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Effect of miR-29c on renal fibrosis in diabetic rats *via* the AMPK/mTOR signaling pathway

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Abstract. – OBJECTIVE: To explore the effect of micro-ribonucleic acid (miR)-29c on renal fibrosis in diabetic rats through the adenosine 5'-monophosphate-activated protein kinase (AMPK)/mammalian target of rapamycin (mTOR) signaling pathway, and to investigate its related mechanism by the research on the effect of miR-29c on the expression of fibrosis-related genes.

MATERIALS AND METHODS: The rat model of diabetic nephropathy (DN) was established, and the levels of fasting blood glucose (FBG), 24 h urine protein (24h-Pro), blood urea nitrogen (BUN), and serum creatinine (sCr) were monitored. After the rats were executed, kidney tissues were dissected, stained with paraffin and embedded in hematoxylin and eosin (H&E). Then, Western blotting was used to detect the levels of miR-29c, phosphorylated-AMPK (p-AMPK), α-smooth muscle actin (α-SMA), tumor necrosis factor-α (TNF-α), and macrophage migration inhibitory factor (MIF). The human renal tubular epithelial HK-2 cell line was treated with high glucose (HG) to simulate DN cell status *in vivo*. After that, the expressions of miR-29c and the renal fibrosis marker α-SMA were examined *via* Western blotting. Finally, the level of α-SMA was measured by Western blotting after HG treatment combined with miR-29c silencing.

RESULTS: The levels of FBG, BUN, sCr, and 24h-Pro in DN model rats were significantly higher than those in rats of control group. The data manifested that the DN model was successfully established. The expression level of miR-29c in DN model rats was markedly increased and that of the downstream protein p-AMPK also exhibited a significantly increasing trend. In addition, the levels of α-SMA, TNF-α, and MIF were elevated. The expression levels of miR-29c and α-SMA were increased markedly after the human renal tubular epithelial HK-2 cell line was treated with HG, but the expression of α-SMA was reduced after HG treatment combined with miR-29c silencing for 24 h.

CONCLUSIONS: MiR-29c is probably related to the occurrence and development of DN. Besides, miR-29c silencing may inhibit the DN renal fibrosis through AMPK/mTOR signals, so miR-29c may be an effective target for the treatment of DN renal fibrosis.

Key Words:
AMPK/mTOR, MiR-29c, Diabetes.

Introduction

Diabetes is one of the metabolic disorders, whose incidence rate has increased year by year, thus seriously affecting human health and quality of life[1]. Diabetic nephropathy (DN) is a frequently occurring complication in the advanced stage, whose incidence rate takes up about half of the total population, showing an increasing trend year by year[2,3]. Renal fibrosis in diabetic patients is often complicated with glomerular basement membrane thickening, sclerosis, and tubulointerstitial fibrosis[4]. Endogenous non-coding micro ribonucleic acids (miRNAs) play a crucial role

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in the growth and development of individuals[5]. Some studies[6,7] have indicated that miRNAs regulate epigenetics, stem cell self-renewal and differentiation, tumor occurrence and development, and metabolic diseases.

According to a number of works, miRNAs play an indispensable role in the pathogenesis of diabetes type 2 (T2D)[8,9]. Massart et al[10] have indicated that, compared with skeletal muscles of healthy people, the level of miR-29c in skeletal muscles of patients is increased; also, the up-regulated gene expression of miR-29c weakened insulin signals and the insulin receptor substrate responses to a certain extent, playing an important role in the occurrence of T2D.

Peng et al[11] found that the level of miR-29c in the urine of T2D patients can be used as a macromolecular marker for exacerbated DN. In addition, Lim et al[12] analyzed the expression difference of miRNAs in skeletal muscles of healthy rats and diabetic rats by gene microarray techniques. The data revealed that the level of miR-29a-c in skeletal muscles of diabetic rats was significantly higher than that in skeletal muscles of healthy rats.

In this work, therefore, α-smooth muscle actin (α-SMA) was selected as a marker for renal fibrosis. Then, the effect of miR-29c on renal fibrosis in DN rats was studied through diabetic model rats, and the action mechanism of miR-29c in diabetes was preliminarily explored, thus providing an important clinical guidance in the early diagnosis and prevention of DN from developing into an end-stage disease.

