

Low expression of miR-203 promoted diabetic nephropathy via increasing TLR4

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Abstract. – **OBJECTIVE:** To investigate the relationship between microRNA-203 (miR-203) and diabetic nephropathy and its potential mechanism.

MATERIALS AND METHODS: The expression of microRNA-203 in mice with diabetic nephropathy and M4200 cells cultured with high glucose was detected by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). Toll-like receptor 4 (TLR4), the target gene of microRNA-203, was predicted and screened by bioinformatics method. Real-time quantitative PCR and Western blot were used to detect the endogenous TLR4 level in renal cortex of db/db mice with diabetic nephropathy and glomerular mesangial cells cultured in high glucose or low glucose. The expression of microRNA-203 and TLR4 mRNA were evaluated by RT-PCR after treatment of miR-203 mimics and inhibitor. The protein of TLR4 level was detected by Western blot. Additionally, the proliferation ability of cells was evaluated by Cell Counting Kit-8 (CCK8). The target relationship between microRNA-203 and TLR4 3' UTR was confirmed by luciferase reporter assay

RESULTS: The expression of miR-203 was significantly decreased in the kidney of mice with diabetic nephropathy and M4200 cells cultured in high glucose. On the contrary, TLR4 expression was significantly increased. Results of *in vitro* experiments showed that miR-203 could bind to 3'UTR region of TLR4. Overexpression of microRNA-203 significantly decreased the levels of TLR4 mRNA and protein. Meanwhile, low expression of miR-203 led to increased TLR4 expression, resulting in an enhanced proliferation of M4200 cells.

CONCLUSIONS: The downregulation of microRNA-203 led to an increased level of TLR4, thus promoting proliferation of M4200 cells in the pathogenesis of diabetic nephropathy.

Key Words:

miR-203, Diabetic nephropathy, TLR4, Cell proliferation.

ated that the number of diabetic patients in the world will exceed 430 million by 2030¹. Diabetic nephropathy (DN) is one of the most serious complications of diabetes. About 40% of diabetic patients may develop diabetic nephropathy, which is the leading cause of end-stage renal disease². Diabetic nephropathy has become a major public health problem on a global scale, bringing huge social and economic burdens to individuals, families and society. Therefore, the study of the molecular mechanisms underlying the development of diabetic nephropathy can not only provide a new perspective on the pathogenesis of diabetic nephropathy, but also provide a more targeted approach for the prevention and treatment of this disease.

During the development of diabetic nephropathy, the final clinical manifestation of the injury of renal structure and function is urinary albumin abnormalities³. In recent years, researchers⁴ have proposed the theory of micro-inflammation in the progression of diabetic nephropathy, and it is believed that diabetic nephropathy is a natural immunological disease with low-level inflammation. In diabetic nephropathy, abnormal transcriptional expression of inflammatory factors, such as toll-like receptor 4 (TLR4), tumor necrosis factor- α (TNF- α), interleukin-18 (IL-18), etc.^{5,6}, has been confirmed to be closely related to the progress of diabetic nephropathy in animal experiments and clinical studies. However, the mechanism of activation of TLR4 in diabetic nephropathy is not clear.

MicroRNA (miRNA) refers to a single-stranded, non-coding short-sequence RNA of about 22 nucleotides in length and is found in all multicellular organisms⁷. MiRNA participates in the regulation of gene expression after transcription, with its site of action located at the 3'UTR region of messenger RNA (mRNA), which results in the reduction of the synthesis of the corresponding protein. The mode of its action includes inhibition of mRNA translation and interference with mRNA stability⁸. The expression of miRNAs and their regulation of target genes mainly occur in

Introduction

Diabetes mellitus is a chronic metabolic syndrome characterized by hyperglycemia and it is esti-

cells. Their binding to target genes at the pre-transcriptional level can result in interference with the expression of target genes⁹.

