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MiRNA-337 leads to podocyte injury in mice with diabetic nephropathy

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Abstract. – OBJECTIVE: To elucidate the function of miRNA-337 in the pathogenesis of diabetic nephropathy (DN) and its underlying mechanism.

MATERIALS AND METHODS: Type 2 diabetes db/db mice were assigned into db/db group, vehicle group, and si-miR group, and age-matched db/m mice were in the db/m group. Differences in mouse serum glucose, body weight, serum creatinine, and albumin/creatinine ratio (ACR) among the four groups were compared at 6 weeks, 10 weeks, 14 weeks, 18 weeks, and 22 weeks of age. The expression level of miRNA-337 in mouse kidney tissues was determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Correlation between miRNA-337 expression with ACR was analyzed. Through Western blot analysis, protein levels of interleukin 6 (IL-6), IL-18, podocin, nephrin, and desmin in mouse kidney tissues were detected.

RESULTS: With the increasing age, serum glucose, body weight, serum creatinine, and ACR in db/db mice gradually increased, which were remarkably higher than age-matched db/m mice. After treatment with miRNA-337 inhibitor in db/db mice, no remarkable changes in serum glucose and body weight were found, while serum creatinine and ACR decreased. Compared with db/m mice, miRNA-337 expression in kidney tissues of db/db mice upregulated, which was positively correlated with ACR. Expression levels of IL-6 and IL-18 in kidney tissues of db/db mice increased relative to db/m mice, but they were downregulated by miRNA-337 inhibitor treatment. Moreover, podocin and nephrin downregulated, while desmin upregulated in kidney tissues of db/db mice than db/m mice. By miRNA-337 inhibitor treatment in db/db mice, levels of podocin and nephrin increased, whereas desmin level decreased. We obtained similar results at their cellular level.

CONCLUSIONS: We showed that miRNA-337 expression increases in db/db mice with diabetic nephropathy, which leads to podocyte injury by upregulating levels of IL-6 and IL-18.

Key Words:
Diabetic nephropathy, MiRNA-337, IL-6, IL-18, Podocyte.

Introduction

Diabetic nephropathy (DN) is one of the global health problems. It is reported[1] that there were approximately 415 million people with diabetes mellitus worldwide in 2015, and the number is expected to reach 642 million by 2040. DN is a kidney damage caused by diabetes, which is one of the major chronic complications of diabetes, and it is also the leading cause of end-stage renal disease. The pathogenesis of DN involves various factors[2]. Podocytes are important components of the glomerular filtration barrier. Almost all glomerular diseases are associated with podocyte injury. In recent years, podocyte injury in the progression of DN has been well concerned[3-5]. High prevalence, multiple complications, low awareness rate, high cost, and frequent recurrence of DN force us to explore its pathogenesis, so as to improve the clinical outcomes of affected people.

MicroRNA is a non-coding, single-stranded microRNA of approximately 22 nucleotides in length, which exerts a post-transcriptional regulatory effect[6]. MicroRNA mainly binds to the 3'untranslated region (3'UTR) of the mRNA, thereby causing translational inhibition and/or degradation of the target mRNA to regulate tar-

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get gene expressions. MicroRNA is involved in the regulation of various biological processes, including apoptosis, proliferation, hematopoiesis, angiogenesis, and histomorphogenesis[7]. A great number of studies have shown that microRNAs are closely related to many human diseases, such as cancer, heart disease, and diabetes[8]. The key role of microRNA in the development of DN has been widely recognized, providing a new target for the diagnosis and prevention of DN[9]. Many studies[10,11] have shown that inhibition of certain microRNAs markedly alleviates the pathological changes of DN in animal models. Therefore, altering the expression pattern of specific microRNAs may be a preventive method for alleviating the development of DN.

In this study, we mainly investigated the role of miRNA-337 in DN and its possible mechanism.

Materials and Methods

Experimental Mice

A total of 60 type 2 diabetes db/db mice with 5 weeks old and 12 age-matched db/m mice were provided by Model Animal Research Center of Capital Medical University. Db/db mice developed spontaneous hyperglycemia at 5-6 weeks of age, and proteinuria and renal dysfunction at 8-10 weeks of age. All experimental mice were housed in the Capital Medical University key laboratory animal center (SPF level), with 5 mice per cage (individually ventilated cage). This study got approval from the Ethics Committee of the Capital Medical University Animal Center.