Materials and Methods

Materials

This investigation was approved by the Animal Ethics Committee of Dalian Medical University Animal Center. A total of 24 healthy male Sprague Dawley (SD) rats weighing 160-180 g were provided by the Laboratory Animal Center of Dalian Medical University, and adaptively fed for 1 week before the experiment. Other materials included: the human renal tubular epithelial HK-2 cell line (Shanghai Yanjing, Shanghai, China), miR-29c, α-SMA, tumor necrosis factor-α (TNF-α), macrophage migration inhibitory factor (MIF) and phosphorylated-adenosine 5'-monophosphate-activated protein kinase (p-AMPK) antibodies (Abcam, Cambridge, MA, USA), Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA), streptozotocin (STZ, Sigma-Aldrich, St. Louis, MO, USA), fetal bovine serum and Opti-MEM (Invitrogen, Carlsbad, CA, USA), a weighing balance, a cell incubator, a Sorvall Evolution RC high-speed freezing centrifuge (Thermo Scientific, Waltham, MA, USA), a Roche glucometer (Roche Diagnostics, Basel, Switzerland), and a Beckman AU480 automatic biochemical analyzer (Beckman, Miami, FL, USA).

DN Model Establishment and Experimental Grouping

The modeling method referred to the literature of Wang et al[13]. After adaptive feeding for 1 week, 6 rats were randomly selected as a normal control group and fed with common feed. The remaining rats were fed with high-fat and high-glucose feed for 4 weeks, fasted for 12 h and intraperitoneally injected with 35 mg/kg STZ. Meanwhile, the rats in normal control group were given the same dose of citric acid buffer. After 72 h, fasting blood glucose (FBG) was measured through blood collection from the tail tip, and FBG ≥16.7 mmol/L indicates that the DN model was successfully established. After the successful modeling, rats were further fed and observed for 4 weeks, during which, blood glucose, urine volume, and body weight were monitored. Finally, DN model rats could be used for experiments after the data were stabilized.

Specimen Collection and Detection

After fasting for 12 h, DN model rats were weighed, blood was collected from the tail tip, and the blood glucose level was measured by a glucometer. Subsequently, after blood and urine specimens were collected, kidneys were taken through dissection, fixed with formaldehyde, embedded in paraffin and observed *via* hematoxylin and eosin (H&E) staining (Boster, Wuhan, China). Ultimately, Western blotting was utilized to examine the expressions of miR-29c, α-SMA, TNF-α, MIF, and p-AMPK.

Cell Model Establishment

The human renal tubular epithelial HK-2 cell line was further cultured for 24 h and treated with high glucose (HG) with reference to Zhao LM's method and recorded as HG group and normal glucose (NG) group, respectively. Cells and supernatants in each group were collected for Western blotting.

Table I. Statistics of FBG, BUN, sCr, and 24h-Pro in diabetic rats.

Group	Control group	DN group
FBG (mmol/L)	5.1 ± 0.68	19.2 ± 2.3[a]
BUN [c/(mmol·L^{-1})]	4.6 ± 0.71	16.3 ± 4.26[a]
SCr [c/(μmol·L^{-1})]	14. 2 ± 1.23	28.9 ± 7.92[a]
24h-Pro [ρ/(mg·L^{-1})]	5.4 ± 1.5	14.1 ± 1.42[a]

Table I shows the statistics of FBG (mmol/L), BUN [c/(mmol·L^{-1})], sCr [c/(μmol·L^{-1})] and 24h-Pro [ρ/(mg·L^{-1})] in the rats of control group and DN group [different lowercase English letters represent significant differences, a: $p<0.05$)].

Cell Transfection

An appropriate number of HK-2 cells in the logarithmic growth phase were collected from the sterile operating room and inoculated into a sterile 6-well plate. When about 60% of cells adhered to the wall, scrambled siRNAs and simiR-29c were transfected into HK-2 cells at a concentration of 55 nmol/L, according to the instructions. After transfection for 6.5 h, the cells were further cultured in a fresh culture medium for 24 h and collected for Western blotting detection.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 16.0 (SPSS Inc., Chicago, IL, USA) was adopted for statistical analysis. All the data were expressed as ($\bar{x} \pm s$) and analyzed by the *t*-test. $p<0.05$ suggested that the difference was statistically significant.

Results

Establishment of the Rat Model of DN

Compared with those in rats in control group, the levels of FBG, blood urea nitrogen (BUN) and serum creatinine (sCr) and 24 h urine protein (24h-Pro) were remarkably increased ($p<0.05$) (Table I). After H&E staining, a glomerular basement membrane thickening was observed in diabetic rats, accompanied by a crescent or glomerular sclerosis (Figure 1).

