MicroRNA-181 exerts an inhibitory role during renal fibrosis by targeting early growth response factor-1 and attenuating the expression of profibrotic markers

XIAOYAN ZHANG[1], ZHENNING YANG[2], YANYAN HENG[1] and CONGXIU MIAO[3]

[1]Department of Nephrology, Heping Hospital Affiliated to Changzhi Medical College, Changzhi, Shanxi 046000; [2]School of Clinical Medicine, Norman Bethune Health Science Center of Jilin University, Changchun, Jilin 130022; [3]Department of Scientific Research, Changzhi Medical College, Changzhi, Shanxi 046000, P.R. China

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Abstract. Progressive renal fibrosis is a common complication of chronic kidney disease that results in end-stage renal disorder. It is well established that several microRNAs (miRs) function as critical regulators implicated in fibrotic diseases. However, the role of miR-181 in the development and progression of renal fibrosis remains unclear, and the precise mechanism has not yet been fully defined. The present study identified the functional implications of miR-181 expression during renal fibrosis. miR-181 exhibited significantly reduced expression in the serum of renal fibrosis patients and in the kidneys of mice with unilateral ureteral obstruction (UUO). In addition, miR-181 downregulated the expression of human α-smooth muscle actin (α-SMA) in response to angiotensin II stimulation. Transfection with miR-181 mimics significantly suppressed the expression levels of α-SMA, connective tissue growth factor, collagen type I α1 (COL1A1) and collagen type III α1 (COL3A1) in NRK49F cells. Notably, early growth response factor-1 (Egr1) was identified as a direct target gene of miR-181. Furthermore, *in vivo* experiments revealed that treatment with miR-181 agonist strongly rescued kidney impairment induced by UUO, as supported by Masson's trichrome staining of kidney tissues and reverse transcription-quantitative polymerase chain reaction analysis of COL1A1 and COL3A1 mRNA levels. Therefore, miR-181 may be regarded as an important mediator in the control of profibrotic markers during renal fibrosis via binding to Egr1, and may be a promising new target in the diagnosis and therapy of renal fibrosis.

Correspondence to: Dr Congxiu Miao, Department of Scientific Research, Changzhi Medical College, 161 Liberation East Street, Changzhi, Shanxi 046000, P.R. China
E-mail: miaocongxiu3264@163.com

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Introduction

Chronic kidney disease is a common cause of morbidity and mortality, and has a prevalence of 8-16% worldwide (1). It is well established that renal fibrosis, considered as the final stage of chronic kidney disease, leads to the impairment of kidney function (2,3). Myofibroblast accumulations, excessive deposition of extracellular matrix (ECM) and renal tubule destruction have been demonstrated to be typical hallmarks of tubule interstitial fibrosis (4,5). Although recent advances in functional genomics have contributed to the understanding of the pathophysiology of renal fibrosis, definitive therapies are not yet available (5-7). Therefore, the absence of a precise target and therapeutic strategy renders this disease a considerable challenge.

Recent studies have demonstrated an association between the expression of microRNAs (miRNAs or miRs) and fibrotic diseases. miRNAs are a class of short noncoding RNAs that regulate gene expression via post-translational modification and induction of mRNA degradation, thus affecting numerous molecular and cellular processes (8-12). Several miRNAs, including miR-29, miR-192 and miR-21, have been reported to be associated with fibrotic processes in various diseases (13-15). These miRNAs can mediate transforming growth factor-β1 (TGF-β1) signaling in renal cells, and normalization of their expression can alleviate fibrosis *in vitro* and *in vivo*, thus making miRNAs potential targets for therapy of renal fibrosis (15).

TGF-β1, as a major profibrotic agent, can trigger renal fibrosis, and blockade of TGF-β1 suppresses progressive kidney fibrosis (16,17). TGF-β1 can induce the transcription of early growth response factor-1 (Egr1), which has been verified to be correlated with excessive production of ECM (18,19). Egr1, a zinc-finger transcription factor that encodes an 80-82 kDa protein targeting a GCGGGGGCG binding site, regulates downstream gene transcription and controls diverse cellular processes, such as cell growth, proliferation and differentiation. It has been reported that Egr1 serves a critical role during the fibrotic process (20-22). However, the function of miR-181 in renal fibrosis remains largely unknown, and the association between miR-181 and Egr1 is not fully understood.