In the early work of the pathogenesis of diabetic nephropathy, the renal tissues in normal mice or the mice with diabetic nephropathy caused by type 2 diabetes were compared by chip analysis. It was found that miR-203 showed abnormally low expression in the kidney of mice with diabetic nephropathy¹⁰. At the same time, miR-203 was found to be involved in tumor formation by affecting cell proliferation and other functions¹¹. The occurrence of diabetic nephropathy is closely associated with the excessive proliferation of mesangial cells¹². Therefore, in this study, we further explored whether miR-203 can participate in the development of DN through causing the excessive proliferation of mesangial cells.

Materials and Methods

Experimental Animals

4-week-old specific-pathogen-free (SPF) diabetic mice (db/db mice) and control normal mice (db/m mice) were purchased from Model Animal Institute, Nanjing University. During the experiment, mice were housed in a temperature-controlled room ($21 \pm 2^\circ\text{C}$) on a 12:12-h light/dark cycle (lights on at 06:00). Tissue collection was as follows: the mice were sacrificed after anesthesia, and their kidneys were rapidly stripped and frozen in liquid nitrogen for later extraction of RNA and protein. This study was approved by the Animal Ethical Committee of Renmin Hospital, Hubei University of Medicine.

Mouse Biochemical Detection

Blood glucose concentration was measured using a fast glucose meter. The mice were fasting within 6 hours, but water was allowed. Then, mouse-tail vein blood was collected for measuring blood glucose. Urine samples were collected using mouse metabolic cage to get 24 h urine. The mice had free diet in metabolic cages, and 0.5 ml of preservative was added to the container before collecting.

Cell Culture

Mouse mesangial cells M4200 were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (FBS, Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 $\mu\text{g}/\text{ml}$ strep-

tomycin. When the degree of cell fusion was about 80% to 90%, trypsin was applied to digest the cells. As soon as the cell morphology changed from a spindle to a circle, the serum was used to stop digestion, and the cells were collected by centrifugation, passaged or frozen. Cells were treated with 5 mM, 15 mM, and 25 mM glucose for 24 h. After different treatments, cells were harvested to extract RNA to detect miR-203 expression.

Transfection of Cells

After the degree of cell fusion was about 50-60%, the medium was removed. The transfection reagent previously mixed with lipo2000 (Invitrogen, Carlsbad, CA, USA) was added to a certain amount of serum-free medium in Petri dish, which was replaced with serum-containing medium 6 h later. The transfection efficiency was confirmed by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR). The miRNA mimics or inhibitor used in this experiment as well as the overexpression plasmid and siRNA of the target gene, were all provided by Shanghai Genepharma (Shanghai, China).

QRT-PCR

Total RNA was extracted by TRIzol according to instructions (Invitrogen, Carlsbad, CA, USA). The RNA precipitate was washed and dried at room temperature. Finally, 20-30 μL of diethyl pyrocarbonate (DEPC) were added to dissolve the precipitate and the concentration of RNA was measured. After that, the RNA was stored in a -80°C freezer until use. Reverse transcription experiments were performed according to the TaKaRa (Otsu, Shiga, Japan) OneStep PrimeScript[®] miRNA complementary Deoxyribose Nucleic Acid (cDNA) Synthesis Kit instructions. The PCR assay was performed with SYBR Green I fluorescent dye (Thermo Fisher Scientific, Waltham, MA, USA). The TLR4 primer sequence was F 5'ATGGCATGGCTTACACCACC3', R 5'GAGGCCAATTTTGTCTCCACA3'. The relative concentration of the sample was calculated using the method of $2^{-\Delta\Delta\text{CT}}$. The same experiment was repeated 3 times.

Reporter Gene Assay

Plasmid construction steps were as follows: the binding site of miR-23a-3p and ZO-1 was predicted by Target Scan. This combination sequence was sent for construction of wild-type and mutant plasmids. Mimics and inhibitors of miR-23a-3p were achieved from Shanghai Genepharma

(Shanghai, China). The relative fluorescence value after plasmid transfection was then measured using a standardized method.