Expression of MiR-29c in Kidney Tissues of DN Model Rats

Two specimens were randomly selected from kidney tissue specimens of DN model rats. Western blotting detection results manifested that the miR-29c expression in DN group was evidently up-regulated compared with that in control group ($^{a}p<0.05$), suggesting that miR-29c may be related to the occurrence of DN (Figure 2).

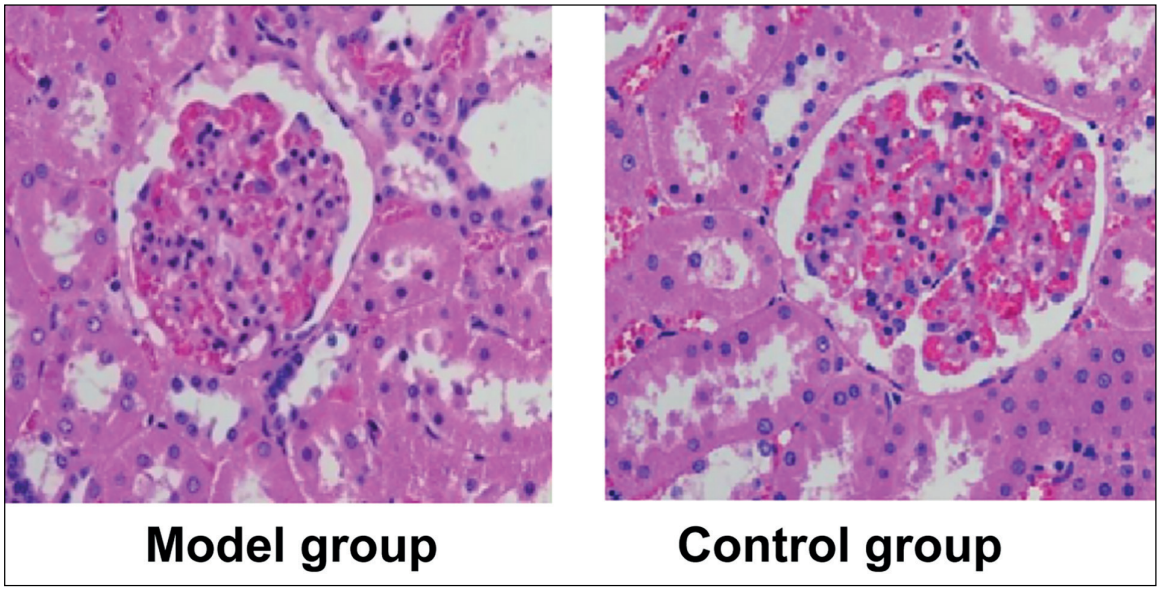

Figure 1. H&E staining of individual kidney tissues of diabetic rats (H&E×400).

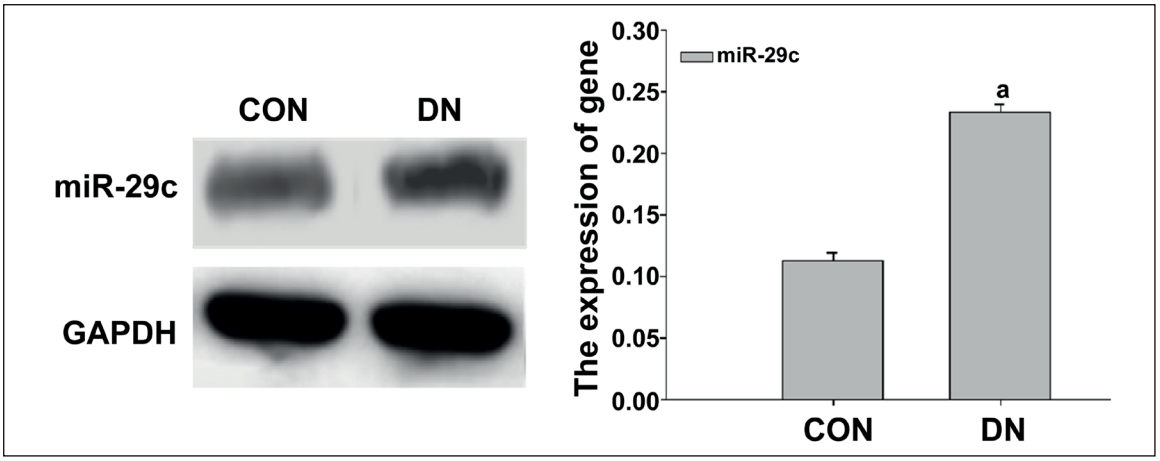

Figure 2. Expression level of miR-29c in kidney tissues of DN model rats. Figure shows the gene expression of miR-29c. CON: control group, and DN: DN group ($^a p<0.05$).

