Original Article
MicroRNA-134-5p promotes high glucose-induced podocyte apoptosis by targeting bcl-2

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Abstract: Podocyte apoptosis is a typical early feature of diabetic nephropathy (DN), with loss of nephrin integrity contributing to increased proteinuria in patients with DN. Emerging evidence shows that microRNAs (miRNAs) play vital roles in the pathogenesis of DN. Thus, we aimed to further elucidate the role of miRNAs in podocyte apoptosis in DN. We used db/db and db/m mice maintained under a continuous feeding regime for 12 weeks. Using microarray analysis, we found several miRNAs potentially related to podocyte apoptosis. In addition, we cultured a conditionally immortalized human podocyte cell line in 30 mM D-glucose and found that miR-134-5p was upregulated in both db/db mice and high-glucose (HG)-treated podocytes. Upregulation of miR-134-5p was accompanied by podocyte apoptosis and downregulation of nephrin. Inhibition of miR-134-5p produced the opposite effect. Dual-luciferase reporter assays showed that miR-134-5p directly targeted the 3'-untranslated region of the B-cell lymphoma-2 gene (BCL2), and further study confirmed an increase in bcl-2 protein level in HG-treated podocytes transfected with anti-miR-134-5p. Knockdown of BCL2 impeded the antiapoptotic effect of anti-miR-134-5p. Finally, we found that miR-134-5p might regulate apoptosis in db/db mice and podocytes by targeting BCL2. Taken together, our findings suggest that miR-134-5p promotes podocyte apoptosis under HG conditions by targeting BCL2. Our study provides a meaningful approach to interpret the mechanisms of action of miRNAs involved in DN.

Keywords: Diabetic nephropathy, apoptosis, podocyte, high glucose, microRNA, BCL2

Introduction

Diabetic nephropathy (DN) is a major debilitating complication of both type 1 and type 2 diabetes that progresses to end-stage renal disease [1]. Persistent microalbuminuria is widely used as a biomarker of early DN, which indicates a progressive decline in renal function [2]. Podocytes are terminally differentiated cells that are unable to proliferate [3], and which form the glomerular filtration barrier, together with endothelial cells and the glomerular basement membrane [4]. Previous research has shown that podocyte apoptosis is associated with decreased expression of podocin, nephrin, and slit-associated proteins [5], resulting in massive proteinuria in DN [6]. Therefore, it is critical to further explore podocyte-based therapies that can prevent or cure DN in the early stages.

MicroRNAs (miRNAs) are single-stranded, small, noncoding RNAs (21-25 nucleotides) [7] that regulate gene expression by binding to the mRNAs of protein-coding genes to inhibit their translation [8]. Cumulative studies suggest that miRNAs are involved in the pathogenesis of various diseases, including kidney disease [9]. MiR-26a [10], miR-192 [11], miR-200 [12], and miR-215 [13] have been shown to be involved in DN in a transforming growth factor (TGF)-β-dependent manner. Emerging evidence suggests that miRNAs also play an important role in podocytes. MiR-29c has been determined to be involved in podocyte apoptosis by targeting Sprouty homolog 1 in diabetic mice [14]. Knockdown of miR-34c in podocytes has been shown to reduce apoptosis by blocking upregulation of proapoptotic factors induced by high-glucose (HG) treatment [15]. MiR-195 has been found to promote podocyte apoptosis by target-

ing BCL2 in cultured podocytes [16]. Our previous studies have found that several miRNAs were differentially expressed in both db/db mice and HG-treated podocytes. Among these, miR-383-5p, miR-205-5p, and miR-134-5p were upregulated. In addition, overexpression of miR-383-5p has been demonstrated to block the increase in autophagy and attenuation of HG-induced apoptosis induced by resveratrol [17]. However, the mechanisms by which miRNAs modulate disease are complex and further investigations are required to clarify the role of these miRNAs in DN.

The BCL2 gene family and its related protein bcl-2 were the first apoptosis-related genes to be studied [18]. Using two miRNA target analyzing databases (TargetScan and miRNAWalk 2.0), we found that the well-known anti-apoptotic gene BCL2 might be a direct target of miR-134-5p. This led us to hypothesize that miR-134-5p might be involved in the pathological process of DN by regulating bcl-2 expression. This study aimed to gain insight into the biological roles of miR-134-5p. We found that miR-134-5p accelerates HG-induced podocyte apoptosis by suppressing its direct target gene, BCL2.

Materials and methods

Animal model of DN

Twenty male mice (eight weeks of age), including ten C57BL/KsJ db/db mice as the experimental group and ten C57BL/KsJ db/m mice as the control group, were purchased from the Experimental Animal Center of Nanjing Medical University (Nanjing, China). Db/db mice are a genetic model of the early-stage type 2 DN, with features of hyperglycemia and urinary albumin excretion enhancement [19]. Animal experiments were conducted in accordance with Nanjing Medical University guidelines and ethical norms for animal care. The mice were adaptively fed for a week and then housed in well-ventilated plastic cages with stainless steel grid tops at 22 ± 2°C with a 12-h light/dark cycle. After 20 weeks of sustained hyperglycemia, all mice were weighed and placed in individual metabolic cages. Urine samples were collected after 24 h to test the urine albumin excretion rate (UAER) using the microalbuminuria ELISA Kit (SenBeiJia Biological Technology Company, Nanjing, China). Blood samples from the orbital vein of mice were used for detection

of blood urea nitrogen, creatinine, and other biochemical parameters. Mice were sacrificed by cervical dislocation, and all renal tissues were isolated immediately. Half of the kidney samples from each mouse were sent at low temperature in formaldehyde to Google Biotechnology Co., Ltd. (Wuhan, China) for staining and immunohistochemistry. Samples of the harvested kidneys were also sent to the kidney laboratory of The Second Affiliated Hospital of Nanjing Medical University for immunofluorescence analysis. The remaining samples were stored at -80°C.