Western Blot

The total protein of mouse kidney and M4200 cells was extracted and quantitated. The protein concentration was adjusted for sodium dodecyl sulphate (SDS) electrophoresis. After electrophoresis, the protein was transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA) and subjected to routine immunostaining. TLR4 primary antibody was used to incubate the membrane (1:500) at 4°C overnight. After that, anti-rabbit secondary antibody was used at 37°C for 2 h (1:1000). These protein bands cut from the membrane were subjected to enhanced chemiluminescence (ECL) luminescence (Thermo Fisher Scientific, Waltham, MA, USA) and then imaged. The same experiment was repeated 3 times and the average was calculated.

Cell Counting Kit-8 (CCK8) Experiment

M4200 cells in logarithmic growth phase were cultured in 96-well plates at a density of 1×10^6 /mL with 100 μ L per well. After 24 h, 10 μ L CCK8 (Dojindo, Kumamoto, Japan) were added to each well. Absorbance was measured at 450 nm after 1 h.

Statistical Analysis

Data analysis was performed using Statistical Product and Service Solutions (SPSS 20.0, Armonk, NY, USA) package and Graphpad software (La Jolla, CA, USA). The measurement data were expressed as mean \pm standard deviation, and Student's *t*-test was used for statistical analysis. When the *p*-value was less than 0.05, the difference was considered statistically significant.

Results

Biochemical Indicators of Mice with Diabetic Nephropathy

Normal db/m mice were taken as control. Body weight, blood glucose and urine microalbumin changes of db/m and db/db mice were detected to determine the nephropathy progress of db/db diabetic mice. As shown in Figure 1A-C, body weight, blood glucose and urinary microalbumin levels were significantly increased in db/db mice at 8th week. From these test indications, mice in our study were confirmed to be in line with the reported

successful pathological model and could provide a good model for later *in vivo* experiments.

High Expression of miR-203 Inhibits M4200 Cell Proliferation

As shown in Figure 2A, miR-203 was significantly downregulated in the kidneys of db/db mice both 8 weeks and 12 weeks of age compared to normal db/m mice, which was consistent with the literature reported. *In vitro* cultured mesangial cells with different concentrations of glucose indicated that microRNA-203 level in M4200 cells (H-MC group) cultured in high concentration of glucose (25 mmol/L) was significantly lower than that in low concentration of glucose (5 mmol/L, L-MC group). Additionally, there was a dose-dependent relationship between miR-203 expression and glucose concentration (Figure 2B). To further investigate the function of microRNA-203, we artificially regulated the expression of miR-203 in cells. Figure 2C indicated that microRNA-203 level in the group of H-MC mimics transfected with miR-203 mimics was significantly higher than that in group H-MC, while in L-MC inhibitor group transfected with miR-203 inhibitor, miR-203 was significantly lower than that in the L-MC group. CCK8 assay results demonstrated that overexpression of miR-203 significantly reduced the proliferation ability; the opposite result was observed after miR-203 expression was inhibited (Figure 2D).

TLR4 is a Potential miR-203 Target Gene

The biological functions of target genes were analyzed by REG-RNA database and GO (Gene Ontology) annotation information. The genes with high conservativeness, low binding free energy and closely related to the development of diabetic nephropathy were screened out as candidate target genes. As a result, it was found that the 3'UTR of the TLR4 gene contains a binding site for miR-203, which is highly conserved in the human and mouse genomes. We first detected the mRNA and protein expressions of TLR4 in the kidney of db/db mice and M4200 cells cultured *in vitro* by qRT-PCR and Western blot, respectively. As shown in Figure 3A and B, TLR4 mRNA and protein expression levels were significantly increased in the kidneys of db/db mice as compared with the normal db/m mice of 12 weeks of age. *In vitro* results showed that endogenous TLR4 mRNA and protein expressions were also significantly up-regulated in H-MC cultured in high