Experimental Grouping

5-week-old experimental mice were adaptively habituated for one week. They were randomly divided into the following four groups: (1) db/m group, no special treatment was performed in 12 db/m mice; (2) db/db group, 20 db/db mice were injected with saline from tail vein at 10 and 14 weeks of age; (3) vehicle group, 20 db/db mice were injected with 3×10^7 TU miR-NC lentivirus from tail vein at 10 and 14 weeks of age; (4) 20 db/db mice were injected with 3×10^7 TU miRNA-337 inhibitor lentivirus from tail vein at 10 and 14 weeks of age. Serum glucose and body weight were regularly recorded from the age of 6 weeks. Mouse blood and urine samples were collected for determining serum creatinine and urine protein. Mice were sacrificed at 10, 14, 18, and 22 weeks of age.

Blood Sample Collection

At 10, 14, 18, and 22 weeks of age, mice received thoracotomy for harvesting blood sample from the heart. Mice were first intraperitoneally injected with 5% chloral hydrate (0.1 mL/kg) and cut open the thoracic cavity along the anterior midline for heart exposure. Blood was slowly extracted from the right ventricle using a 1 mL syringe and centrifuged at 3000 rpm for 15 minutes for collecting the supernatant. The serum sample was preserved at -80°C.

Urine Sample Collection

Mice were individually housed in a metabolic cage for about 5 hours in a fasting state before sacrifice, and they were given free access to water. An appropriate amount of antiseptic was added to the collection tube. Mouse urine was collected, centrifuged at 2000 rpm for 10 minutes and stored at -80°C.

Blood Glucose Determination

Blood glucose was determined using a blood glucose meter. Mouse blood glucose was detected at 6, 10, 14, 18, and 22 weeks of age for consecutive 3 days. The final blood glucose was the average level from three independent records.

Isolation of Primary Cells

22-week-old db/m and db/db mice were anesthetized and sacrificed. About 1 mm kidney tissue was harvested, digested in 0.25% trypsin at 37°C for 20-40 minutes, and shaken every 5 minutes. Trypsin digestion was terminated by adding 3-5 mL of Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA). The suspension was centrifuged at 1000 rpm for 10 minutes. The obtained HMCs were adjusted at a density of 5×10^5/mL and cultured in a flask at 37°C. HMCs isolated from db/m mice were treated with low-glucose, whereas those isolated from db/db mice were treated with high-glucose.

Cell Culture and Transfection

HMCs were cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin. Cell transfection was performed according to the instructions of Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA). Cells in logarithmic growth were inoculated into 6-well plates with 1×10^5 cells/well one day prior to transfection. Until 60-70% of confluence, cells were transfected with miRNA-337 mimics or negative control for 48 hours.

RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA from kidney tissues or HMCs was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), quantified by the ultraviolet spectrophotometer and those with 1.8-2.1 of A260/280 value were considered to be qualified. Subsequently, qualified RNA was reversely transcribed into complementary deoxyribose nucleic acid (cDNA). QRT-PCR was performed according to the instruction of the SYBR fluorescence quantitative premixing kit with pre-denaturation at 95°C for 30 s, 95°C for 5 s, and 60°C for 31 s, for a total of 40 cycles. U6 was used as the internal reference. Primer sequences were as follows: miRNA-337, F: CCTGAACTAATAGACAGACGG, R: TTGTATTCACCTTCGCTCACACG; U6, F: CTCGCTTCGGCAGCACA, R: AACGCTTCACGAATTTGCGT.

Western Blot

The total protein was extracted using radioimmunoprecipitation assay (RIPA; Beyotime, Shanghai, China) and denaturated at 100°C for 5 minutes. The protein sample was loaded for electrophoresis. After transferring on a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) at 300 mA for 100 minutes, it was blocked in 5% skim milk for 2 hours, incubated with primary antibodies at 4°C overnight and secondary antibodies for 2 hours. Bands were exposed by enhanced chemiluminescence (ECL) and analyzed by Image Software.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 (IBM, Armonk, NY, USA) was used for all statistical analyses. Data were represented as mean ± SD. The *t*-test was used for analyzing the difference between the two groups. The Pearson correlation analysis was conducted to evaluate the correlation between miRNA-337 expression and ARC. $p<0.05$ indicated the significant difference.

