miR-206 inhibits high glucose-induced cell proliferation and extracellular matrix accumulation in mice glomerular mesangial cells

Juan Liu*, Changyan Li, Fang Gu

Department of Nephrology, Chengdu Fifth People's Hospital, Chengdu 611130, China

* Corresponding author
E-mail: liujuanguai@126.com

Abstract: The aim of this study was to investigate the effects of miR-206 on the proliferation and extracellular matrix (ECM) excessive accumulation of diabetic renal mesangial cells in mice with diabetic nephropathy. Mesangial cells (MCs) were cultured with high glucose to mimic diabetes mellitus. MCs were then transfected with miR-206 mimics. MTT and flow cytometry were used to detect the effects of miR-206 on the proliferation and cell cycle of mesangial cells. The impact of miR-206 level on expression of ERK signal pathway proteins and extracellular matrix proteins such as collagen IV, fibronectin were detected by real-time PCR and western blot. The results showed that high glucose could activate ERK signal transduction pathway in MCs, resulting in increased the expression levels of collagen IV, fibronectin. However, the overexpression of miR-206 obviously inhibited the proliferation of ECM cells, significantly decreased the phosphorylation level of ERK signaling pathway (P<0.05), and effectively decreased the secretion and expression of ECM protein (P<0.05). In conclusion, miR-206 may be a potential target for the prevention and therapy of diabetic nephropathy by inhibiting the accumulation of ECM via ERK signaling pathway activated by high glucose condition.

Keyword: miR-206, Diabetic nephropathy, Mesangial cells, Extracellular matrix, ERK
Introduction

Diabetic nephropathy (DN) is a major microvascular complications of diabetes mellitus, and it is the leading cause of end-stage renal disease (ESRD) in diabetic patients, which is associated with high cardiovascular morbidity and mortality (1). DN develops in approximately 40% of patients with T1DM, and also common in those with T2DM (2). Meanwhile, these DN patients accounts for 40% of end-stage renal disease patients in the United States and Europe (3). Mesangial cells (MCs) proliferation and extracellular matrix (ECM) accumulation are the major of pathologic features in the early stage of DN (4). In the state of high glucose, the dysfunction of MCs causes the imbalance between the secretion and degradation of ECM protein, leading to their abundant deposition in mesangium and glomerular basement membrane, consequently, resulting in the pathological changes of morphological and functional in glomerulus, which eventually leads to the occurrence of glomerulosclerosis (5,6). However, the specific mechanism of MCs dysfunction is not yet clear. Although we know that sustained hyperglycemia is an important inducement of DN, but the mechanism of hyperglycemia leading to DN has not been fully elucidated.

MicroRNA (miRNA) has a different expression in different tissues, and its expressed changes involve in the happening and development of a variety of human diseases, such as tumor, cardiovascular disease, diabetes, kidney disease, etc (7-9). Recently several studies have demonstrated that DN has yielded interesting findings related to miRNA expression, and kidney specific miRNAs plays a regulatory role in the signaling pathway of pathological changes of DN (10). In previously reported studies of kidney diseases, miR-206 has been recognized as an important role in the epithelial-mesenchymal transformation of stem cells in renal cell carcinoma, and had effects to regulate cell proliferation and apoptosis in cancer cells (11). Recent clinical trial results have shown that miR-206 expression was down-regulated in skeletal muscle of patients with type II diabetes, being associated with skeletal muscle insulin resistance (12). Moreover, miR-206 was involved in improving insulin signaling, and
protected against fructose-induced glomerular podocyte injury, and proteinuria, which may provide new evidence regarding its effects on fructose-associated kidney injury (13). However, the role of miRNA-206 on mesangial cells in diabetic nephropathy remains unknown.

In this study, mice mesangial cells were cultured under high glucose conditions to establish a cell model of DN, and to investigate the role of miR-206 on the proliferation of mesangial cells and the accumulation of ECM via ERK signal pathway in the development of diabetic nephropathy. Thus, the results may provide new insight into its molecular mechanism.