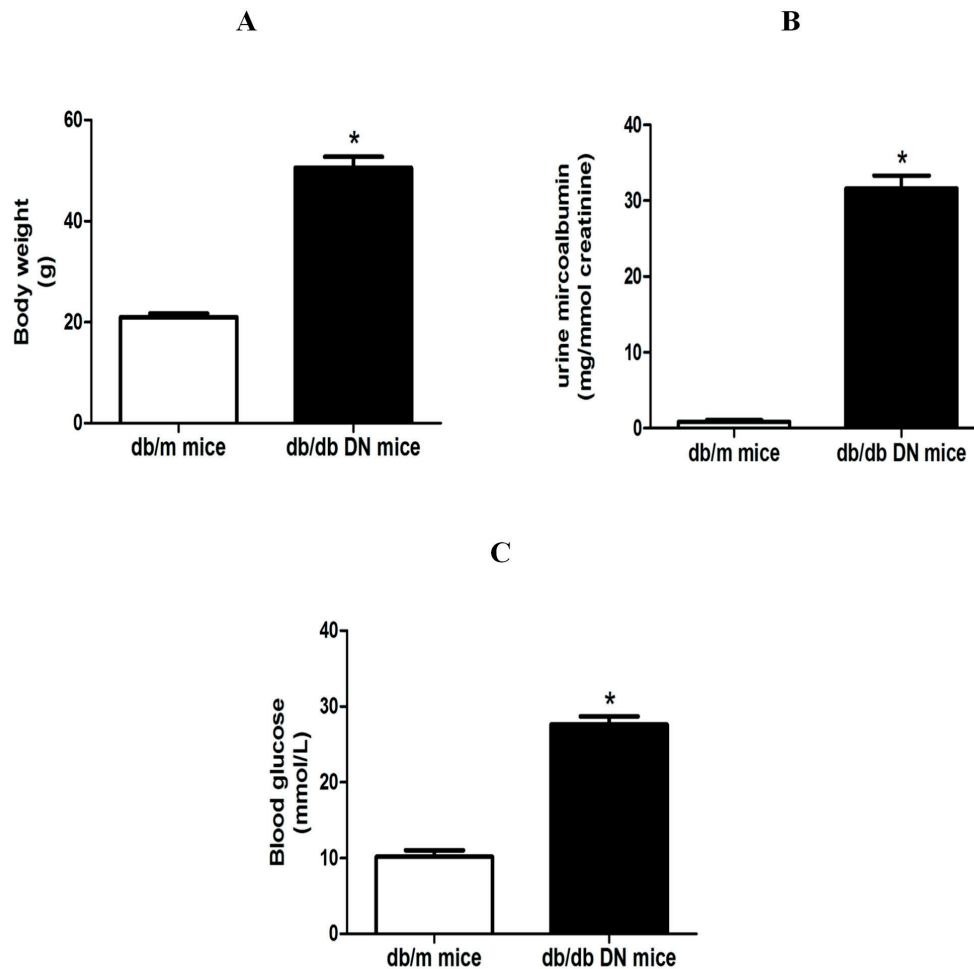


Figure 1. Body weight (A), urine microalbumin (B), and blood glucose (C) levels were measured.

glucose (Figure 3C, 3D). Thus, TLR4 was considered as a potential miR-203 target gene.

MiR-203 Regulates Proliferation of M4200 Cells Through TLR4

Based on the REG-RNA database, we obtained the binding sites of miR-203 and TLR4 3'UTR (Figure 4A). It was confirmed by the reporter gene results that miR-203 could bind to TLR4 (Figure 4B). As shown in Figure 4C, qRT-PCR results demonstrated that high expression of miR-203 significantly inhibited the level of TLR4 RNA. At the same time, this was confirmed by Western blot (Figure 4D). In order to further verify the function of TLR4, we transfected TLR4 siRNA and negative control (NC) into H-MCs with high expression of TLR4 to artificially down-regulate the expression of TLR4. TLR4 overexpression plasmid and NC were transfected into L-MCs

with low expression of TLR4 to upregulate TLR4 expression. The results of qRT-PCR implied that TLR4 was markedly lower in H-MC siRNA group than that in H-MC group. However, in the L-MC OE group transfected with TLR4 overexpression plasmid, TLR4 level was significantly higher than that in the L-MC group (Figure 4E). Next, CCK8 experiments revealed that in the H-MC siRNA group, the proliferative capacity of mesangial cells decreased significantly; conversely, the ability was enhanced in the L-MC OE group. Therefore, miR-203 may regulate M4200 cell proliferation *via* targeting TLR4 (Figure 4F).