Results

Comparison of Mouse Basic Indexes

Serum glucose and body weight of db/m mice maintained stable throughout the experiment, while mice in the other three groups (db/db group, vehicle group, and si-miR group) showed gradual increases in serum glucose and body weight with the aging. Compared with age-matched db/m mice, db/db mice in other three groups showed higher serum glucose and body weight at the appointed time points. However, we did not observe changes in serum glucose and body weight in si-miR group relative to db/db group and vehicle group at each time point (Figure 1A, 1B). Serum creatinine and ACR of db/m mice were stable during the whole experiment. Increases in serum creatinine and ACR were seen in db/db group and vehicle group with mouse aging. At 6 weeks of age, no remarkable differences in mouse serum creatinine and ACR were found among the four groups. However, all mice in db/db group, vehicle group, and si-miR group showed higher serum creatinine and ACR than those in db/m group at 10, 14, 18, and 22 weeks. No significant differences in serum creatinine and ACR were found in mice of si-miR group compared with those of db/db group and vehicle group at 6 and 10 weeks. At 14 weeks, although serum creatinine in mice of si-miR group declined relative to age-matched mice in db/db group and vehicle group, the difference was not statistically significant. Remarkable reductions in serum creatinine and ACR were shown at 18 and 22 weeks in mice of si-miR group than those in db/db group and vehicle group (Figure 1C, 1D).

MiRNA-337 Was Highly Expressed in Kidney Tissues of DN Mice

Kidney tissues of mice at 10, 14, 18, and 22 weeks of age were harvested for detecting miRNA-337 expression. In comparison with db/m mice, the expression level of miRNA-337 in the mouse kidney tissues of db/db group and vehicle group markedly increased at the appointed time points. However, we did not find a significant difference in the kidney expression of miRNA-337 between si-miR group with db/db group and vehicle group at 10 weeks of age, which was remarkably downregulated at 14, 18, and 22 weeks of age (Figure 2A). Moreover, the miRNA-337 expression in mouse kidney tissues was positively correlated to ACR (Figure 2B). Subsequently, the primary HMCs isolated from mice of db/db group and db/m group were subjected to a high-glucose and low-glucose treatment, respectively. MiRNA-337 was highly expressed in high-glucose group relative to low-glucose one (Figure 2C). To further elucidate the biological function of miRNA-337, miRNA-337 mimics was constructed and tested for its transfection efficacy. The transfec-