Podocyte culture and treatment

Conditionally immortalized human podocytes were kindly provided by Dr. Junwei Yang (Center for Kidney Disease, Second Affiliated Hospital of Nanjing Medical University, Nanjing, China). Podocytes were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (Invitrogen, Grand Island, NY, USA) at 33°C in a humidified atmosphere of 5% CO_2. After culturing to a confluence of 80%-90%, the podocytes were subcultured under similar conditions for 10-14 days to induce cell differentiation. After serum starvation for 12 h, the cells were exposed to the indicated high-glucose (HG, 30 mM D-glucose) or normal glucose (NG, 5 mM D-glucose) conditions for 24, 48, or 72 h.

qRT-PCR

Total RNA was extracted with TRIzol reagent (Invitrogen, Shanghai, China). Gene expression was detected by qRT-PCR using the Mir-X miRNA First-Strand Synthesis and SYBR qRT-PCR Kits (Takara, Nanjing, China). The primers used were purchased from GeneCopoeia (Rockville, MD, USA). U6 was used as an internal reference for quantification of the relative expression of mRNA and miRNA using the $2^{-\Delta\Delta Ct}$ method.

Cell transfection

Hsa-miR-134-5p mimic, hsa-miR-134-5p inhibitor, and hsa-miR-ctrl were chemically synthesized by GenePharma (Shanghai, China). Human bcl-2 and bcl-2 shRNA plasmids were constructed and supplied by GenePharma. Human BCL2 cDNA was cloned into the pcDNA-vector to generate pcDNA-bcl2 or pcDNA-sh-bcl2 recombinant plasmids. All oligonucleotides and plasmids were transfected into cells using Lipo-
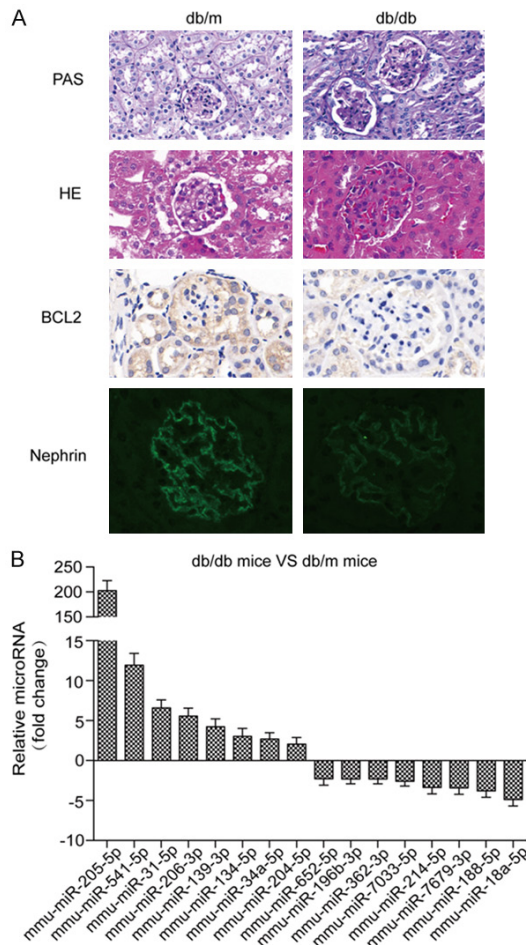
Figure 1. Characteristics of db/db and db/m mouse kidney samples used for miRNA microarray analysis. A. Compared with db/m mice, glomerular hypertrophy, mesangial matrix expansion, and capillary basement membrane thickening changes were apparent in HE-stained and PAS-stained tissue sections from kidney specimens from db/db mice. Immunohistochemistry and immunofluorescence were used to detect bcl-2 and nephrin protein expression in renal tissue. B. Differentially expressed miRNAs are shown from microarray miRNA profiling.

fectamine 2000 Transfection Reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions.

Western blot analysis

Proteins from podocytes were extracted using RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) and the protein concentration was determined by the BCA Protein Assay Kit (Beyotime Biotechnology). Proteins were separated by electrophoresis on 10% SDS-polyacrylamide denaturing gels and transferred to

nitrocellulose membranes, which were then blocked in 5% non-fat dry milk for 2 h, followed by overnight incubation at 4°C with primary antibodies against nephrin (0.5-1 µg/ml; ab-58968 Abcam, Cambridge, MA, USA), Bcl-2 (1:1000; #2872 Cell Signaling Technology, Danvers, MA, USA), cleaved caspase-3 (1:1000, #29034, SAB), and GAPDH (1:1000, #21612, SAB). After washing in TBS-Tween buffer, the membranes were incubated with peroxidase-conjugated goat anti-rabbit IgG (1:3000) for 1 h at room temperature. Protein bands were visualized using an imager. The intensities of the identified bands were quantified.