Materials and methods

Cell culture and grouping

Isolation of mice mesangial cells (MCs) was performed as previous described. Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS, Gibco, USA), 100 IU/ml penicillin and 100 μg/ml streptomycin (Thermo, USA), and were kept in a humidified incubator that was maintained at 37 °C and supplied with 5% CO₂ and 95% air. The culture media were purchased from Gibco (Thermo Fisher Scientific, Carlsbad, CA, USA). The cells were respectively divided into 4 groups: Normal culture group (NG), Simple high glucose culture group (HG), High glucose +miRNA-206 mimics group (miR-206), High glucose+ negative control mimics group (NC-miR).

Transfection

The miR-206 mimics and miR-206 mimics negative control (NC) oligonucleotides were synthesized by GenePharma (Shanghai, China). At confluence, cells were seeded at the density of 100,000 cells/well and transfected with of miR-206 mimics or NC with Lipofectamine 2000 (Thermo Fisher Scientific, Massachusetts, USA) for 48h. The transfections were performed by using Lipofectamine 2000 reagent (Invitrogen®, Thermo Fisher Scientific). Cells were harvested 48 hours after transfection for further assays.

Cell proliferation analysis
For proliferation assay, MCs were transfected with miR-206 mimics or NC-miR. The number of viable cells was measured by using cell counting kit-8 (CCK-8, Beyotime Biotechnology, Shanghai, China) at different time points. The cell density was determined by the absorbance at 490 nm on a microplate reader (Bio-Rad, Hercules, CA, USA).

**Cell cycle by flow cytometry**

The cells of difference groups were cultured for 24 h, after that, the cells were collected, centrifugal for 5 min as 1000 r/min, rejection the supernatant, washing by PBS at 2 times, centrifugal for 5 min as 1200 r/min, rejection the supernatant, adding the 70% ethanol (4°C) to fix, color by Propidium iodide (PI) for 30 min, The distribution of cell cycle was analyzed by flow cytometry and MUTCYCLE software.

**Real-time quantitative PCR**

The total RNAs were extracted from the cells and tissues samples with TRIzol (Invitrogen, Massachusetts, USA). The expression of miR-206 was determined by using the Hairpin-it™ miRNAs qPCR Quantitation Kit (GenePharma, Shanghai, China). U6 (RNU6B; GenePharma) served as the internal control. The expression of p-ERK, ERK, Bcl-2, Bax, fibronectin, and type IV collagen was determined by YBR ExScript™ RT-PCR kit (Takara, Dalian, China), and GAPDH was used as the internal control. Real-time quantitative PCR(qRT-PCR) was performed with ABI 7500 Real-Time PCR System (Applied Biosystems, CA, USA).

**Western blot analysis**

Cells were lysed by RIPA Lysis Buffer, and the concentration of the protein was determined by the BCA Protein Assay Kit (Beyotime, Shanghai, China). Then electrophoresis was performed to separate the proteins, and the proteins were then transferred onto PVDF membranes and blocked with 5% non-fat milk. The membranes were then incubated with primary antibodies (1:1000 dilutions, all purchased from Abcam, MA, USA) at 4°C overnight; in the following day, the membranes were washed and then incubated with the HRP-conjugated secondary antibodies (1:2000 dilutions, Abcam, MA, USA), and the enhanced chemiluminescent
reagent (Beyotime, Shanghai, China). Finally, the signals were detected by ChemiDoc™ XRS+ imaging system (Bio-Rad, CA, USA).

Statistical analysis

All the experiments were repeated at least three times. Representative results were shown in this paper. Continuous variables are presented as mean ± standard deviation. Comparisons between two groups were analyzed by Student t-test. Comparisons among multiple groups were analyzed by one-way analysis of variance(ANOVA) with Tukey test. \( P \) values < 0.05 were considered as statistical significance.