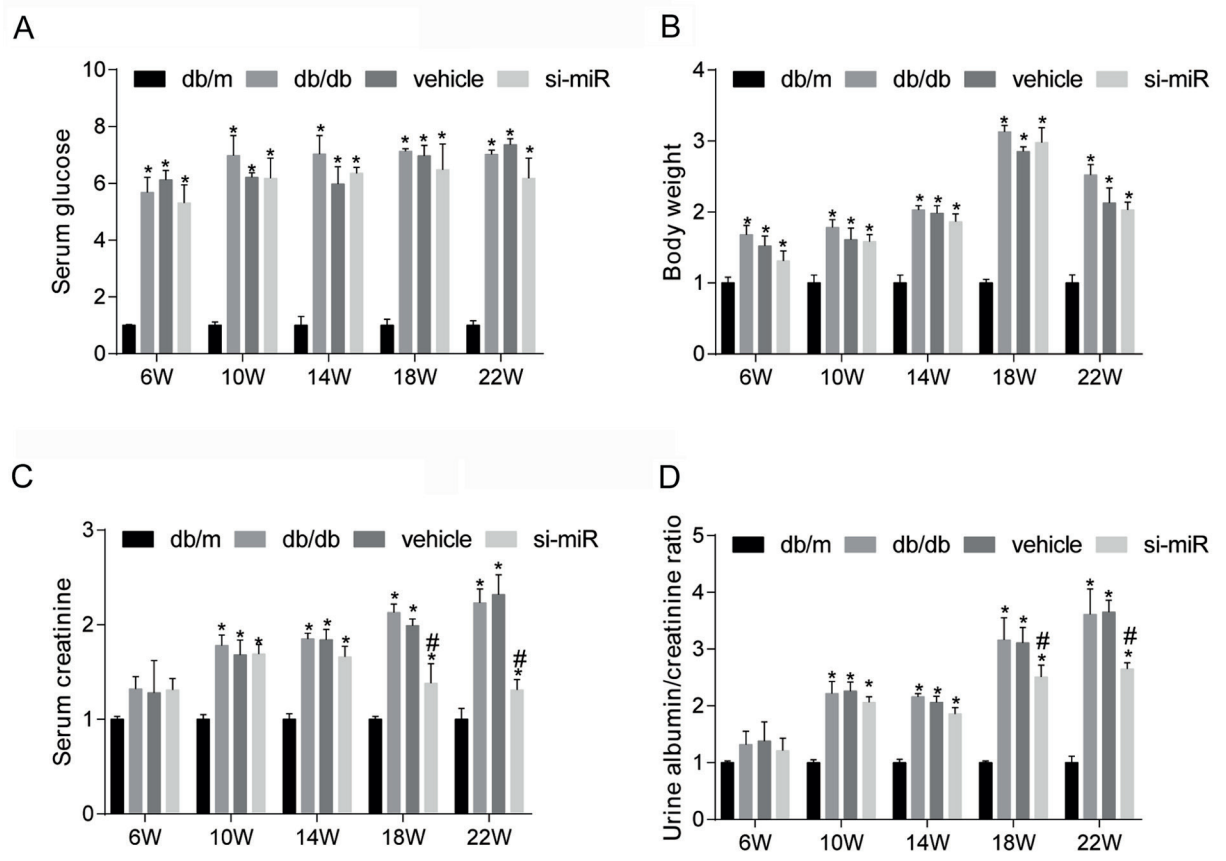

Figure 1. Establishment of DN mice. Mice were assigned into db/m group, db/db group, vehicle group, and si-miR group. **A,** Serum glucose of mice at 6, 10, 14, 18, and 22 weeks of age. **B,** Body weight of mice at 6, 10, 14, 18, and 22 weeks of age. **C,** Serum creatinine of mice at 6, 10, 14, 18, and 22 weeks of age. **D,** Urine albumin/creatinine ratio of mice at 6, 10, 14, 18, and 22 weeks of age. *$p<0.05$ *vs.* db/m group, #$p<0.05$ *vs.* db/db group.

tion of miRNA-337 mimics in HMCs effectively upregulated miRNA-337 expression, showing a sufficient transfection efficacy (Figure 2D).

MiRNA-337 Regulated Expressions of IL-6 and IL-18

At 22 weeks of age, expression levels of IL-6 and IL-18 in the mouse kidney tissues of db/db group and vehicle group markedly increased compared with db/m mice. Mice in si-miR group showed lower levels of IL-6 and IL-18 than age-matched mice in db/db group and vehicle group (Figure 3A). *In vitro* experiments confirmed the similar results. The protein levels of IL-6 and IL-18 were upregulated in HMCs overexpressing miRNA-337 (Figure 3B).

MiRNA-337 Impaired Podocytes in db/db Mice

At 22 weeks of age, the protein levels of podocin and nephrin in the mouse kidney tissues of db/db group and vehicle group downregulated, while the desmin level upregulated relative to db/m group. However, mice in si-miR group showed upregulated podocin and nephrin, as well as downregulated desmin in comparison with age-matched mice in db/db group and vehicle group (Figure 4A). After transfection of miRNA-337 mimics in HMCs, the protein levels of podocin and nephrin decreased, while the desmin level increased (Figure 4B).

Discussion

Recent studies have reported that the podocyte-specific deletion of dicer in microRNA biosynthesis leads to serious kidney injury in diabetic mice, suggesting the crucial role of microRNA in the development of DN[12]. The downregulation of miR-25 and miR-146a in rat primary endothelial cells and high-glucose-induced endothelial cells increases Nox4 expression and further aggravates