Analysis of apoptosis by flow cytometry

Apoptosis of the podocytes was evaluated 48 h after their exposure to the various treatments. Briefly, podocytes were trypsinized and centrifuged at 1000 rpm for 5 min at room temperature. Next, 1× binding buffer was added to the precipitate followed by Annexin V Fluorescein isothiocyanate (FITC) stock solution (Annexin V-FITC Apoptosis Detection Kit, Sigma). Cells were incubated for 10 min at 4°C. Propidium iodide (PI) was added immediately before flow cytometric analysis. A total of 20,000 cells per sample were employed for flow cytometry analysis. Flow cytometric data were used to determine the percentage of cells undergoing apoptosis.

Dual luciferase reporter assay

To validate whether miR-134-5p directly targets the bcl-2 3'-untranslated region (3'-UTR), we performed a firefly luciferase reporter assay. Wildtype (WT) and mutated (mut) putative miR-134-5p seed-matching sites in bcl-2 3'-UTRs were amplified from human cDNA by PCR and inserted into the Sac I and Hind III restriction enzyme sites of the pmiRNA-Report vector (Genechem, Shanghai, China). Podocytes were seeded in a 24-well plate and co-transfected with WT or mut reporter plasmid, Renilla luciferase (pRL) plasmids, or miR-134-5p mimic or miR-ctrl. After transfection for 24 h, the cells were harvested and luciferase activity was analyzed with the Dual Luciferase Reporter Assay System (Promega, Madison, WI, USA).

Statistical analysis

All data are presented as the means ± S.E.M. of at least three independent assays, each per-
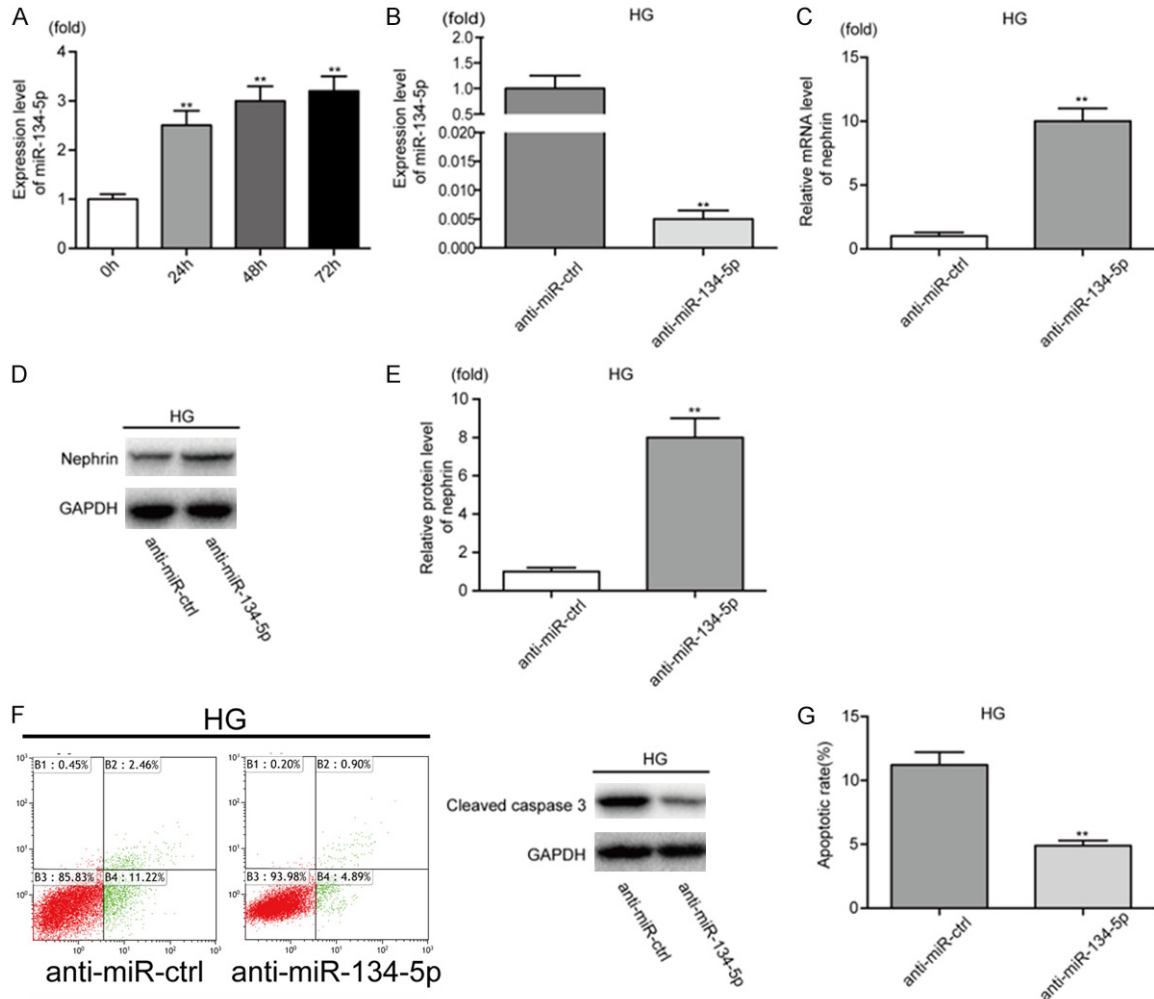
Figure 2. MiRNA-134-5p is upregulated in HG-treated podocytes and promotes podocyte apoptosis. A. The expression of miR-134-5p in podocytes treated with HG (30 mM) at different time points (0-72 h) was analyzed by qRT-PCR. B. The expression of miR-134-5p was analyzed by qRT-PCR after anti-miR-134-5p transfection in HG-treated podocytes. C. The relative mRNA level of nephrin was analyzed by qRT-PCR in HG-treated podocytes after transfection with anti-miR-ctrl or anti-miR-134-5p. D, E. The expression level of nephrin in HG-treated podocytes was analyzed by western blotting. F, G. Apoptosis in HG-treated podocytes was measured by western blot analysis and flow cytometry analysis of Annexin V and PI double staining. **$P < 0.01$. Data are presented as the mean ± SE.