Results

High glucose influences cell proliferation, apoptosis, and cell cycle in MCs

First of all, we tested whether high glucose had an effect on the proliferation, apoptosis, and cell cycle of MCs in vitro. Cell proliferation, apoptosis, and cell cycle potential were examined. As shown in Figure 1A, Growth curve assay by CCK-8 method showed that high glucose induced significant increase in the proliferation of MCs\( (P < 0.05, \text{ respectively}) \). The cell apoptosis rate of high glucose culture group (HG group) was significantly decreased compared with that of normal culture group(NG group) in MCs (Figure 1B). The G1 phase rate of HG group was significantly decreased compared with NG group in MCs \( (P < 0.05, \text{ respectively}) \). The relative data were shown in Figure 1C. The results indicated that high glucose does affect the cell cycle, proliferation and apoptosis of mesangial cells, thus affecting DN development.
Figure 1. High glucose influences cell proliferation, apoptosis, and cell cycle in MCs

*: $P < 0.05$, compared with NG group

High glucose induces ECM proteins expression by activated ERK signal pathway

To further confirm that the accumulation of ECM was dysregulated during DN development, mice mesangial cells (MCs) were challenged with high glucose. The results were shown that expressions of ECM proteins such as collagen IV, fibronectin in high glucose culture group were remarkably elevated compared with normal culture group by western blot measurement($P < 0.05$, respectively). Our data revealed that over expression of collagen IV, fibronectin were a response to high glucose.

Moreover, we observed that the phosphorylation of ERK signaling pathway in mesangial cells was stimulated by high glucose. Compared with normal culture group, the phosphorylation level of p-ERK was up-regulated in high glucose culture group, meanwhile, the expressions of ERK remained no significant change between HG and
NG groups ($P < 0.05$, respectively).

Furthermore, the treatments with high glucose influenced the protein levels of pro-apoptotic and anti-apoptotic in MCs. The pro-apoptotic protein expression of Bax was down-regulated in high glucose culture group compared with those in NG group, by contrast, the incubations with high glucose markedly increased the anti-apoptotic protein level of bcl-2 compared with NG group ($P < 0.05$, respectively). The analytical results indicated that high glucose could accelerate the rate of apoptosis in MCs. The data was shown in Figure 2.

![Graph showing protein expression](image)

**Figure 2.** High glucose induces the relative proteins expression in MCs

*: $P < 0.05$, compared with NG group

**High glucose inhibits miR-206 expression in MCs**

Next, we examined the effect of high glucose culture on the expression of miR-206 in glomerular mesangial cells. As shown in Figure 3, the expression
of miR-206 was inhibited in high glucose culture group, compared with NG group \((P < 0.05, \text{respectively})\).

![Graph showing miR-206 expression](image)

**Figure 3.** High glucose inhibits miR-206 expression in MCs

\*: \(P < 0.05\), compared with NG group

**Impact of miR-206 on cell proliferation, apoptosis, and cell cycle under high glucose in MCs**

To further investigate the influence of introducing miR-206 on cellular proliferation, apoptosis, and cell cycle, MCs were transfected with miR-27b mimic or NC-miR under high glucose. CCK-8 analysis (Figure 4A) showed that the cell viability was lower in the miR-206 mimics group, compared with those of control groups in MCs \((P < 0.01, \text{respectively})\). Next, the effect of miR-206 on the apoptosis of MCs was examined by flow cytometry methods under high glucose. It was observed that transfection of miR-206 mimics induced significant increase in the apoptosis of MCs in vitro \((P < 0.05)\)(Figure 4B). The G1 phase rate of miR-206 mimics group was significantly improved compared with that of NC groups \((P < 0.05, \text{respectively})\). The relative data were shown in Figure 4C.
Figure 4. Impact of miR-206 on cell proliferation, apoptosis, and cell cycle under high glucose in MCs (*: $P < 0.05$, vs NC group)

The expression of ECM proteins were directly down-regulated by miR-206 via inhibiting ERK signaling pathway

Finally, we performed the western blot experiment to verify the effects of miR-206 on extracellular matrix (ECM) proteins, ERK signal pathway proteins, and apoptosis related proteins. As illustrated in Figure 5, Western blot showed that transfection of miR-206 mimics obviously inhibited the phosphorylation level of p-ERK, and eventually lead to significant decrease in the expression of collagen IV, fibronectin. In consistent the effects of miR-206 on cellular proliferation and apoptosis, the protein levels of pro-apoptosis factor Bax was elevated in the miR-206 mimic group, whereas anti-apoptosis factor Bcl-2 was decreased when miR-206 was over-expressed. The results suggested that the regulation of miR-206 on the accumulation of ECM, cellular growth and apoptosis was achieved by modulating ERK signaling pathway.
Figure 5. Impact of miR-206 on the relative proteins expression under high glucose in MCs (*: $P < 0.05$, vs NC group)