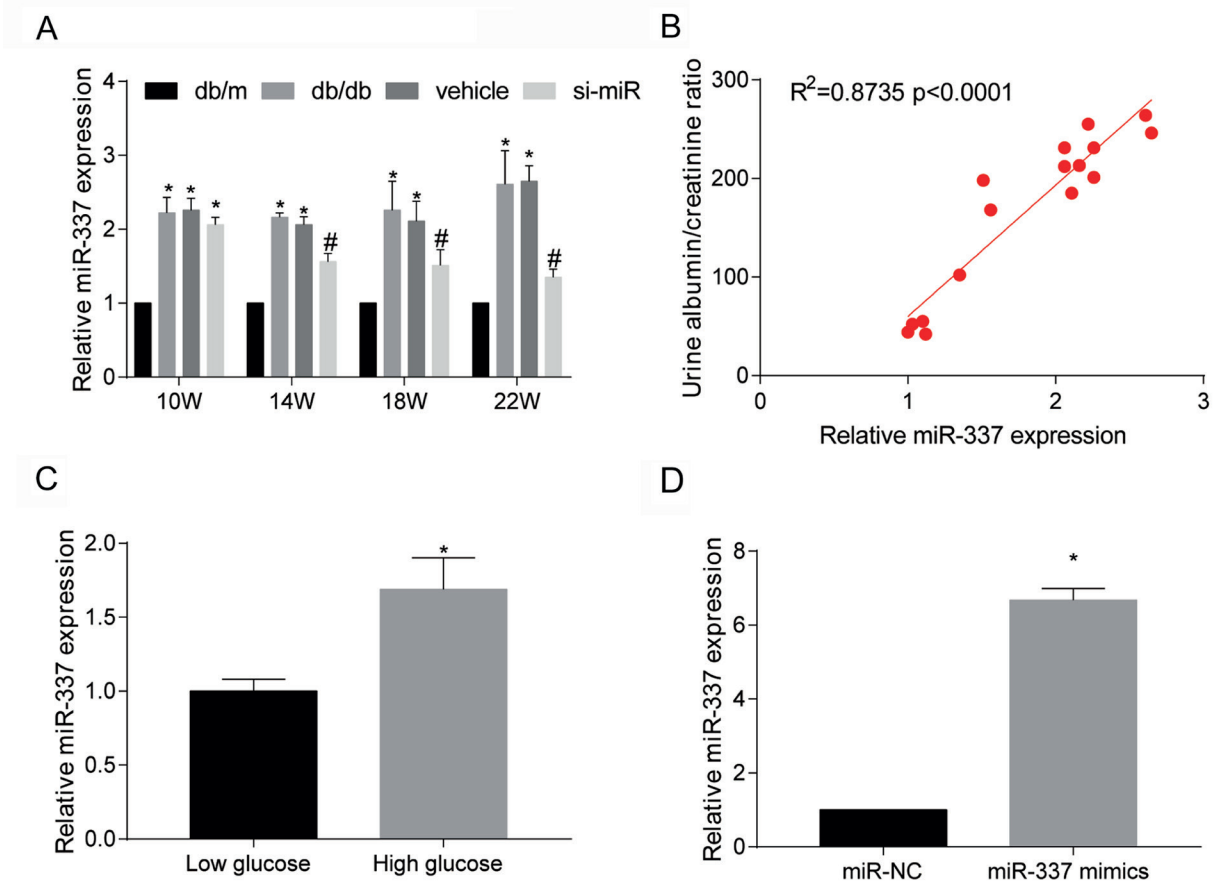

Figure 2. MiR-337 was highly expressed in kidney tissues of DN mice. Mice were assigned into db/m group, db/db group, vehicle group, and si-miR group. **A,** Expression level of miR-337 in kidney tissues of each group. **B,** Correlation between miR-337 expression with ARC (R^2=0.8735, p<0.001). **C,** Expression level of miR-337 in HMCs treated with high-glucose and low-glucose. **D,** Transfection efficacy of miR-337 mimics in HMCs. *p<0.05 *vs.* db/m group, #p<0.05 *vs.* db/db group.

DN-induced oxidative stress and kidney injury[13,14]. He et al[15] have reported that the inhibition of certain microRNAs alleviates DN, providing novel directions for clinical treatment of DN. MiRNA-337 is confirmed to be related to the inflammatory response. In a rat spinal cord injury model, miRNA-337 attenuates apoptosis, inflammation, and oxidative stress by targeting Nrf2[16].

IL-6 is an inflammatory factor with multiple roles in the development of DN, and its expression is remarkably elevated in DN patients compared with diabetic patients without kidney disease[17]. Hyperglycemia induces IL-6 production from podocytes, mesangial cells, mesenchymal tissues, and renal tubules, leading to local and systemic inflammatory processes in DN[18]. IL-6 is responsible for multiple DN-related kidney injuries, including increased glomerular endothelial cell permeability, mesangial cell expansion, fibronectin upregulation, and GBM thickening[19]. IL-18 is a potent inflammatory cytokine with multiple effects. It induces the production of interferon-γ (IFN-γ), IL-1, and TNF-α, upregulates expressions of chemokine receptors and ICAM-1, and regulates apoptosis of endothelial cells[20-22]. *In vitro* stimulation of podocytes with pro-inflammatory factors upregulates IL-18 level in a time- and dose-dependent manner, further aggravating podocyte injury[23].

