Int J Clin Exp Pathol 2018;11(8):4004-4011
www.ijcep.com /ISSN:1936-2625/IJCEP0073249

Original Article

Downregulation of miR-181a alleviates renal fibrosis in diabetic nephropathy mice

Jia Zhang[1], Cong Wu[2*], Jie Dong[2], Jian Liu[3*], Xiaowen Wei[2]

[1]School of Medicine, Shandong University, Jinan 250012, China; [2]Department of Geriatric Endocrinology, The First Affiliated Hospital of Zhengzhou University, Zhengzhou 450003, China; [3]Zhengzhou University, Zhengzhou 450001, China. [*]Equal contributors.

Received January 22, 2018; Accepted March 8, 2018; Epub August 1, 2018; Published August 15, 2018

Abstract: Accumulating evidence suggests that microRNAs are important regulators in the pathology of diabetes and its relevant renal injures. Little is known about the role of miR-181a in development of diabetic nephropathy. The aim of our present study was to investigate levels of miR-181a in diabetic nephropathy and explore its underlying mechanism. In the present study, Db/db and db/m mice were randomized into groups with 12 mice in each: db/m group, db/db group, and antagomiR-181a-treated db/db group. Changes in renal cortical sections were studied by histopathology. Mouse mesangial cells transfected with miR-mimic or miR-inhibitor and cell growth was measured using MTT assay. Levels of miR-181a expression were detected using qRT-PCR under different conditions. Indexes were measured using qRT-PCR and Western blot. Our results show that downregulation of miR-181a could alleviate pathological changes of diabetic nephropathy in mice. miR-181a expression was significantly upregulated in mouse mesaginal cells *in vitro* ($P<0.05$). Overexpression of miR-181a promoted extracellular matrix under high glucose by measuring related indexes such as collagen I, collagen IV, and fibronectin, which could be reversed by miR-181a inhibitors ($P<0.05$). Upregulation of miR-181a suppressed expression of TβRIII by binding with 3'-UTR. These findings suggest miR-181a plays as an important role in renal fibrosis of diabetic nephropathy in an animal model.

Keywords: Diabetic nephropathy, miR-181a, mouse mesangial cells

Introduction

Diabetes mellitus, commonly referred to as diabetes, is a metabolic disease characterized by hyperglycemia due to insulin-secreting or insulin-action disorders [1]. Persistent hyperglycemia and chronic metabolic disorders can lead to systemic organ dysfunction and failure, especially in the eyes [2], kidneys [3], cardiovascular [4] and nervous [5] systems.

Diabetic nephropathy (DN) is one of the most serious and dangerous chronic complications caused by diabetes. Pathophysiologic abnormalities in diabetic nephropathy begin with long-standing poorly controlled blood glucose levels. This is followed by multiple changes within the glomerulus which include a thickening of the basement membrane, a widening of slit membranes of the podocytes, an increase in the number of mesangial cells, and an accumulation of extracellular matrix proteins [6, 7].

microRNAs (miRNAs) are a class of endogenous non-coding RNAs (about 21-24 nucleotides) that exhibit biological functions by binding to the 3' untranslated region (3'-UTR) of target genes [8]. miRNAs could participate in a series of physiological and pathological processes by inhibiting target gene expressions including cell differentiation, metabolism, proliferation, and apoptosis [9].

Accumulating evidence suggests that miRNAs are important regulators in the pathology of diabetes and its relevant renal injures [10-12]. Recent studies have shown that miR-181a expression was upregulated in diabetes mellitus and inhibition of miR-181a might be a potential new strategy for treating insulin resistance and type 2 diabetes mellitus [13, 14].

Fibroblast growth factor 1 (FGF1) is a prototypic member of the FGF family of proteins, which contains 22 members in humans and in mice

[15]. Recombinant FGF1 could normalize blood glucose levels and restore insulin sensitivity in diabetic rodents [16]. Guang Liang et al. have demonstrated that FGF1 could attenuate development of diabetic nephropathy by preventing inflammatory responses [17].

The aim of our present study was to investigate levels of miR-181a in development of DN and explore its underlying mechanism.