formed in duplicate. Means and standard error of the mean or standard deviation were subjected to the Student's t-test for pairwise comparison or ANOVA for multivariate analysis using Graphpad Prism 5 software. The level of statistical significance was set at $P < 0.05$.

Results

Establishment of the DN mouse model

Hematoxylin-eosin (HE) and Periodic acid-Schiff (PAS) tissue staining were used for morphological analysis of all mice at 20 weeks of age (**Figure 1A**). All ten db/db mice had early symptoms of DN, including glomerular hypertrophy, mesangial matrix expansion, and capillary basement membrane thickening. In contrast, the renal function of db/m mice was normal. The expression of nephrin and bcl-2 in glomeruli was significantly decreased in db/db mice compared with that in db/m mice.

Microarray miRNA profiling

To investigate the potential function of miRNAs during DN, we conducted a microarray analysis of the renal tissues from the two groups of mice. Compared with the db/m group, 43 miR-NAs were differentially expressed in the db/db
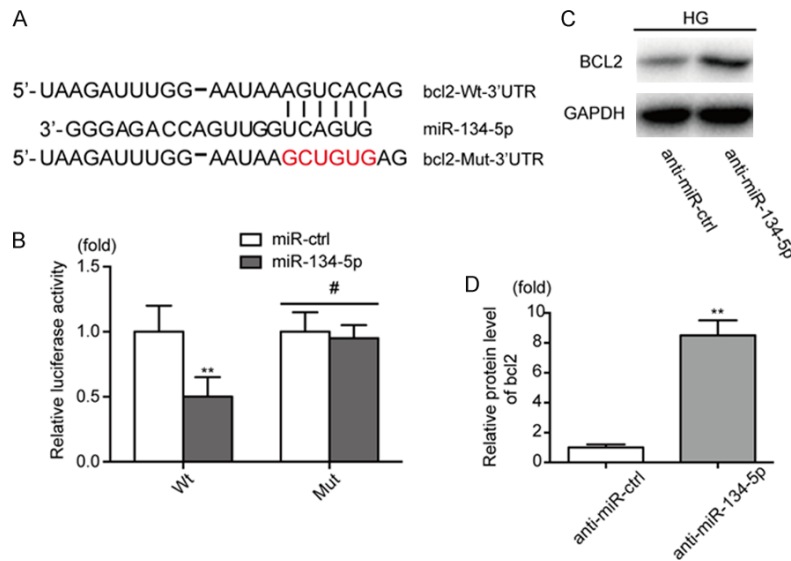
Figure 3. BCL2 is a direct target of miR-134-5p. A. Alignment of miR-134-5p with Bcl-2 3'-UTR sequences. B. Relative luciferase activity of reporters containing wild-type or mutated type with miR-134-5p target sites in NG-treated podocytes. C, D. The expression level of bcl-2 was analyzed by western blotting after co-transfection with anti-miR-ctrl or anti-miR-134-5p in HG-treated podocytes. **P < 0.01. Data are presented as the mean ± SE.

examine the effect of miR-134-5p on the expression of nephrin, a key marker of podocytes in the filtration slits. Notably, mRNA and protein levels of nephrin were significantly increased in HG-treated podocytes transfected with anti-miR-134-5p compared with that in cells transfected with anti-miR-ctrl (**Figure 2C-E**). In addition, we performed flow cytometry and western blotting to detect apoptosis in podocytes. We found that the apoptosis rate and levels of cleaved caspase 3 decreased in HG-treated podocytes transfected with anti-miR-134-5p, compared with that in cells transfected with anti-miR-ctrl (**Figure 2F**, **2G**). In contrast, NG-treated podocytes trans-fected with miR-ctrl or miR-134-5p showed the opposite results (Figure S1). These results indicate that miR-134-5p acts as a promoter of podocyte apoptosis.

group, of which 28 were significantly upregulated and 15 were significantly downregulated. The top 16 up- or down-regulated miRNAs are presented in **Figure 1B**.