Expression Levels of α-SMA, TNF-α, MIF and p-AMPK in DN Model Rats

In the detection *via* Western blotting, it was found that the expression levels of α-SMA, TNF-α, MIF, and p-AMPK in DN group were significantly up-regulated compared with those in control group ($p<0.05$), indicating that renal injury and renal injury-related inflammatory responses occurred in the DN model, which may be associated with miR-29c (Figure 3).

Expression Levels of MiR-29c and α-SMA in HK-2 Cells Cultured Under the Condition of HG

According to the Western blotting results, the protein levels of miR-29c and α-SMA in HK-2 cells under the HG culture conditions were notably up-regulated ($^a p<0.05$), further verifying the *in vivo* experimental results (Figure 4).

Construction of HK-2 Cells With MiR-29c Silencing

Western blotting results demonstrated that the expression of miR-29c in HK-2 cells transfected with simiR-29c was significantly reduced compared with those in HK-2 cells transfected with scrambled siRNAs ($p<0.05$), indicating the successful establishment of cells (Figure 5).

Changes in the Levels of Extracellular Signal-Regulated Kinase (ERK) and IL-8 Proteins in HK-2 Cells After MiR-29c Silencing Under the HG Culture Condition

The HK-2 cells treated with miR-29c silencing and ERK inhibitors were cultured in HG. Western blotting analysis showed that the levels of the downstream protein of miR-29c, p-ERK, α-SMA, TNF-α, MIF, and p-AMPK were significantly decreased ($p<0.05$). The above results indicate that silencing miR-29c expression can inhibit the

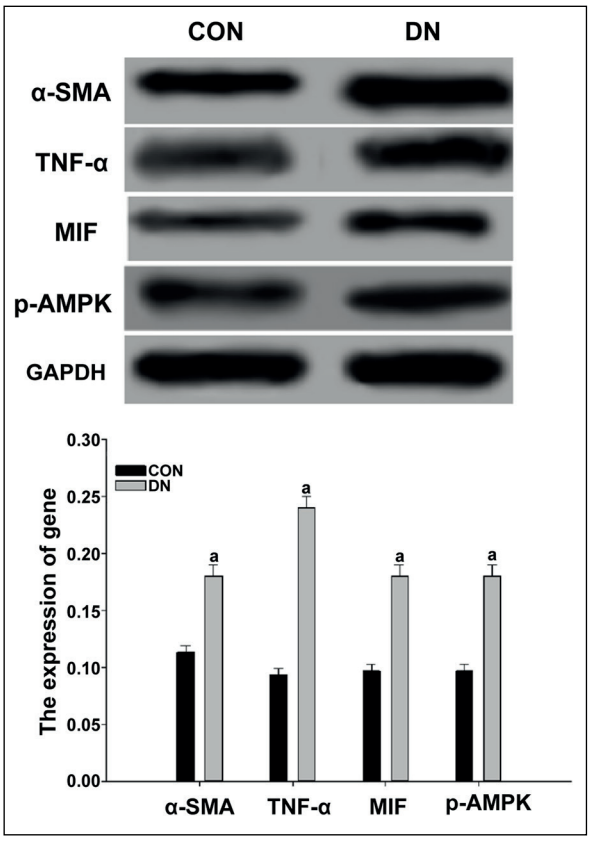

Figure 3. Protein levels of renal inflammatory cytokine and renal injury molecule-1 in DN model rats. Figure displays the gene expression levels of α-SMA, TNF-α, MIF, and p-AMPK ($^a p<0.05$).