Materials and methods

Animals

C57BL/6 db/db and control db/m mice (8 weeks old, nine each group) were acquired from the laboratory animal center of Zhengzhou University. The animal room was controlled at 20-22°C and 40-60% humidity with a 12 hour light/12 hour dark cycle. Mice were given standard chow and autoclaved water ad libitum. Mouse models of diabetes were constructed by intraperitoneal STZ injections (100 mg/kg) for three consecutive days [18]. Db/db and db/m mice were randomized into groups with 12 mice in each: (1) db/m group, (2) db/db group, and (3) antagomiR-181a-treated db/db group. Tail vein blood glucose levels >300 mg/dl was considered diabetic, measured by Glucose LiquiColor Test (Stanbio Laboratory, Boerne, TX, USA) every 4 weeks. Urinary samples (24 hours) were collected by metabolic cage every 4 weeks and urine albumin concentration was determined by competitive ELISA, according to manufacturer instructions (Exocell, PA). Renal cortical tissues were dissected from kidneys, as previously described [19]. One piece of fresh kidney tissue sample was fixed by neutral formalin and the remaining tissues were stored at -80°C for future analysis. All animal experiments were performed according to institutional, local, and national guidelines on animal research and ethics.

Histological studies

Renal cortical tissues were fixed for 48 hours, dehydrated through a graded series of ethanol, embedded in paraffin wax, and cut into 4 μm sections. The samples were stained with hematoxylin-eosin (HE) and periodic acid Schiff (PAS) to observe glomerular morphological changes. Four random fields were chosen by light microscope.

Cell culture and transfection

293T cells were cultured in DMEM with 5 mmol/l glucose and 10% fetal bovine serum at 37°C in a humidified 5% CO_2 atmosphere. Mouse mesangial cells (MMCs) cultured in DMEM were treated with 25 and 5 mmol/l glucose respectively to mimic diabetic pathological and normal physiological environments, as previously described [20]. For transfection, cells were seeded into plates and transfected with miRNAs or siRNAs (Gene Pharm, Shanghai, China) mixed with Lipofectamine 2000 reagent (Invitrogen, Carlsbad, MA, USA), according to manufacturer protocol.

Cell proliferation assay

Cells (3×10^3) were cultured in 96-well plates, incubated for 24 hours, and stained with 0.5 mg/ml MTT for 4 hours. Supernatant was discarded and 200 μl of dimethylsulfoxide (DMSO) was added to dissolve precipitates. Absorption values were measured at 490 nm.

Real time quantitative PCR analysis

Total RNA was isolated from tissues and cells using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), according to manufacturer protocol. Complementary DNA (cDNA) was synthesized from 50 ng of total RNA using a miRCURY LNA Universal cDNA synthesis kit (Exiqon, Vedbaek, Denmark). β-actin was used as an internal control gene to normalize target genes. All primers were designed and synthesized by GenePharma (Shanghai, China). Relative levels of gene expression were expressed relative to β-actin and calculated using 2-ΔΔCt method.

Luciferase activity assay

The 3' untranslated region (UTR) of FGF1 containing miR-181a binding sites was amplified and cloned into the psiCHECK-2 luciferase vector (Promega, USA). Similarly, mutant 3'-UTR of FGF1 was cloned into the same vector. Cells maintained in 96-well plates were co-transfected with miR-141a mimic or miR-NC. Transfected cells were detected using Dual-Luciferase Reporter Assay System (Promega) 48 hours later.

Western blotting analysis

Cultured cells were washed with ice-cold PBS and lysed in RIPA buffer supplemented with