The present study aimed to investigate the role of miR-181 *in vitro* and *in vivo*, and to further explore its underlying mechanism in renal fibrosis. It was observed that miR-181 functioned as an important factor in the modulation of the fibrotic process in the kidneys, and Egr1 was identified as a potential target of miR-181. Thus, the miR-181/Egr1 signaling pathway may serve as a novel target for the diagnosis and prognosis of renal fibrosis.

Materials and methods

Clinical samples. A total of 58 patients with renal fibrosis (46 males and 12 females) with a mean age of 51.2±6.9 years (age range, 43.5-65.1 years), who were admitted to the Heping Hospital Affiliated to Changzhi Medical College (Changzhi, China) to undergo therapy, were enrolled into the present study. All patients were diagnosed with renal fibrosis by biopsy (23). In addition, 10 normal control subjects (8 males and 2 females) with a mean age of 54.9±7.1 years were included in the study. Blood samples were collected from the subjects and centrifuged at 1,000 x g for 10 min at 4˚C to separate the serum, which was stored at -80˚C for subsequent assays. The present study was approved by the Ethics Committee of Heping Hospital Affiliated to Changzhi Medical College, and written informed consent was provided by all the participants in the study.

Experimental animals. A unilateral ureteral obstruction (UUO) kidney disease model was established in C57Bl/6J mice (Jackson Laboratory, Bar Harbor, ME, USA) by left ureteral ligation as previously described (18). These mice were housed five per cage under the following conditions: Constant temperature, 22˚C; humidity, 35-75%; free access to food and water; 12-h light/dark cycle. Briefly, male mice (age, 10-12 weeks; weight, 30-40 g) were randomly divided into three groups, as follows: Sham-operated group (n=6), in which mice underwent the same surgical procedure as the observation group, with the exception of ureteral ligation; UUO model group (n=8), in which mice with UUO were injected with the negative control (NC) vector (Biomics Biotechnologies, Guangzhou, China); and observation group (n=8), in which mice with UUO were injected with a miR-181 agonist (Biomics Biotechnologies). The NC vector and miR-181 agonist were administered 1 week after surgery. Mice were sacrificed 14 days after injection, and their kidney tissues were collected and processed to evaluate the expression levels of collagen type I α1 (COL1A1), collagen type III α1 (COL3A1), α-smooth muscle actin (α-SMA) and connective tissue growth factor (CTGF) by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). All the procedures were performed in accordance with national and international laws and policies, and were approved by the Heping Hospital Affiliated to Changzhi Medical College Animal Care and Use Ethics Committee.

Masson's trichrome staining. Changes in renal morphology were examined with Masson's trichrome staining. Briefly, the kidney tissues were fixed in 4% paraformaldehyde solution for 6-8 h at room temperature, fully automatically dehydrated, paraffin embedded and sliced into 4-μm tissue sections. The slices were then stained with Masson's trichrome (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) according to the manufacturer's protocol.

Bioinformatics analysis. The targets and binding sites of miR-181 were predicted using numerous online databases with different algorithms, TargetScan (http://www.targetscan.org/).

Cell culture and transfection. Rat kidney NRK49F cells (American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA), supplemented with 10% fetal calf serum (Gibco; Thermo Fisher Scientific, Inc.), 1% glutamine (Gibco; Thermo Fisher Scientific, Inc.), 1% nonessential amino acids (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin and streptomycin (Gibco; Thermo Fisher Scientific, Inc.) in a humidified incubator containing 5% CO_2 at 37˚C. Angiotensin II (10 μM; AngII; R&D Systems, Inc., Minneapolis, MN, USA) was used to stimulate the cells, in order to establish a fibrosis model (14). Subsequently, NRK49F cells ($5x10^5$ cells/well) were transfected with 30 nM miR-181 mimics or NC miRNA mimics (both Ambion; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in 6-well plates using siPORT NeoFX Transfection Agent (Ambion; Thermo Fisher Scientific, Inc.), in accordance with the manufacturer's protocol. Following transient transfection for 24 h, the cells were synchronized by culture in low-glucose medium without serum for 24 h.