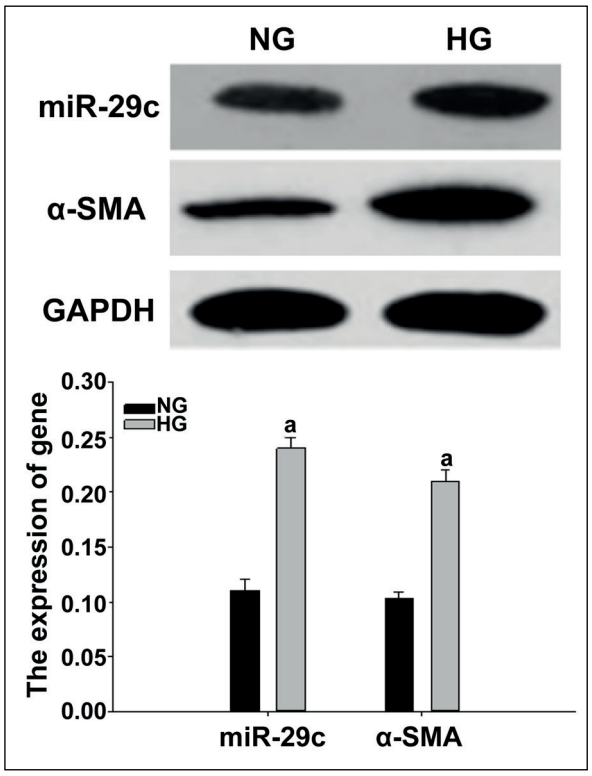

Figure 4. Expression levels of miR-29c and α-SMA in HK-2 cells cultured under the condition of HG. Figure reveals the gene expression levels of miR-29c and α-SMA ($^a p<0.05$).

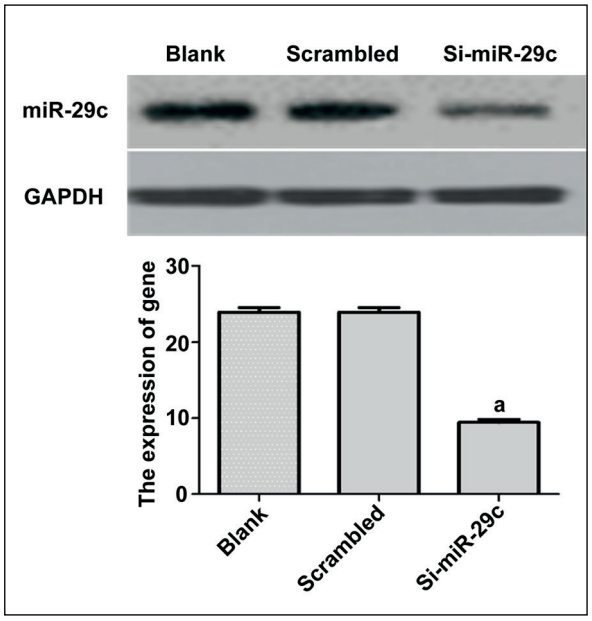

Figure 5. MiR-29c silencing in HK-2 cells. Figure illustrates the gene expression of miR-29c. Blank: blank control group and simiR-29c: gene silencing group ($^a p<0.05$).

secretion of cytokines by renal tubular epithelial cells and their injury ability by blocking ERK signals (Figure 6).

Discussion

DN is a frequently occurring complication in the advanced stage, whose incidence rate accounts for about half of the total population, displaying an increasing trend year by year. Renal fibrosis in diabetic patients is often complicated with a glomerular basement membrane thickening, sclerosis, and tubulointerstitial fibrosis[12]. MiRNAs take up about 3% of the total number of human genes, but they exert a crucial effect on the protein coding[12] and regulate epigenetics, stem cell self-renewal and differentiation, tumor occurrence and development, and metabolic diseases[13,14].

Xing et al[14] measured the expression of miR-29c in the serum of patients with liver cancer, liver cirrhosis, hepatitis B virus (HBV), and