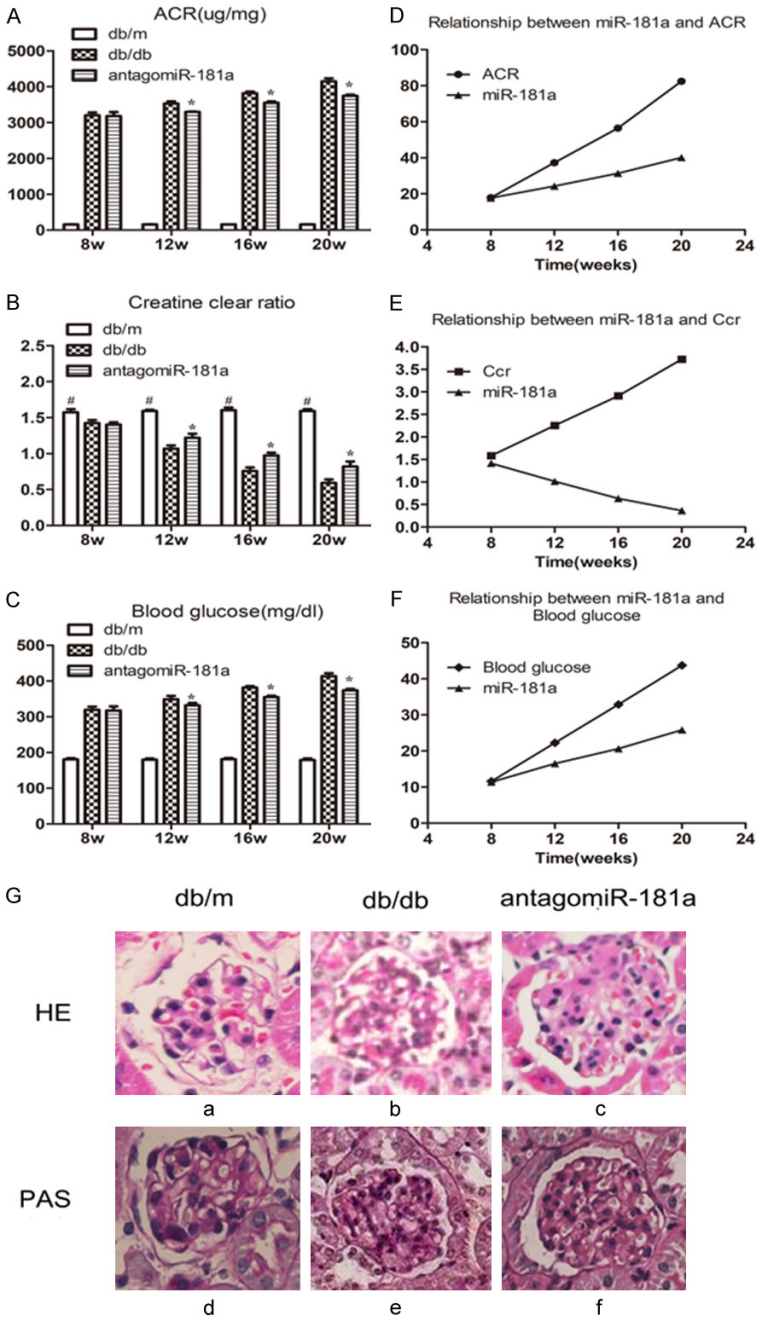

Figure 1. Role of miR-181a in renal function and pathological changes of DN in db/db mice. A, B: The ACR increased and the Ccr decreased significantly with the progression of DN. When db/db mice were injected with antagomiR-181a, there was a significant decrease of ACR and an increase in Ccr (P<0.05). C: The level of blood glucose was upregulated remarkably in db/db mice, which was significantly improved by antagomiR-181a (P<0.05). D-F: miR-181a expression was positively correlated with ACR and blood glucose (r=0.689, r=0.759; P<0.05) and negatively correlated with Ccr (r=-0.685, P<0.05). G: Specimens showed increased mesangial matrix in db/db mice and the degree of pathological changes was lightened when db/db mice received antagomiR-181a treatment.

protease inhibitor mixture. After electrophoresis, the protein samples were incubated with primary antibody (anti-FGF1, anti-Collagen I, anti-Collagen IV, and anti-fibronectin, 1:1000 dilution). Samples were incubated with secondary antibodies conjugated by HRP. Bands were quantified using ImageJ software.

Statistical analysis

Results are expressed as mean ± standard error. Student's t-test and ANOVA were performed among the different groups. All calculations were performed using SPSS 17.0 software (IBM Software, Chicago, IL, USA) and GraphPad (vision 6.0, USA). Corr-elation analysis of continuous variables was based on Spearman's test correlation method. A value of P<0.05 indicated a statistically significant difference.

Results

Role of miR-181a in renal function and pathological changes of DN in db/db mice

ACR and Ccr were considered clinical indexes of renal function in DN [11, 21]. In order to examine miR-181a function in progression of DN, expression of these indexes was analyzed using qPCR. Results showed that ACR increased and Ccr decreased significantly with progression of DN (P<0.05) (**Figure 1A**, **1B**). When db/db mice were injected with antagomiR-181a, there was a significant decrease of ACR and increase in Ccr (P<0.05) (**Figure 1A**, **1B**). At the same time, we also detected changes in blood glucose. The data of our present study demonstrated that levels of blood glucose were upregulated remarkably in db/db mice, significantly improved by antagomiR-181a (P<0.05) (**Figure 1C**). Overall,