RNA extraction and RT-qPCR. Total RNA from human blood samples, mouse renal tissues and NRK49F cells was extracted using TRIzol® reagent (Zoonbio Biotechnology Co., Ltd., Nanjing, China) in accordance with the manufacturer's protocol. RNA concentration was measured using a spectrophotometer (NanoDrop® ND-1000; NanoDrop Technologies; Thermo Fisher Scientific, Inc., Wilmington, DE, USA); RNA with an optical density (OD)260/OD280 range between 1.8 and 2.0 was used. Subsequently, cDNA was synthesized using the TransScript miRNA RT Enzyme Mix (TransGen Biotech Co., Ltd., Beijing, China), according to the manufacturer's protocol, as follows: RT at 50˚C for 60 min and inactivation of reverse transcriptase at 70˚C for 15 min. For the detection of miR-181 expression, a miRNA-specific TaqMan MicroRNA assay (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used. U6 was used as an internal control for normalization of miRNA expression. GAPDH was used as an internal control for normalization of mRNA expression. For the detection of mRNA expression, SYBR Premix Ex Taq™ II assay (Takara Biotechnology Co., Ltd., Dalian, China) was conducted using a 20 μl reaction volume, under the following conditions: First step, initial denaturation at 95˚C for 30 sec; second step, denaturation at 95˚C for 30 sec and primer annealing at 60˚C for 30 sec, this step was repeated 35 times. The experiments were performed in triplicate. Primer sequences are provided in Table I. The $2^{-\Delta\Delta Cq}$ method was used to calculate the results (23).

Western blotting. Transfected NRK49F cells were collected according to the previously described protocols (14). Subsequently, western blot analysis was conducted as previously described (24). Total protein concentration was quantified using a spectrophotometer (NanoDrop® ND-1000;

Table I. Primer information.

Gene	Sequence	Primer length (bp)	Annealing temperature (˚C)
miR-181	Forward, 5'-GTGGATCCGACATTCATTTGAGTCTGCTTGT-3'	31	62.0
	Reverse, 5'-GCGAATTCTCATCATGGACTGCTCTTAC-3'	28	60.2
Egr1	Forward, 5'-GCAGGCTCGCTCCCACGGTC-3'	20	62.0
	Reverse, 5'-GGGGTTGGCCGGGTTACATG-3'	20	58.8
U6	Forward, 5'- GCTTCGGCAGCACATATACTAAAAT-3'	25	54.6
	Reverse, 5'-CGCTTCACGAACCGCGTGTCA-3'	21	58.3
α-SMA	Forward, 5'-CATCACGAACTGGGATGACATG-3'	22	55.8
	Reverse, 5'-CATCTTCTCCCTGTTGGCTTTAG-3'	23	55.1
CTGF	Forward, 5'-TCCTTTCTGAGCAATTCACCAAG-3'	23	60.7
	Reverse, 5'-GCACACTCCGTCTTTTTCCTC-3'	21	61.2
COL1A1	Forward, 5'-GAGGGCCAAGACGAAGACATC-3'	21	62.5
	Reverse, 5'-CAGATCACGTCATCGCACAAC-3'	21	61.6
COL3A1	Forward, 5'-GGAGCTGGCTACTTCTCGC-3'	19	62.2
	Reverse, 5'-GGGAACATCCTCCTTCAACAG-3'	21	60.0
GAPDH	Forward, 5'-TGTGGGCATCAATGGATTTGG-3'	21	60.9
	Reverse, 5'-ACACCATGTATTCCGGGTCAAT-3'	22	61.4

α-SMA, α-smooth muscle actin; Egr1, early growth response factor-1; COL1A1, collagen type I α1; COL3A1, collagen type III α1; CTGF, connective tissue growth factor; miR, microRNA.