HG induces higher expression of miR-134-5p

To explore the potential function of miR-134-5p in HG-treated podocytes, we used qRT-PCR to examine the expression of miR-134-5p at different timepoints (0, 24, 48, and 72 h) following HG (30 mM) stimulation. Notably, miR-134-5p levels in podocytes were upregulated after HG treatment in a time-dependent manner (**Figure 2A**), indicating that miR-134-5p might play a critical role in HG-treated podocytes.

MiR-134-5p promotes apoptosis of podocytes

Podocytes are thought to be injured at an early stage in DN; therefore, we investigated the functional role of miR-134-5p in podocyte apoptosis. First, anti-miR-ctrl or anti-miR-134-5p was transfected into HG-treated podocytes prior to analysis by qRT-PCR. Importantly, the relative expression of miR-134-5p in HG-treated podocytes transfected with anti-miR-134-5p was significantly reduced compared with that in cells transfected with anti-miR-ctrl (**Figure 2B**). Next, we used qRT-PCR and western blotting to

Luciferase assay validates bcl-2 as a direct target of miR-134-5p

To explore the mechanism by which miR-134-5p promotes HG-induced apoptosis of podocytes, we searched two miRNA target analyzing databases (TargetScan and miRNAWalk 2.0) and found that bcl-2 is a latent target of miR-134-5p. We then performed luciferase reporter assays. The sequence of the 3'-UTR of bcl-2 mRNA matched the seed sequence of miR-134-5p. To test the functional significance of this finding, the 3'-UTR sequences, containing putative binding sites of the WT or mut for the seed matching sites, were introduced into a luciferase reporter vector (**Figure 3A**) and each was co-transfected into podocytes with the miR-134-5p mimic or miR-ctrl (**Figure 3B**). The results demonstrated that bcl-2 is a direct target of miR-134-5p. To further examine the effect of miR-134-5p on bcl-2, we transfected HG-treated podocytes with anti-miR-134-5p or anti-miR-ctrl. Western blotting was performed to assess bcl-2 protein levels. The results showed that bcl-2 protein levels were increased
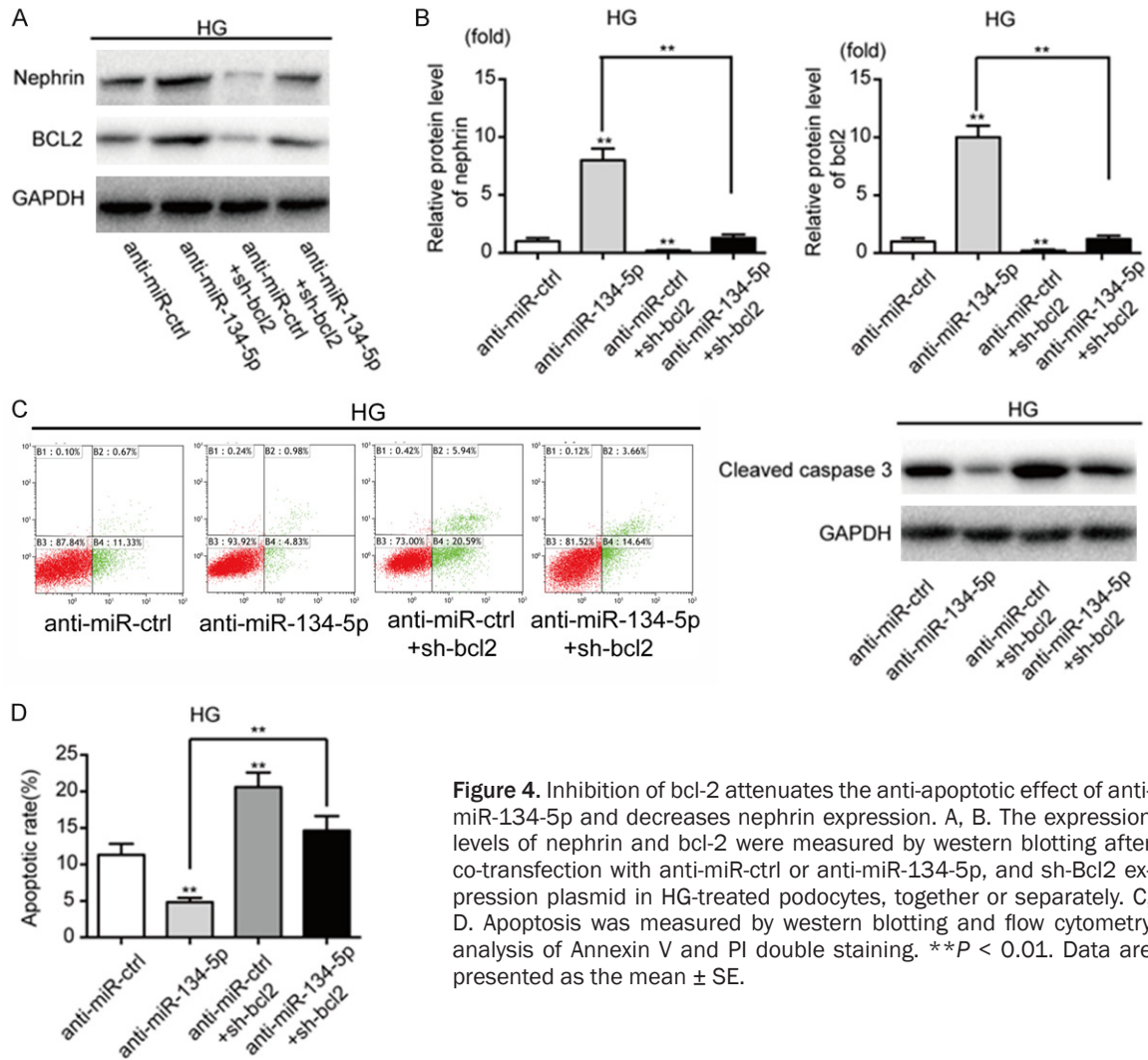
Figure 4. Inhibition of bcl-2 attenuates the anti-apoptotic effect of anti-miR-134-5p and decreases nephrin expression. A, B. The expression levels of nephrin and bcl-2 were measured by western blotting after co-transfection with anti-miR-ctrl or anti-miR-134-5p, and sh-Bcl2 expression plasmid in HG-treated podocytes, together or separately. C, D. Apoptosis was measured by western blotting and flow cytometry analysis of Annexin V and PI double staining. **$P < 0.01$. Data are presented as the mean ± SE.