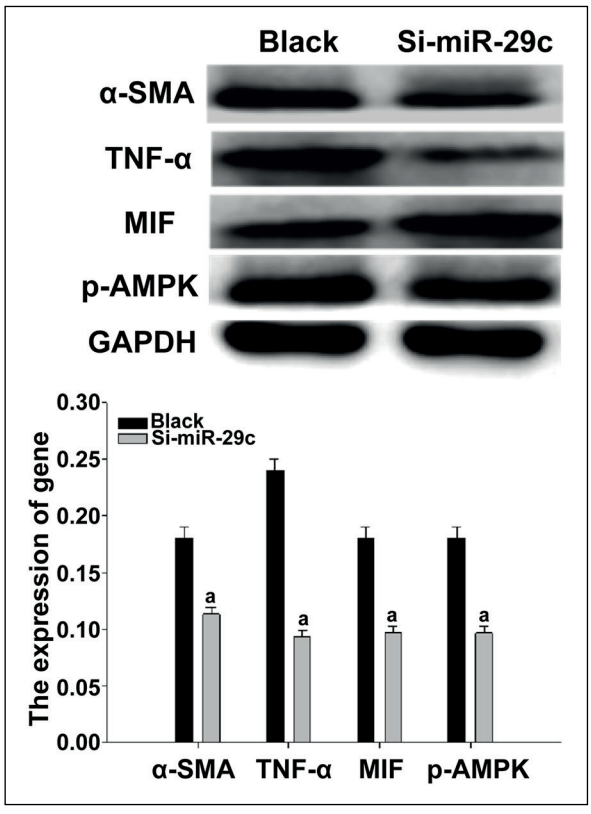

Figure 6. Expression levels of α-SMA, TNF-α, MIF, and p-AMPK in cells with miR-29a silencing. Figure shows the gene expressions of α-SMA, TNF-α, MIF, and p-AMPK. Black bars: blank group, and gray bars: simiR-29c gene silencing group ($^a p<0.05$).

chronic hepatitis B as well as healthy people by RT-PCR, respectively. The results revealed that the gene expression level of miR-29c in the serum of patients with chronic hepatitis B is linearly correlated with HBV DNAs, so it is inferred that the expression changes in miR-29c are related to liver fibrosis. Roderburg et al[15] investigated the effect of miR-29c on liver fibrosis by establishing a mouse model with miR-29c overexpression. The data showed that miR-29c regulates the degree of liver fibrosis by regulating the collagen expression[16]. Other researchers[17] analyzed islet tissues of non-obese diabetic mice using gene microassay techniques and discovered that the expression level of miR-29a-c is markedly elevated, compared with that in healthy mice. Moreover, the overexpression of miR-29a/b/c in MIN6 cells destroys the glucose homeostasis in the cell environment, which is generally maintained by insulin secreted by islet tissues. When the expression level of miR-29a-c is up-regulated, the expression levels of apoptotic proteins will be decreased, promoting cell apoptosis to some extent[17].

In this study, the rat model of DN was established by the intraperitoneal injection of STZ. Through detection, miR-29c was found to be highly expressed in DN kidney tissues. Meanwhile, it was also found that the expression of the fibrosis marker α-SMA was significantly increased. Fibrosis in the fibroblast compartment is partly mediated by activating resident fibroblasts, which secrete inflammatory cytokines and further stimulate the proximal tubular epithelium to secrete pro-inflammatory cytokines, which mediate local inflammation and fibrosis. Furthermore, chemotactic cytokines, secreted by fibroblasts and epithelial cells, provide a directional gradient which directs infiltration of monocytes/macrophages and T cells into the tubulointerstitium[18]. Fibrosis refers to the processes of wound repairing and healing when the body is injured to maintain the integrity of the original tissue structure and function. α-SMA is a marker of myofibroblasts[19]. According to some reports[20,21], inflammation plays a key role in fibrosis of DN, and inflammatory cells such as neutrophils, macrophages, and lymphocytes infiltrate into glomeruli and tubulointerstitial areas, indicating that the overexpression of miR29c is indeed related to the occurrence of diabetic renal fibrosis and can aggravate renal fibrosis. In this work, the expression of α-SMA, a marker of renal fibrosis, was detected, and it was found that the expression level of α-SMA in DN model rats was significantly increased. The above results further pointed out the speculation that miR-29c is related to the occurrence and development of diabetic renal fibrosis. The expressions of TNF-α and MIF were also increased. After HG treatment of cells in the human renal tubular epithelial HK-2 cell line, the expression levels of miR-29c and α-SMA were evidently up-regulated. Besides, HG combined with miR-29c silencing for 24 h reduced the level of α-SMA. These data can indeed indicate to some extent that miR-29c may be associated with the development of DN, and miR-29c silencing may inhibit DN renal fibrosis through AMPK/mTOR signals. Therefore, miR-29c may be an effective target for the treatment of DN renal fibrosis.

Conclusions

We found that miR-29c is probably related to the occurrence and development of DN. Besides, miR-29c silencing may inhibit DN renal fibrosis through AMPK/mTOR signals, so miR-29c may be an effective target for the treatment of DN renal fibrosis.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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