5p targeted 3'-UTR of Becn1, and its overexpression did significantly down-regulate Becn1 expression. indicating that miR-30a-5p target and suppress the expression of Becn1 in injured podocytes.

It is generally believed that podocyte loss and apoptosis in early DN are closely related to its progression, and largely contribute to the development of proteinuria and glomerulosclerosis [32]. Apoptosis is considered to be the main cause of podocyte depletion in initial and early pathological DN [33]. The level of inflammatory factors, such as IL-6 and TNF-α as well as oxidative stress injury indicator, such as SOD, ROS and MDA could reflect the disease development of podocyte injury [34, 35]. In this study, both up-regulation of miR-30a-5p and inhibition of Becn1 promote proliferation, inhibit apoptosis, with elevation on SOD level, and reduction on IL-6 and TNF-α levels as well as ROS and MDA levels. However, after transfecting miR-30a-5p mimic, the podocytes presented with a reversed trend on above indices when additionally receiving overexpression of Becn1. All these results indicated that miR-30a-5p could promote proliferation, and inhibit apoptosis and inflammation in the podocytes, especially offer protection against oxidative stress injury by reducing the expression of Becn1.

However, to date, the signaling axis in the downstream in the regulation on DN by Becn1 gene

has not been clearly understood. In addition, the molecular mechanism by which Becn1 regulates rat podocyte injury is unclear, and there is a lack of verification from clinical data.

This study investigated the function of miR-30a-5p in rat podocytes, and confirmed that miR-30a-5p can increase the growth of rat podocytes and inhibit the apoptosis, therefore ameliorating podocyte injury induced by high glucose. Thus, miR-30a-5p can play a protective role in DN. Further study on the role of miR-30a-5p in podocytes of DN patients can provide a better understanding of the molecular mechanism of DN development and progression. These results will provide a broader perspective for intervention, prevention and treatment strategies among DN patients.

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Disclosure of conflict of interest

None.

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