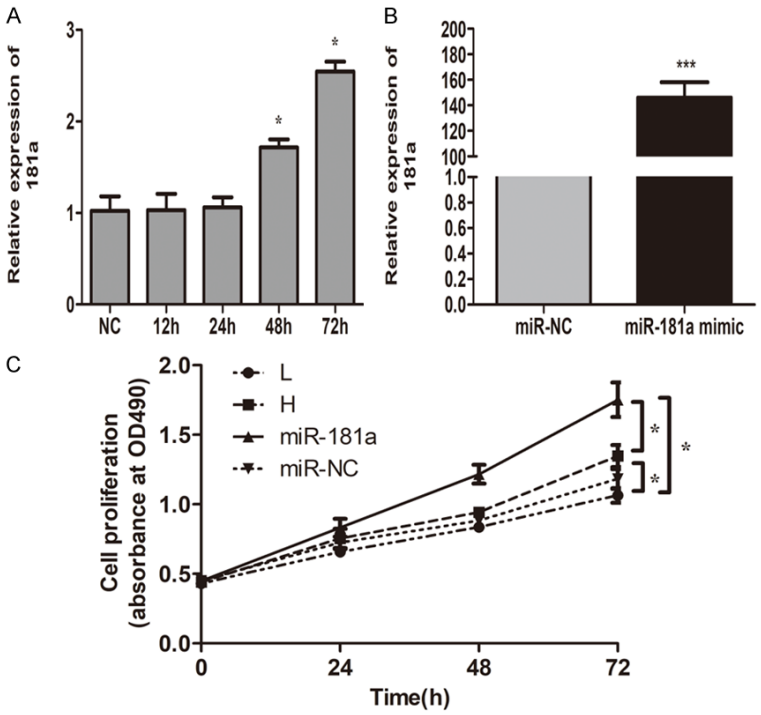

Figure 2. Expression of miR-181a in MMCs under high glucose conditions. MMCs were induced by high glucose (25 mM) for 12, 24, 48, and 72 hours. A: Showed that miR-181a mRNA expression was significantly upregulated in MMCs under high glucose conditions. B: miR-181a level was significantly elevated in MMCs transduced with miR-181a mimic compared with miR-NC (P<0.001). C: The growth rate of MMCs in high glucose conditions was increased, which could be further elevated by BANCR over-expression (P<0.05).

miR-181a expression was positively correlated with ACR and blood glucose (r=0.689, r= 0.759; P<0.05) (**Figure 1D**, **1E**) and negatively correlated with Ccr (r=-0.685, P<0.05) (**Figure 1F**). To characterize the role of miR-181a in renal fibrosis in DN, specimens obtained from renal cortex at 20 weeks showed increased mesangial matrix in db/db mice. PAS and HE staining also showed the degree of pathological changes as they were lightened when db/db mice received antagomiR-181a treatment (**Figure 1G**).

Expression of miR-181a in MMCs under high glucose condition

To further investigate effects of miR-181a on development of DN, expression of miR-181a in MMCs was analyzed by RT-qPCR. MMCs were induced by high glucose (25 mM) for 12, 24, 48, and 72 hours. **Figure 2A** shows that miR-181a mRNA expression was significantly upregulated in MMCs under high glucose conditions, implying the elevated level of miR-181a mRNA induced by high glucose was time-dependent. Moreover, miR-18-1a-overexpression in MMCs was firstly established by retrovirus infection. Results of qRT-PCR manifested that miR-181a levels were significantly elevated in MMCs transduced with miR-181a mimic compared with miR-NC (P<0.001) (**Figure 2B**). To explore the role of miR-181a in cell proliferation, MMT assay was performed to evaluate viability of MMCs under high and low glucose conditions, which mimic diabetic pathological and normal physiological environments, respectively. The results indicated that the growth rate of MMCs in high glucose conditions was increased, which could be further elevated by BANCR overexpression (P< 0.05) (**Figure 2C**).

miR-181a promotes extracellular matrix accumulation in vitro

As major components of ECM in MMCs, expressions of collagen I, collagen IV, and fibronectin were detected to predict the accumulation of ECM under high glucose conditions by RT-PCR and Western blot. The results indicated that expression of these indexes mRNA was significantly increased in MMCs (P<0.05) (**Figure 3A**). Additionally, overexpression of miR-181a elevated levels of these indexes (P<0.05) (**Figure 3D**). This means that overexpression of miR-181a aggravated fibrosis of MMCs. Also, a small interfering RNA (si-RNA) against miR-181a was successfully designed and assessed by qRT-PCR (P<0.05) (**Figure 3B**). Results of qRT-PCR confirmed that expression of these indexes was significantly reduced in MMCs (P<0.05) (**Figure 3C**, **3D**). Taken together, this suggests that miR-181a regulates fibrosis of MMCs *in vitro.*

miR-181a targets with 3'-UTR of FGF1

Bioinformatics analysis was used to predict candidate targets of miR-141. Results revealed