in HG-treated podocytes transfected with anti-miR-134-5p compared with that in cells transfected with anti-miR-ctrl (**Figure 3C**, **3D**). Overexpression of miR-134-5p by miR-134-5p mimic transfection in podocytes reduced the expression of bcl-2 under NG conditions (Figure S2). Together, these results indicate that bcl-2 is a direct target of miR-134-5p.

Inhibition of bcl-2 enhances the proapoptotic effect of miR-134-5p, while overexpression of bcl-2 attenuates it

To investigate the contribution of Bcl-2 to the proapoptotic effect of miR-134-5p in podocytes, we co-transfected human sh-bcl2 plasmids and anti-miR-134-5p into HG-treated podocytes. The effects of miR-134-5p on apoptosis were enhanced following reduction in

bcl-2 levels in HG-treated podocytes (**Figure 4**). In addition, downregulation of bcl-2 alone markedly enhanced the effects of miR-134-5p on HG-treated podocytes. We found that the effects of miR-134-5p on apoptosis were inhibited by bcl-2 overexpression in NG-treated podocytes (Figure S3). Our results suggest that miR-134-5p functionally promotes podocyte apoptosis in a bcl-2-dependent manner.

Discussion

In this study, we successfully established a DN model in C57BL/KsJ db/db mice and found that glomerular miR-134-5p is differentially expressed in the mouse kidney in diabetes, with or without proteinuria. To further explore the roles of miRNAs, we cultured cells from a conditionally immortalized human podocyte

cell line in medium supplemented with 30 mM D-glucose to mimic conditions in diabetes. Our data showed that miR-134-5p expression is higher in high-glucose than in normal (non-diabetic) glucose conditions. We found that miR-134-5p mimics can reduce bcl-2 protein levels when transfected into podocytes. As a result, the podocyte-specific biomarker nephrin was downregulated and the podocytes became apoptotic. The reduced nephrin levels could lead to the impairment of podocyte function and structure. In contrast, miR-134-5p inhibitors conferred protection from apoptosis, even under hyperglycemic conditions.

MiRNAs are a class of non-coding small RNA molecules consisting of 21-25 nucleotides, which not only cause the degradation of target mRNAs, but also inhibit translation through target mRNA-specific base pairing [20, 21]. Recent studies have shown that miRNAs are essential regulators of expression and important therapeutic targets in DN [22-24]. Studies have also demonstrated that miRNAs negatively regulate protein-coding genes at the posttranscriptional level, primarily by binding to their 3'-UTR [20, 21, 25]. Therefore, identification of the key roles of miRNAs in apoptosis, via modulation of various targets, has promoted research into miRNAs as biomarkers and therapeutic targets for the treatment of DN. Using miRNA target gene prediction tools and bioinformatics-based analyses, we found that miR-134-5p, which is expressed in cultured podocytes dependent on the extracellular glucose concentration, might complement a single site in the 3'-UTR of transcripts of BCL2, FUT2, ESRRG, TMEM184A, DRP2, PUSL1, and PXMP4. Among these genes, we validated that BCL2 was a direct target of miR-134-5p and intrinsically linked to apoptosis.