NanoDrop Technologies; Thermo Fisher Scientific, Inc.) to ensure that sample quantity was consistent. Subsequently, proteins were denatured at 95˚C for 15 min in a water bath. The proteins (20 μg/well) were separated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane at 200 mA for 3 h. The membranes were blocked with 5% skimmed milk powder for 2 h at room temperature, after which, they were incubated with the primary antibody overnight at 4˚C. Anti-Egr1 was used as the primary antibody (1:1,000; cat. no. MAB2818; R&D Systems, Inc.) and GAPDH (1:500; cat. no. AF5718; R&D Systems, Inc.) was used as a protein loading control. Tris Buffered Saline with 0.1% Tween 20 (TBST; cat. no. 28358; Thermo Fisher, Scientific, Inc., Waltham, MA, USA) was used to wash the membranes three times (10 min/wash), and they were then incubated with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:20,000; cat. no. HAF008; R&D Systems, Inc.) at room temperature for 2 h. Finally, the membranes were washed with TBST three times (10 min/wash). The images were developed with SuperSignal West Pico Chemiluminescent Substrate (cat. no. 34080; Pierce; Thermo Fisher Scientific, Inc.) using a ChemiDoc XRS (cat. no. 1708265; Bio-Rad Laboratories, Inc., Hercules, CA, USA), and analyzed with Gel-Pro analyzer (version 4.0, Media Cybernetics, Inc., Rockville, MD, USA).

Dual-luciferase reporter assay. NRK49F cells were plated in a 24-well plate (1x10^6 cells/well), and then co-transfected with a luciferase reporter plasmid [pMIR-Egr1-wild-type (WT) or pMIR-Egr1-mutant (Mut)] together with miR-181 or NC mimics and the pRL-TK vector encoding *Renilla* luciferase (Promega Corporation, Madison, WI, USA) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). After 48 h, the original culture medium was discarded and the cells were washed gently three times with PBS (cat. no. AM9624; Thermo Fisher Scientific, Inc.), after which, 20 μl passive lysis buffer (cat. no. E1910; Promega Corporation) was added and the cells were agitated at room temperature for 20 min. The luciferase activity was analyzed using a Dual-Luciferase Reporter Assay system (Promega Corporation) according to the manufacturer's recommendations.

Statistical analyses. All quantitative data for statistical analyses were obtained from at least three independent experiments. Data are presented as the mean ± standard deviation. Comparisons between two groups were performed using Student's t-test, while paired Student's t-test was used to analyze paired data. Comparisons among three or more groups were performed by analysis of variance test, with Bonferroni correction used as a post hoc test. Statistical analyses were performed with IBM SPSS version 22.0 software (IBM Corp., Armonk, NY, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

miR-181 expression is downregulated in patients and mice with renal fibrosis. To explore whether miR-181 affects the progression of kidney fibrosis, miR-181 expression was detected in clinical blood samples by RT-qPCR. As shown in Fig. 1A, the expression of miR-181 was significantly reduced in the serum of patients with renal fibrosis as compared with the controls. Consistent with this observation, the results of RT-qPCR assay in kidney samples of sham-operated and UUO mice revealed that miR-181 exhibited a markedly lower expression

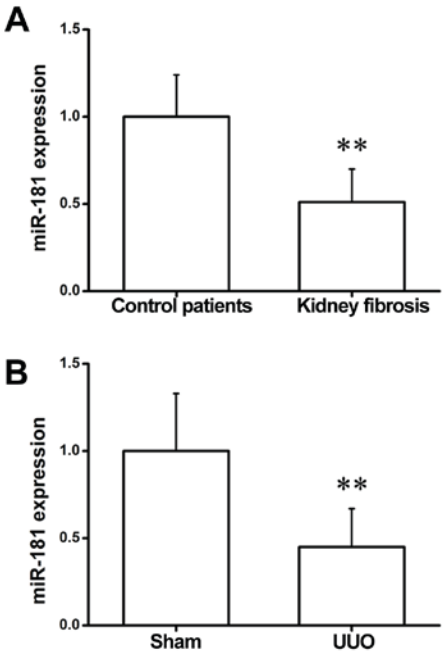

Figure 1. Expression of miR-181 is downregulated in patients and mice with renal fibrosis. (A) Serum miR-181 expression in patients with kidney fibrosis and controls was determined by RT-qPCR assays. (B) miR-181 expression in the kidney of mice in the sham surgery and UUO groups was determined by RT-qPCR. $^{**}P<0.01$ vs. control or sham group. miR, microRNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; UUO, unilateral ureteral obstruction.

in UUO kidneys in comparison with the sham-operated group (Fig. 1B). These observations suggest that miR-181 may serve a critical role in the development of fibrotic responses in the kidney.