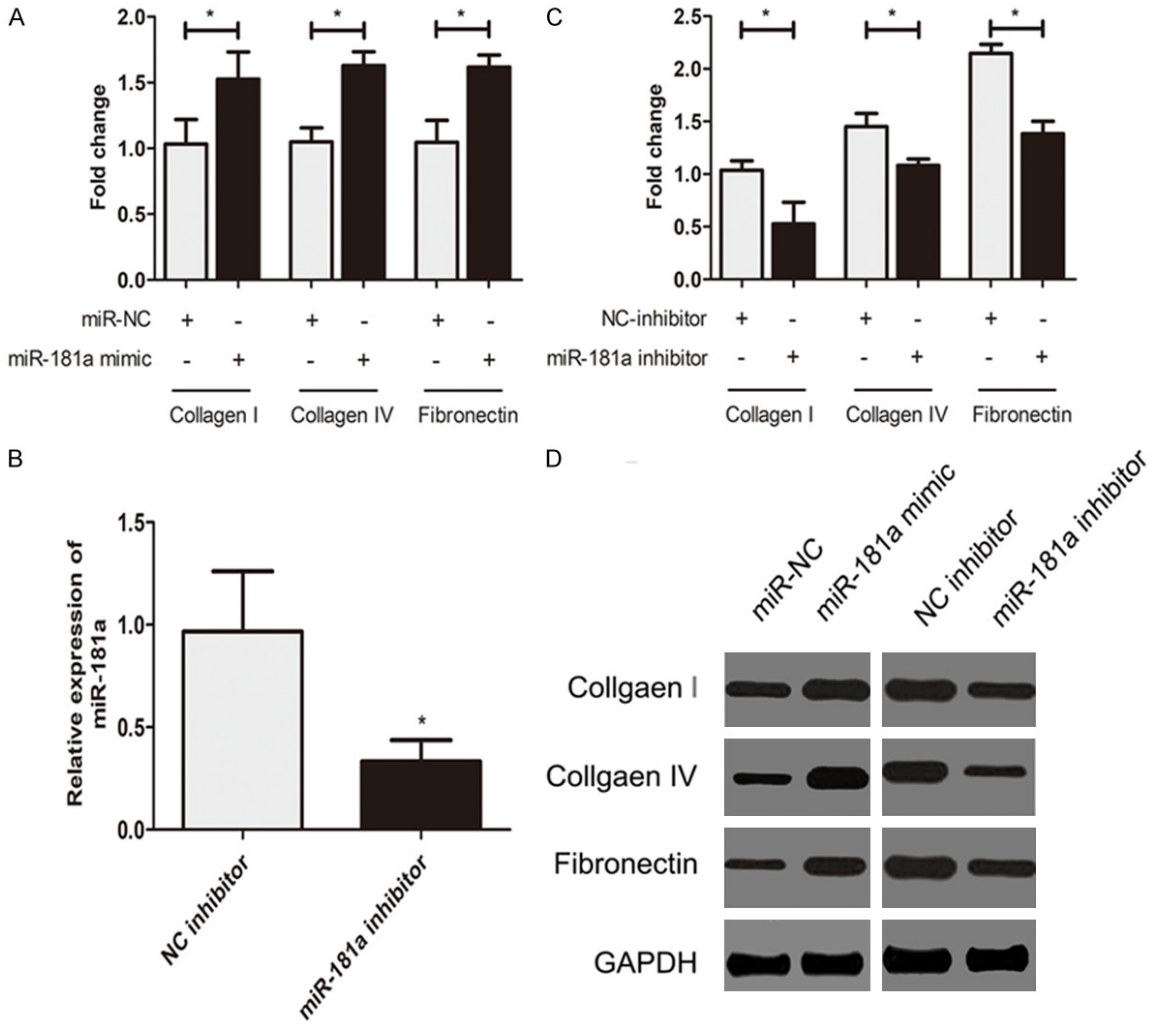

Figure 3. miR-181a promotes extracellular matrix accumulation *in vitro*. A: Expression of collagen I, collagen IV, and fibronectin mRNA was significantly increased in MMCs ($P<0.05$). Meanwhile, overexpression of miR-181a elevated levels of these indexes ($P<0.05$). B: A small interfering RNA (si-RNA) against miR-181a was successfully designed and assessed by qRT-PCR ($P<0.05$). C, D: Expression of these fibrotic indexes was significantly reduced in MMCs ($P<0.05$).