Discussion

Although many investigations¹³⁻¹⁶ have showed the relationship between miRNA and diabetic

nephropathy, the specific role of single miRNA in pathological process of diabetic nephropathy still needs further investigation. Through consulting early biochip data, we found the abnormally expressed miR-203, which is associated with diabetic nephropathy. In this experiment, the significant decrease in the level of miR-203 in renal tissues of mice with DN and high-glucose cultured M4200 cells was verified by qRT-PCR. It has been shown in studies that hyperglycemia plays an important role in the functional and structural abnormalities of mice with DN¹⁷. We also found that glucose treatment caused the down-regula-

tion of miR-203 in a dose-dependent manner *in vitro* in a simulated high glucose environment. The higher the glucose concentration, the lower the expression of miR-203.

Researches have shown that inflammation is of great importance in the early development of diabetic nephropathy¹⁸⁻²⁰. Therefore, finding a direct relationship between miR-203 and inflammation facilitates us to better study the relationship between miRNA and DN. Through target gene prediction software and database of microRNA, all possible target genes of miR-203 were predicted. Among, those genes, associated with

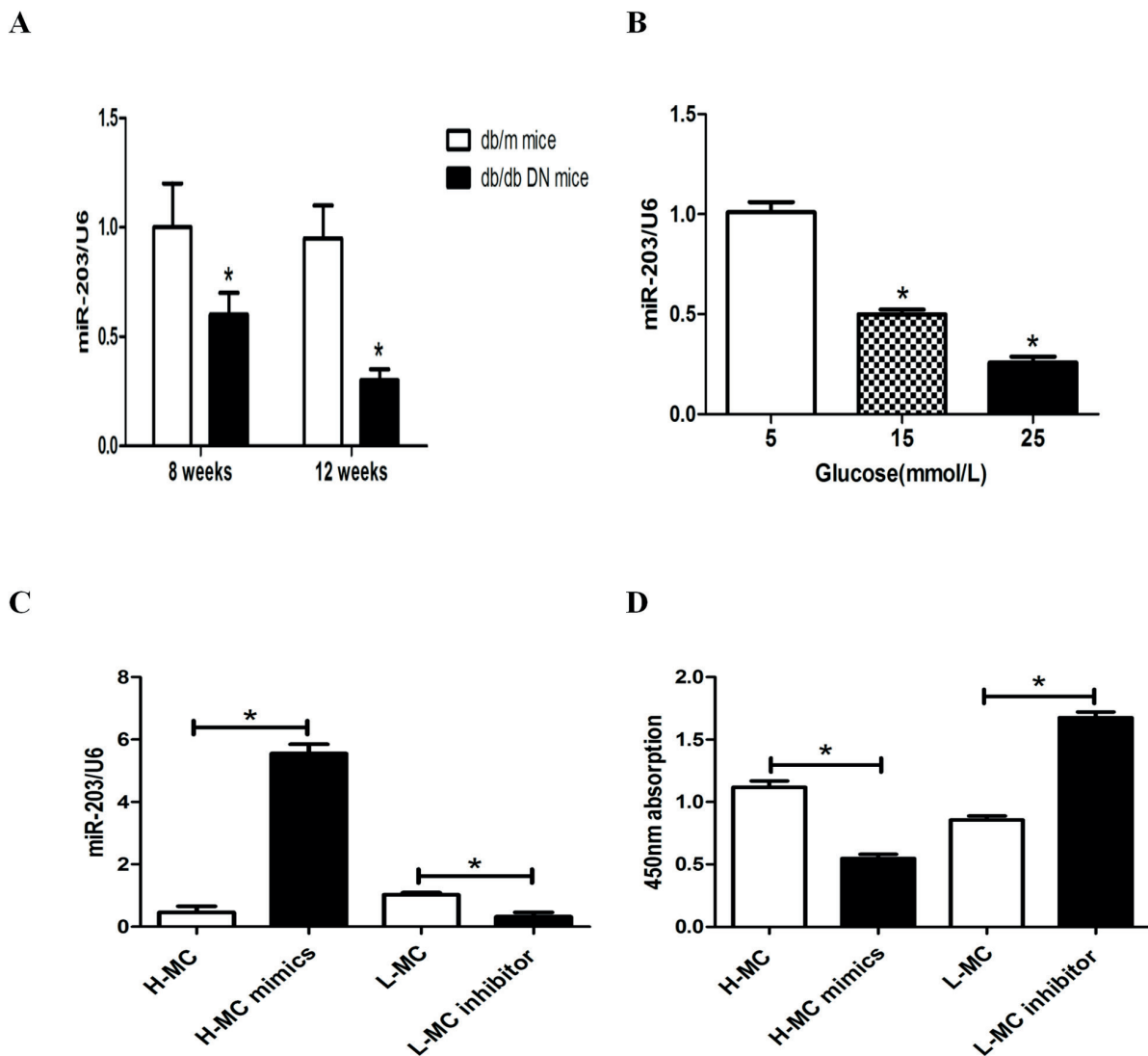


Figure 2. The relative expression level of miR-203 in renal cortex of mice with diabetic nephropathy (A), the expression of miR-203 in M4200 cells cultured at different glucose concentrations (B), the expression of miR-203 after transfected with mimics and inhibitor in M4200 cells (C), proliferation ability of M4200 cells after transfected with miR-203 mimics and inhibitor (D).