Nephrin and podocin are the major proteins on the slit diaphragm of podocytes, which are characterized by podocyte-specific expression. Nephrin (135 kD) is a product of NPHS1 gene belonging to the immunoglobulin superfamily. It is a transmembrane protein on podocyte SD[24]. Podocin (42 kD) is a product of the NPHS2 gene belonging to the stomatin family. It inserts into the slit diaphragm of podocytes in a hairpin-like structure and is linked to nephrin *via* CD2AP[25]. Nephrin and podocin are involved in the development of

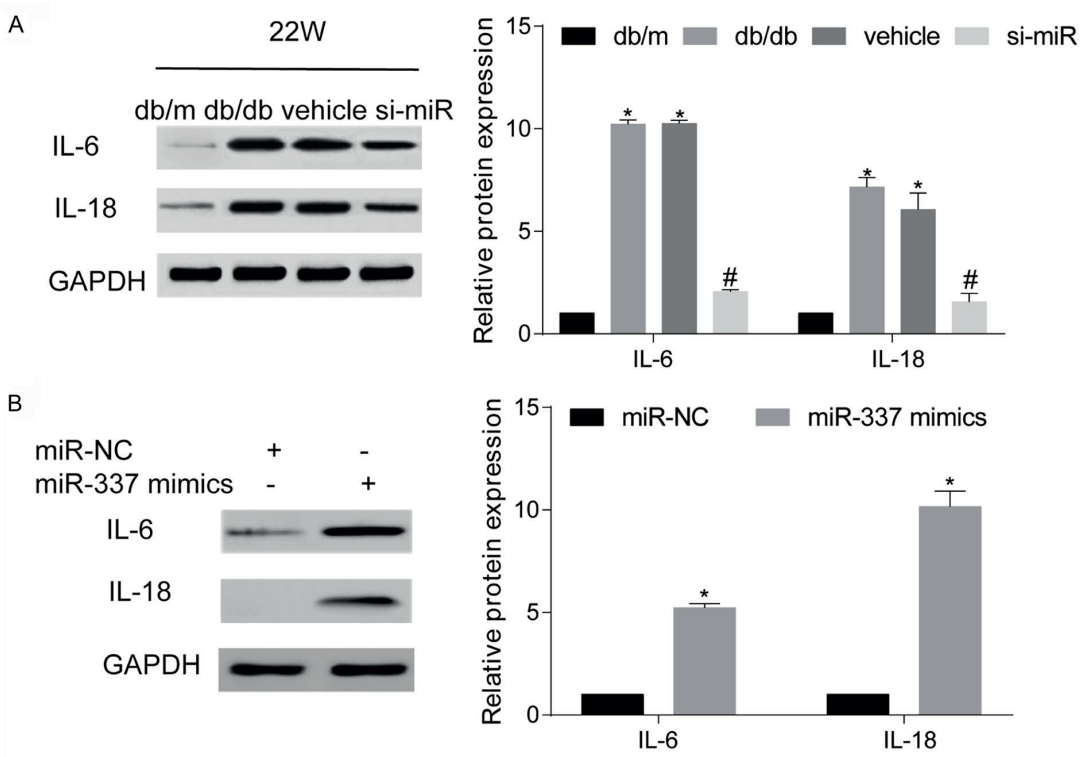

Figure 3. MiR-337 regulated expressions of IL-6 and IL-18. Mice were assigned into db/m group, db/db group, vehicle group, and si-miR group. **A,** Expression levels of IL-6 and IL-18 at 22 weeks of age. *p<0.05 *vs.* db/m group, #p<0.05 *vs.* db/db group. **B,** Expression levels of IL-6 and IL-18 in HMCs transfected with miR-NC or miR-337 mimics. *p<0.05.