3'UTR of FGF1 was highly conserved to bind with miR-181a. The 3'-UTR binding sites can be seen in **Figure 4A**. Luciferase reporter assay showed transfection of miR-181a could significantly restrict relative luciferase activity in MMCs ($P<0.05$) (**Figure 4B**), suggesting that miR-181a has inhibitory effects on FGF1 expression via interaction with 3'-UTR of FGF1. After cells were transfected with miR-181a mimic, FGF1 expression levels were significantly downregulated ($P<0.05$) (**Figure 4C**). Overall, our study discovered that miR-181a suppressed expression of FGF1 by binding with 3'-UTR.

Discussion

microRNAs (miRNAs) are small non-coding RNA molecules that function in RNA silencing and post-transcriptional regulation of gene expression [22]. miR-181a is derived from an overlapping gene locus that is highly conservative among mammalian species [23]. miR-181a participates in various gene regulatory processes such as development, differentiation, and immune modulation [24]. Zhou, B et al. reported that miR-181a could improve hepatic insulin sensitivity and glucose homeostasis, perhaps providing a potential new therapeutic strategy

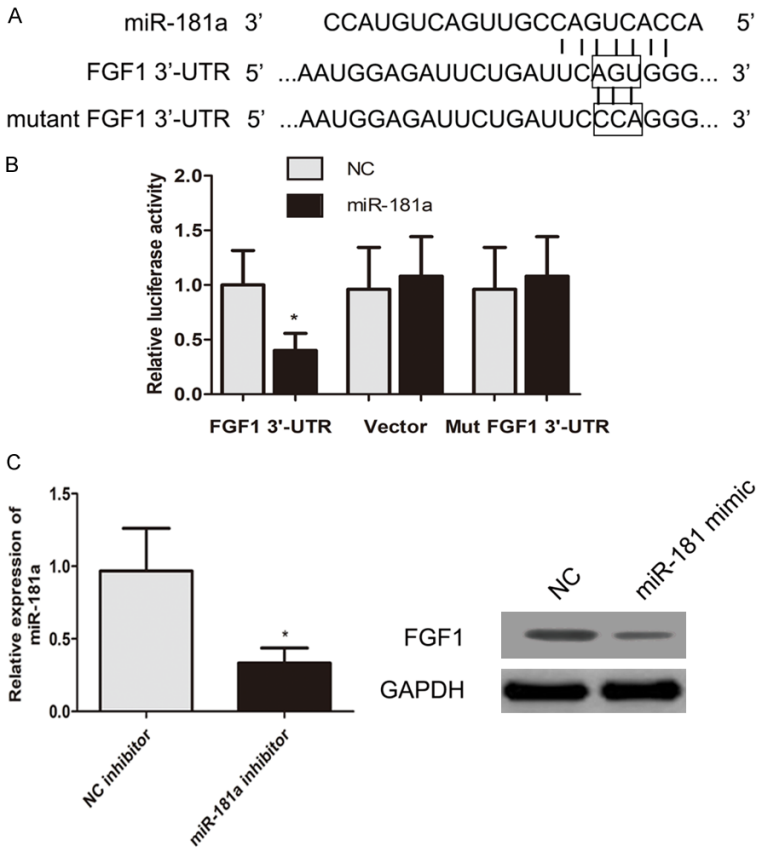

Figure 4. miR-181a targets with 3'-UTR of FGF1. Bioinformatics analysis was used to predict the candidate targets of miR-141. The 3'-UTR binding sites can be seen in A. B: Luciferase reporter assay showed transfection of miR-181a could significantly restrict relative luciferase activity in MMCs ($P<0.05$), suggesting miR-181a has inhibitory effects on FGF1 expression via interaction with the 3'-UTR of FGF1. C: After cells were transfected with miR-181a mimic, FGF1 expression levels were significantly downregulated ($P<0.05$).

for treating insulin resistance and type 2 diabetes [14]. However, to date, few studies have evaluated the role of miR-181a in DN development.