Discussion

Mesangial cells (MCs) are intrinsic cells with active function in the glomerulus. Inhibition of MCs proliferation and extracellular matrix (ECM) deposition play an important role in preventing the occurrence and development of glomerulosclerosis. Especially in the early stage of diabetic nephropathy, increased mesangial cell proliferation is the initiating factor in DN development (14). In this study, we found that high glucose significantly increased the proliferation of mesangial cells, inhibited cell apoptosis and promoted cell cycle. Meanwhile, our data revealed that high glucose inhibits the expression of miR-206 in MCs. So, it is not strange to find that transfection of miR-206 mimics could effectively inhibit the high glucose-induced proliferation of mesangial cells, promote the apoptosis of mesangial cells, and block the cell cycle in G1 phase. This result is consistent with the reported improvement of insulin signal transduction by miR-206 and its protective against fructose-induced
glomerular podocyte injury, and proteinuria (13). Our research indicated that miR-206 over-expression had effects to suppress the proliferation of mesangial cells, suggesting that miR-206 could act as a potential role in the prevention and treatment of diabetic nephropathy in the early stage.

Deposition of extracellular matrix (ECM) from mesangial cells plays a key role in the progress of DN. In healthy condition, ECM is composed of collagen, fibronectin (FN), laminin, elastin, proteoglycan, adhesion glycoprotein, etc (15). Fibronectin (FN) is the main component of ECM in glomeruli and plays an important role in mediating mesangial cell proliferation and deposition of other ECM components (16). In addition, collagen IV is another major component of ECM. Related studies have shown that collagen content in the matrix is above 50% (17). FN is the scaffold that forms the basement membrane network of the glomerulus, firstly appeared in glomerulosclerosis caused by immune and non-immune injury. Subsequently, collagen IV and V increased dramatically and deposited at the bottom of the scaffold, resulting in a thickening of the glomerular basement membrane (18). In human and animal disease models, increased expression of ECM protein in mesangial cells stimulated by high glucose leads to DN progression and ultimate glomerulosclerosis. Our findings support this point. In this study, we found that the expressions of ECM components such as collagen IV, fibronectin in high glucose culture group were remarkably elevated compared with normal culture group by western blot, then transduction by miR-206 significant decrease in the expression of collagen IV, fibronectin. Thus, the present findings suggest that miR-206 regulation of ECM accumulation in mesangial cells was also a mechanism of its involvement in DN.

The functional changes of different cells are related to their specific intracellular signal transduction pathways and corresponding gene changes. In the development of mesangial cell proliferation and extracellular matrix deposition induced by diabetic nephropathy, several signaling pathways have been identified to be involved, such as PI3K/Akt, TGF-β1/Smad, and MAPK, etc (19,20). So which signaling pathway does
miR-206 play its role? Report showed that high glucose and Ang II, two main pathogenic factors of diabetic nephropathy, induced nuclear translocation and activation of ERK in mesangial cells, which activates ERK in the cytoplasm and translocates it into the nucleus, lead to modulate the activity of transcription factors in the nucleus to induce mesangial cell hypertrophy (21). Consistent with previous studies on the prevention of fructose-induced glomerular podocyte injury by miR-206 through ERK signaling pathway (13), our data revealed that transfection of miR-206 mimics obviously inhibited the phosphorylation level of p-ERK, and eventually lead to significant decrease in the expression of collagen IV, fibronectin, anti-apoptosis factor Bcl-2, but improved the protein levels of pro-apoptosis factor Bax. These findings suggest that miR-206 on the accumulation of ECM, cellular growth and apoptosis was achieved by modulating ERK signaling pathway. Thus, this inhibiting effect was probably related with ERK signaling pathway.

In conclusion, our studies had shown that the activation of ERK pathway under high glucose conditions lead to the accumulation ECM protein including collagen IV (Col IV) and fibronectin (FN), thus inhibiting the degradation of ECM. Conversely, the over expression of miR-206 inhibit the MCs proliferation and extracellular matrix (ECM) deposition by modulating ERK signaling pathway under high glucose, suggesting the important role for the prevention and therapy of diabetic nephropathy.

Reference