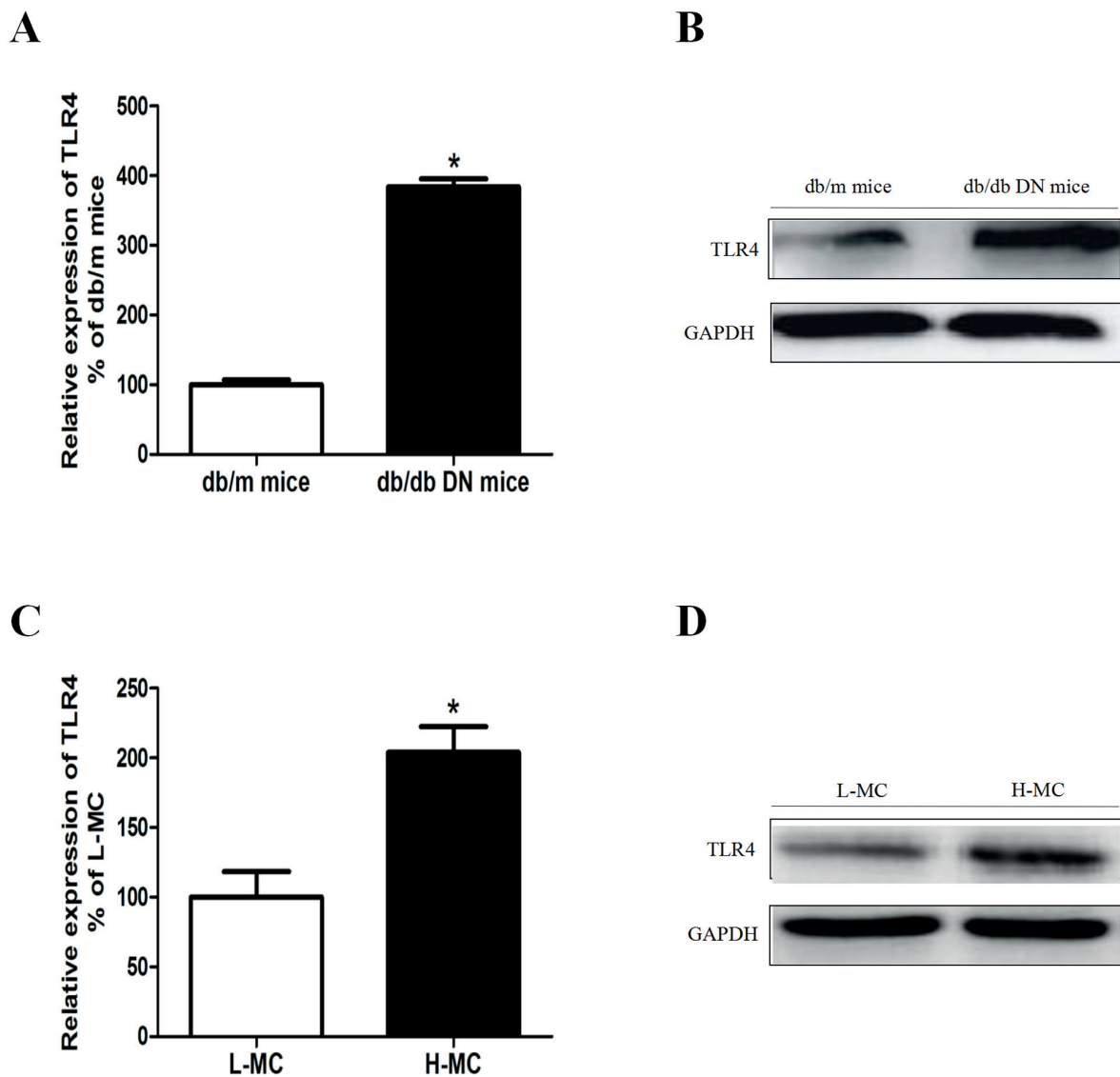


Figure 3. The mRNA expression (A) and protein expression of TLR4 in the kidney of diabetic mice (B). The mRNA expression (C) and protein expression of TLR4 in M4200 cells cultured at different glucose concentrations (D).

inflammation, were classified according to GO annotation information. Finally, the most direct inflammation-related gene was found out. Under such a train of thought, we found that the 1-10th nucleotide sequence at 5'-base of miR-203, which was the "seed sequence", was completely complementary to the 3' UTR of the TLR4 mRNA. We further confirmed the prediction results by experimental evidence that the TLR4 gene was indeed the target gene of miR-203, which could directly regulate TLR4 transcription and translation through binding to the 3'UTR region of TLR4. This result suggested that miR-203 may be invol-

ved in the inflammation of diabetic nephropathy through TLR4.

Toll-like Receptor 4 (TLR4) is a family of pattern receptors that are highly conserved. They can recognize conservative pathogens-related molecular pattern, and therefore represent the first line of defense. TLR4 is considered as a recognition receptor for Gram-positive bacterial lipopolysaccharide²¹. In addition, it links endogenous molecules caused by inflammatory lesions. Hence, TLR4 is a key receptor mediated by proinflammatory responses induced by both exogenous and endogenous ligands and exerts a

key role as inflammation amplifier^{22,23}. Recently, TLR4 has been widely demonstrated to be involved in the pathogenesis of diabetes^{24,25}. In this study, we also verified that the protein expression of TLR4 was conspicuously upregulated in the kidneys of mice with DN and glucose-cultured mesangial cells, while the reduction of TLR4 markedly inhibited cell proliferation, indicating

that TLR4 is essential in the development of diabetic nephropathy.