Our present study focused on the role of miR-181a in renal fibrosis of DN in db/db mice. Results showed that miR-181a expression was positively correlated with ACR and negatively correlated with Ccr, indicating the value of miR-181a as a predictor for progression of DN. When db/db mice received antagomiR-181a injections, renal fibrosis histopathological changes were slightly alleviated. miR-181a mRNA expression was significantly upregulated in MMCs under high glucose conditions. MMT assay showed that overexpression of miR-181a accelerated proliferation of MMCs. Furthermore, upregulated miR-181a promoted expression of fibrotic indexes mRNA and protein levels. This was reversed, however, by using knockdown of miR-181a. These data suggest that miR-181a promotes renal fibrosis in development of DN.

FGF-1 belongs to FGF family and was discovered as a mitogen for cultured fibroblasts [25]. FGF-1 has traditionally been believed to be a profibrotic factor promoting fibroblasts proliferation [25, 26]. Nevertheless, strong evidence has demonstrated that FGF-1 has an anti-fibrotic role. FGF-1 could induce fibroblast apoptosis and inhibit type I collagen expression in lung fibrosis [27, 28]. As for diabetic nephropathy, bioinformatics analysis showed miR-181a targets 3'-UTR of FGF1 closely. These results demonstrate that overexpression of miR-181a could lower FGF1 mRNA and related protein production. These data suggest that miR-181a may induce renal fibrosis in DN through modulation of FGF1.

Our present study characterizes the role of miR-181a in the pathogenesis of DN and provides a novel therapeutic target for treatment of DN.

Acknowledgements

This work was supported by The First Affiliated Hospital of Zhengzhou University.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Cong Wu, Department of Geriatric Endocrinology, The First Affiliated Hospital of Zhengzhou University, No. 1 Jianshe East Road, Zhengzhou 450003, China. E-mail: cong0805@qq.com; Dr. Jian Liu, Zhengzhou University, No. 100 Science Road, Zhengzhou 450001, China. E-mail: liujian_MD@163.com

References

[1] Rother KI. Diabetes treatment--bridging the divide. N Engl J Med 2007; 356: 1499-1501.

[2] Zhang X, Zhao L, Deng S, Sun X and Wang N. Dry eye syndrome in patients with diabetes mellitus: prevalence, etiology, and clinical characteristics. J Ophthalmol 2016; 2016: 8201053.

[3] Alicic RZ and Tuttle KR. Novel therapies for diabetic kidney disease. Adv Chronic Kidney Dis 2014; 21: 121-133.

[4] Nakanishi T and Kato S. Impact of diabetes mellitus on myocardial lipid deposition: an autopsy study. Pathol Res Pract 2014; 210: 1018-1025.

[5] Dehghani C, Srinivasan S, Edwards K, Pritchard N, Russell AW, Malik RA and Efron N. Presence of peripheral neuropathy is associated with progressive thinning of retinal nerve fiber layer in type 1 diabetes. Invest Ophthalmol Vis Sci 2017; 58: BIO234-BIO239.

[6] Wang M, Wang S, Yao D, Yan Q and Lu W. A novel long non-coding RNA CYP4B1-PS1-001 regulates proliferation and fibrosis in diabetic nephropathy. Mol Cell Endocrinol 2016; 426: 136-145.

[7] Tervaert TW, Mooyaart AL, Amann K, Cohen AH, Cook HT, Drachenberg CB, Ferrario F, Fogo AB, Haas M, de Heer E, Joh K, Noel LH, Radhakrishnan J, Seshan SV, Bajema IM and Bruijn JA. Pathologic classification of diabetic nephropathy. J Am Soc Nephrol 2010; 21: 556-563.

[8] Valencia-Sanchez MA, Liu J, Hannon GJ and Parker R. Control of translation and mRNA degradation by miRNAs and siRNAs. Genes Dev 2006; 20: 515-524.

[9] Rigoutsos I and Furnari F. Gene-expression forum: decoy for microRNAs. Nature 2010; 465: 1016-1017.

[10] Conserva F, Pontrelli P, Accetturo M and Gesualdo L. The pathogenesis of diabetic nephropathy: focus on microRNAs and proteomics. J Nephrol 2013; 26: 811-820.

[11] Wang J, Gao Y, Ma M, Li M, Zou D, Yang J, Zhu Z and Zhao X. Effect of miR-21 on renal fibrosis by regulating MMP-9 and TIMP1 in kk-ay diabetic nephropathy mice. Cell Biochem Biophys 2013; 67: 537-546.