Apoptosis is a defined set of molecular cascades that result in lethal changes to the cell, including membrane blebbing, mitochondrial breakdown, and DNA fragmentation [26, 27]. Podocyte apoptosis has been reported to play a key role in the pathophysiology of DN, both dependent and independent of miRNAs [28-34]. In addition, podocyte apoptosis not only causes podocytopenia but also affects the glomerular slit diaphragm by downregulating podocyte biomarkers such as nephrin, which is expressed in mature renal podocytes [35-37]. All of these changes cause a critical impair-

ment of podocytes and subsequently the function of the filtration barrier, which would be at least partially responsible for proteinuria in DN. BCL2 is a well-known prosurvival gene that plays a critical role in a variety of cell systems, including podocytes [38]. Bcl-2 regulates cell death primarily by controlling mitochondrial membrane permeability and functions together with caspases and other proteins in a feedback loop system [16, 39]. Hundreds of discrete miRNA sequences have the potential to inhibit BCL2 [40-42]. Among these miRNAs, miR-134-5p may inhibit bcl-2 expression and increase apoptosis in HG-cultured podocytes. miR-134-5p regulates BCL2 at the post-transcriptional level and induces apoptosis in podocytes under HG conditions by inhibiting bcl-2. Apoptotic podocytes fail to synthesize sufficient levels of specific functional proteins and subsequently undergo actin rearrangement, thus resulting in functional and structural defects that are similar to those observed in humans and animal models with DN. BCL2 shRNA plasmids can also induce podocyte apoptosis, although not at the post-transcriptional level, by targeting BCL2 expression.

Taken together, our findings demonstrate that miR-134-5p promotes podocyte apoptosis under HG conditions by reducing the expression level of bcl-2. This study provides a useful approach for deciphering the mechanisms used by miRNAs in the pathogenesis of DN.

Disclosure of conflict of interest

None.

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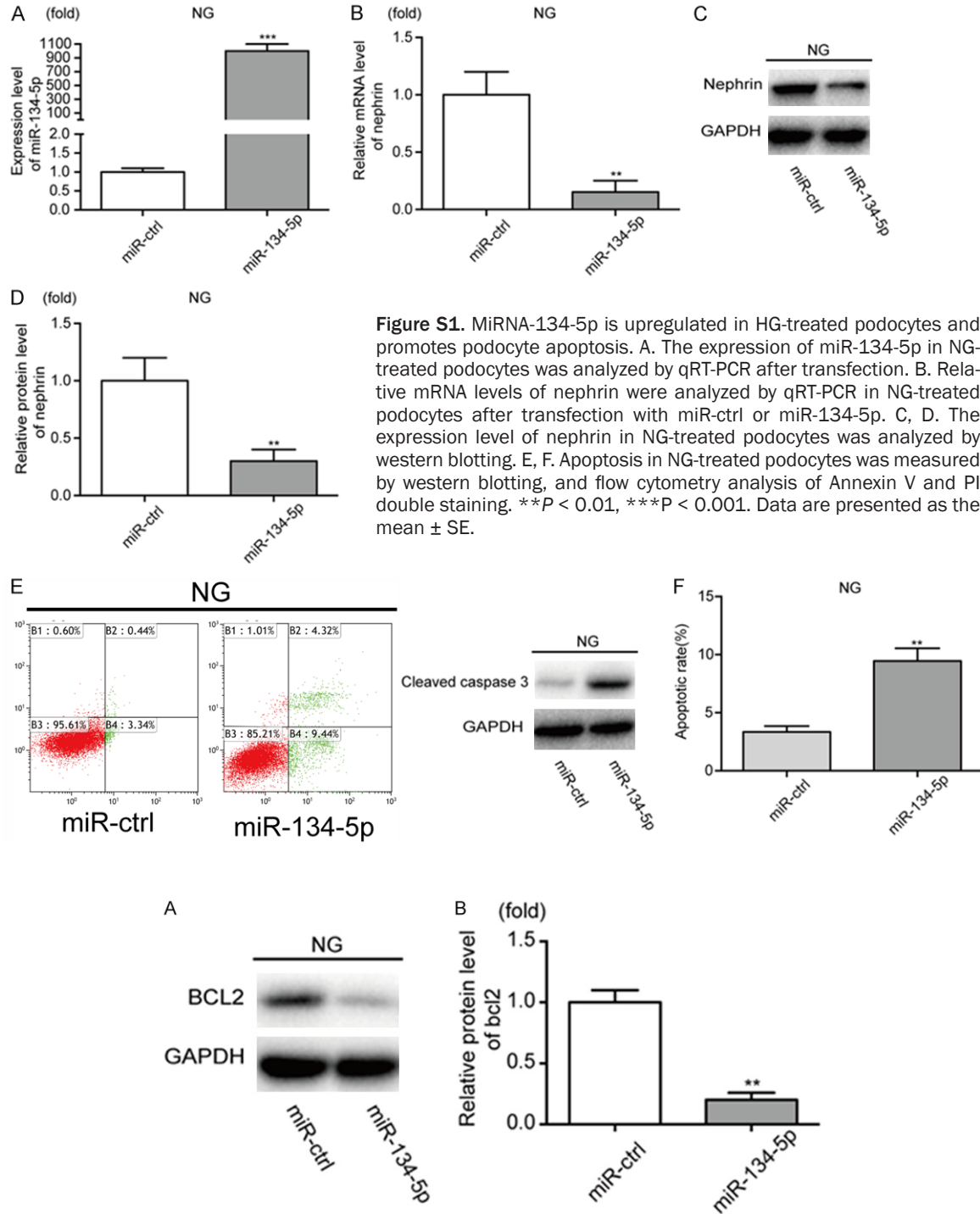
Figure S1. MiRNA-134-5p is upregulated in HG-treated podocytes and promotes podocyte apoptosis. A. The expression of miR-134-5p in NG-treated podocytes was analyzed by qRT-PCR after transfection. B. Relative mRNA levels of nephrin were analyzed by qRT-PCR in NG-treated podocytes after transfection with miR-ctrl or miR-134-5p. C, D. The expression level of nephrin in NG-treated podocytes was analyzed by western blotting. E, F. Apoptosis in NG-treated podocytes was measured by western blotting, and flow cytometry analysis of Annexin V and PI double staining. **P < 0.01, ***P < 0.001. Data are presented as the mean ± SE.