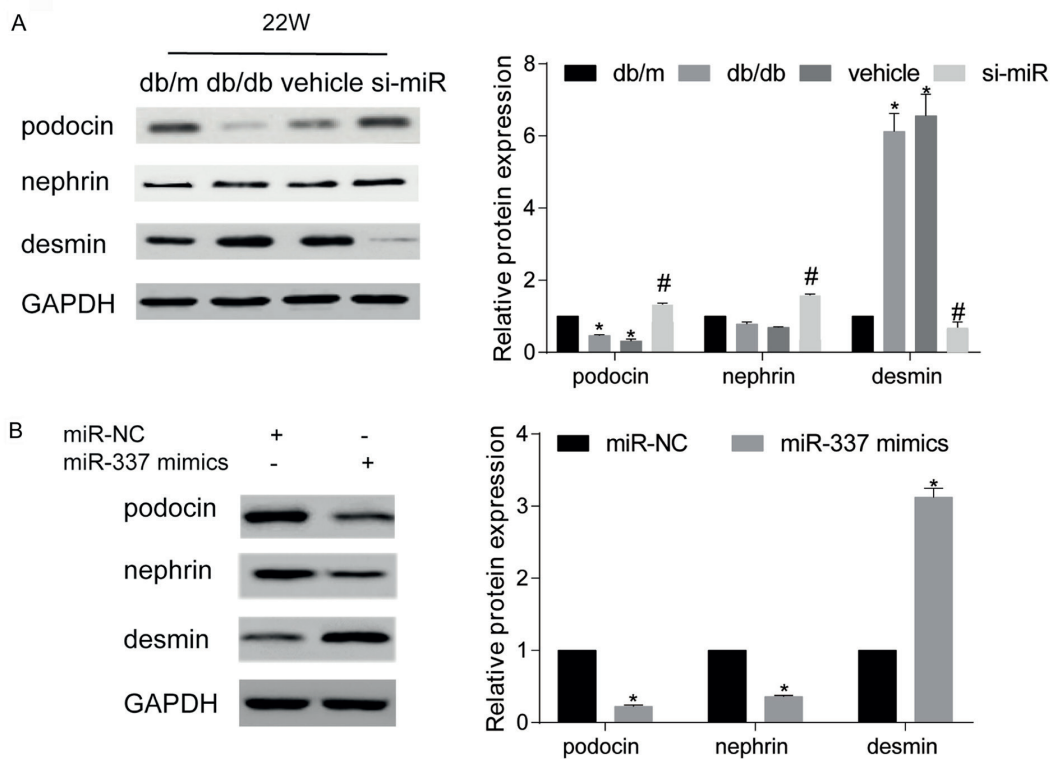

Figure 4. MiR-337 impaired podocytes in db/db mice. Mice were assigned into db/m group, db/db group, vehicle group, and si-miR group. **A,** Expression levels of podocin, nephrin, and desmin at 22 weeks of age. *p<0.05 *vs.* db/m group, #p<0.05 *vs.* db/db group. **B,** Expression levels of podocin, nephrin, and desmin in HMCs transfected with miR-NC or miR-337 mimics. *p<0.05.

proteinuria in both congenital nephrotic syndrome and acquired glomerular disease proteinuria[26]. Their expressions are downregulated after podocyte injury. Desmin belongs to the podocyte cytoskeletal protein. Under normal conditions, it is mainly expressed in mesangial cells but rarely expressed in podocytes. Podocyte injury stimulates the abundance of desmin in podocytes[27].

In this work, we first verified the successful establishment of DN model in db/db mice. MiRNA-377 was highly expressed in kidney tissues of db/db mice, which was positively correlated to ACR. After miRNA-337 inhibition, serum creatinine and ACR decreased. Both IL-6 and IL-18 levels were upregulated in kidney tissues of db/db mice, which were markedly suppressed by the miRNA-337 inhibitor. *In vitro* experiments revealed that miRNA-337 expression remained higher in HMCs with high-glucose treatment relative to those with low-glucose treatment. Of note, protein levels of IL-6 and IL-18 increased by miRNA-337 overexpression in HMCs with low-glucose treatment. Downregulated podocin and nephrin, as well as upregulated desmin, were seen in kidney tissues of db/db mice. More importantly, miRNA-337 inhibitor reversed the changing trends of podocin, nephrin, and desmin.

Conclusions

We showed that the miRNA-337 expression increases in db/db mice with diabetic nephropathy, leading to podocyte injury by upregulating levels of IL-6 and IL-18.

Conflicts of interest

The authors declare no conflicts of interest.

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