[12] Zhang L, He S, Guo S, Xie W, Xin R, Yu H, Yang F, Qiu J, Zhang D, Zhou S and Zhang K. Downregulation of miR-34a alleviates mesangial proliferation in vitro and glomerular hypertrophy in early diabetic nephropathy mice by targeting GAS1. J Diabetes Complications 2014; 28: 259-264.

[13] Collares CV, Evangelista AF, Xavier DJ, Rassi DM, Arns T, Foss-Freitas MC, Foss MC, Puthier D, Sakamoto-Hojo ET, Passos GA and Donadi EA. Identifying common and specific microRNAs expressed in peripheral blood mononuclear cell of type 1, type 2, and gestational diabetes mellitus patients. BMC Res Notes 2013; 6: 491.

[14] Zhou B, Li C, Qi W, Zhang Y, Zhang F, Wu JX, Hu YN, Wu DM, Liu Y, Yan TT, Jing Q, Liu MF and Zhai QW. Downregulation of miR-181a upregulates sirtuin-1 (SIRT1) and improves hepatic insulin sensitivity. Diabetologia 2012; 55: 2032-2043.

[15] Itoh N and Ornitz DM. Evolution of the Fgf and Fgfr gene families. Trends Genet 2004; 20: 563-569.

[16] Holmes D. Therapy: FGF1 restores blood glucose levels and insulin sensitivity in diabetic mice. Nat Rev Endocrinol 2014; 10: 576.

[17] Liang G, Song L, Chen Z, Qian Y, Xie J, Zhao L, Lin Q, Zhu G, Tan Y, Li X, Mohammadi M and Huang Z. Fibroblast growth factor 1 ameliorates diabetic nephropathy by an anti-inflammatory mechanism. Kidney Int 2018; 93: 95-109.

[18] Yokoi H, Mukoyama M, Mori K, Kasahara M, Suganami T, Sawai K, Yoshioka T, Saito Y, Ogawa Y, Kuwabara T, Sugawara A and Nakao K. Overexpression of connective tissue growth factor in podocytes worsens diabetic nephropathy in mice. Kidney Int 2008; 73: 446-455.

[19] Putta S, Lanting L, Sun G, Lawson G, Kato M and Natarajan R. Inhibiting microRNA-192 ameliorates renal fibrosis in diabetic nephropathy. J Am Soc Nephrol 2012; 23: 458-469.

[20] Mahimainathan L, Das F, Venkatesan B and Choudhury GG. Mesangial cell hypertrophy by high glucose is mediated by downregulation of the tumor suppressor PTEN. Diabetes 2006; 55: 2115-2125.

[21] Viberti G and Wheeldon NM. Microalbuminuria reduction with valsartan in patients with type 2 diabetes mellitus: a blood pressure-independent effect. Circulation 2002; 106: 672-678.

[22] Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004; 116: 281-297.

[23] Pichler M, Winter E, Ress AL, Bauernhofer T, Gerger A, Kiesslich T, Lax S, Samonigg H and Hoefler G. miR-181a is associated with poor clinical outcome in patients with colorectal cancer treated with EGFR inhibitor. J Clin Pathol 2014; 67: 198-203.

[24] Tekirdag KA, Korkmaz G, Ozturk DG, Agami R and Gozuacik D. MIR181A regulates starvation- and rapamycin-induced autophagy through targeting of ATG5. Autophagy 2013; 9: 374-385.

[25] Gospodarowicz D and Moran JS. Stimulation of division of sparse and confluent 3T3 cell popu-

lations by a fibroblast growth factor, dexamethasone, and insulin. Proc Natl Acad Sci U S A 1974; 71: 4584-4588.

[26] Armelin HA. Pituitary extracts and steroid hormones in the control of 3T3 cell growth. Proc Natl Acad Sci U S A 1973; 70: 2702-2706.

[27] Becerril C, Pardo A, Montano M, Ramos C, Ramirez R and Selman M. Acidic fibroblast growth factor induces an antifibrogenic phenotype in human lung fibroblasts. Am J Respir Cell Mol Biol 1999; 20: 1020-1027.

[28] Ramos C, Montano M, Becerril C, Cisneros-Lira J, Barrera L, Ruiz V, Pardo A and Selman M. Acidic fibroblast growth factor decreases alpha-smooth muscle actin expression and induces apoptosis in human normal lung fibroblasts. Am J Physiol Lung Cell Mol Physiol 2006; 291: L871-879.