Figure S2. BCL2 is a direct target of miR-134-5p. A, B. The expression level of bcl-2 in NG-treated podocytes was analyzed by western blotting after co-transfection with miR-ctrl or miR-134-5p. **P < 0.01. Data are the mean ± SE.
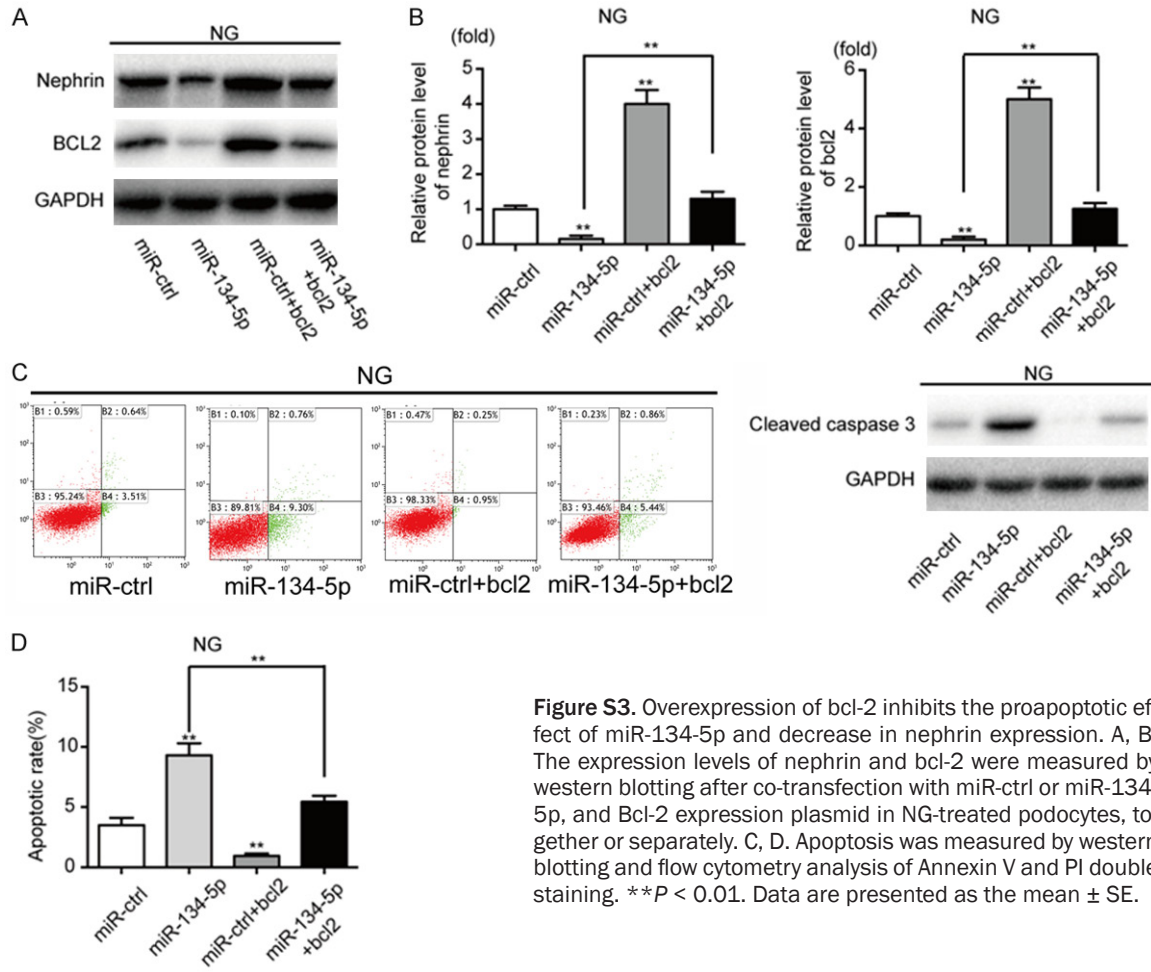
Figure S3. Overexpression of bcl-2 inhibits the proapoptotic effect of miR-134-5p and decrease in nephrin expression. A, B. The expression levels of nephrin and bcl-2 were measured by western blotting after co-transfection with miR-ctrl or miR-134-5p, and Bcl-2 expression plasmid in NG-treated podocytes, together or separately. C, D. Apoptosis was measured by western blotting and flow cytometry analysis of Annexin V and PI double staining. $**P < 0.01$. Data are presented as the mean ± SE.