Conclusions

We demonstrated that low expression of miR-203 can lead to excessive proliferation of mesangial cells

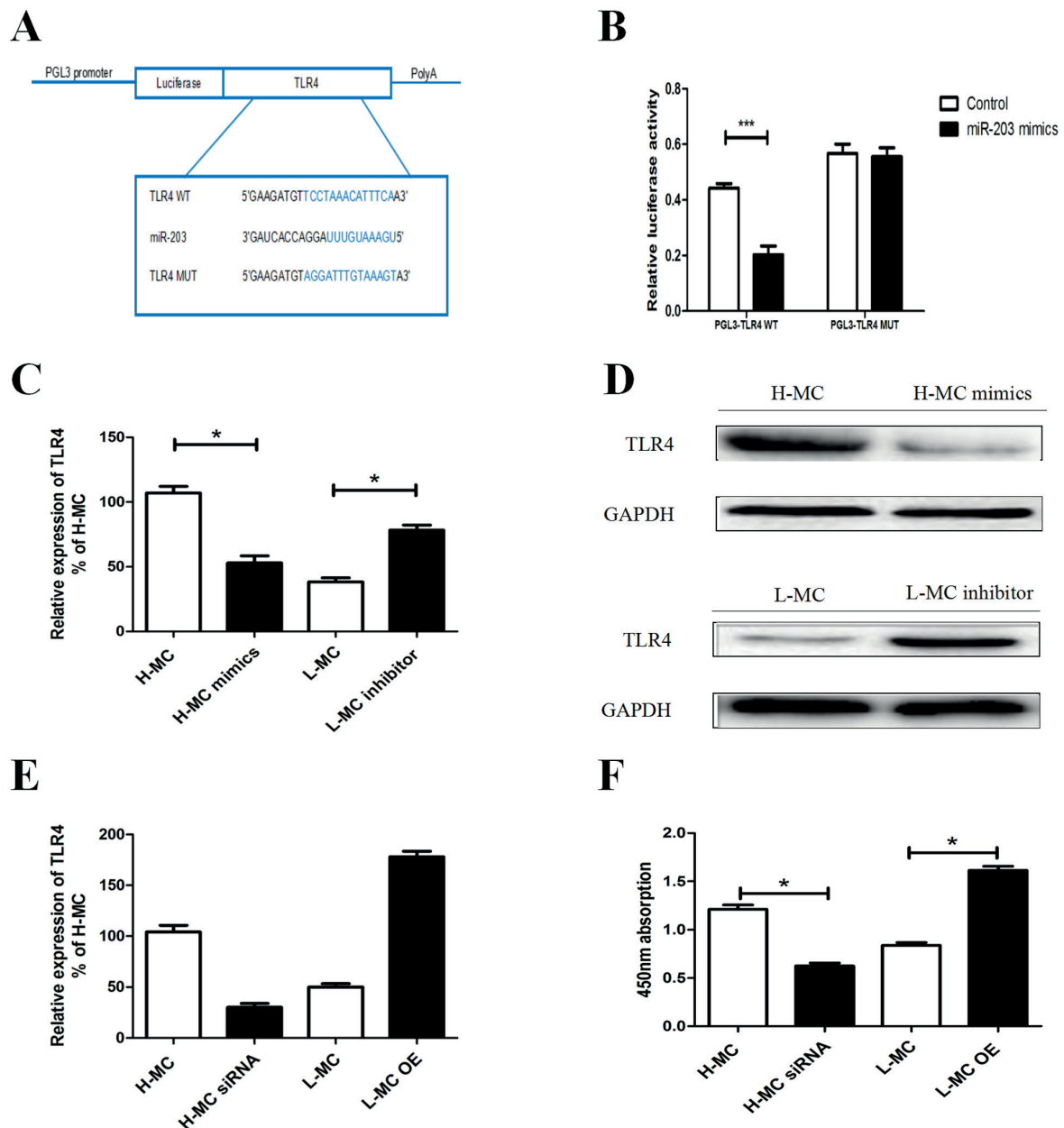


Figure 4. Prediction for the binding site of miR-203 to TLR4 (A). Luciferase reporter gene results (B). The effect of miR-203 mimics and inhibitor on TLR4 mRNA expression in M4200 cells (C) and protein expression (D) was examined. The effects of transfection of TLR4 siRNA and overexpression plasmid on TLR4 mRNA expression in M4200 cells (E) and mesangial cell proliferation ability were examined (F).

by promoting TLR4 expression, thereby involving in the occurrence of diabetic nephropathy.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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