miR-181 downregulates α-SMA expression in vitro. NRK49F, a normal rat renal fibroblast cell line, was employed in the present study to gain insights into the functional significance of miR-181 in renal fibrosis. It is well established that AngII contributes to tubule interstitial injury and fibrosis in kidney diseases (25,26). To identify whether the AngII stimulation has an effect on miR-181 expression during renal fibrotic disease, NRK49F cells were exposed to AngII for 36 h. Using RT-qPCR, miR-181 expression was determined and was observed to be significantly reduced in response to AngII treatment in comparison with the control group (Fig. 2A). Additionally, NRK49F cells were transfected with miR-181 mimics (Fig. 2B) and then stimulated with AngII. Compared with the group treated with NC miRNA mimic, the group treated with miR-181 mimic exhibited downregulation of α-SMA protein expression; however, the group treated with AngII exhibited upregulation of α-SMA protein expression; this effect was suppressed by miR-181 (Fig. 2C). These data suggest that miR-181 can suppress α-SMA expression induced by AngII in fibrotic kidney progression.

Egr1 is a direct target of miR-181. The predicted target sequence of miR-181 was evaluated using the accessible TargetScan Human database (http://www.targetscan.org/vert_72/). As shown in Fig. 3A, a potential binding site for miR-181 was identified in the 3'-untranslated region (UTR) of Egr1. To further investigate whether Egr1 is a direct binding target of miR-181, the WT or Mut miR-181 target sequences in the 3'-UTR of Egr1 were fused into a luciferase reporter gene. Subsequently, pMIR-Egr1-WT or pMIR-Egr1-Mut reporter was co-transfected into NRK49F cells together with miR-181 mimics or miR-NC, and a luciferase reporter assay was performed. As presented in Fig. 3B, NRK49F cells transfected with miR-181 mimics exhibited markedly reduced luciferase activity induced by pMIR-Egr1-WT compared with the control vector. However, transfection with miR-181 mimics failed to inhibit the luciferase activity of the Mut reporter gene. Taken together, these observations indicate that miR-181 can directly bind to the 3'-UTR of Egr1.

miR-181 regulates Egr1 expression at the mRNA and protein level. To further investigate whether miR-181 successfully regulated Egr1 expression in NRK49F cells, Egr1 expression in cells transfected with miR-181 mimics or miR-NC was examined. As shown in Fig. 4A, RT-qPCR assay indicated that transfection with miR-181 significantly inhibited the expression of Egr1 at the mRNA level compared with the NC group. In addition, western blot analysis revealed that NRK49F cells transfected with miR-181 mimics exhibited a significant reduction in comparison with the NC group (Fig. 4B). Taken together, these results suggest that miR-181 suppresses Egr1 expression at the mRNA and protein levels in NRK49F cells by directly binding to the 3'-UTR of Egr1.

miR-181 inhibits the progression of renal fibrosis via downregulation of profibrotic markers. A previous study demonstrated that accumulation of α-SMA, collagen and CTGF can contribute to renal fibrosis (27). To investigate the role of miR-181 in the renal fibrotic response, RT-qPCR was conducted to determine the expression levels of α-SMA, CTGF, COL1A1 and COL3A1 in NRK49F cells. As presented in Fig. 5A-D, NRK49F cells transfected with miR-181 mimics exhibited a significant reduction in the expression levels of α-SMA, CTGF, COL1A1 and COL3A1 at the mRNA level as compared with the NC group. These data indicated that miR-181 exerts an inhibitory effect during renal fibrosis by suppressing the levels of profibrotic markers.

Renal fibrosis morphology in UUO mice is associated with miR-181 regulation. Based on the *in vitro* data indicating that miR-181 serves a suppressive role in the progression of renal fibrosis, an *in vivo* study was conducted to validate whether miR-181 is able to mediate the consequent fibrosis. Upon establishment of a UUO model, an miR-181 agonist was delivered into the mice through an injection in the tail vein, and the histopathological morphology of the kidneys was observed by Masson's trichrome staining. As indicated in Fig. 6A, the expected clear structure of renal tubules and glomerulus was identified in the sham-operated mice, with no evident interstitial cell infiltration observed. However, renal glomerular dilatation, and epithelial cell degeneration and necrosis were detected in the UUO mice, although this response was rescued by treatment with miR-181 agonist. The injection of miR-181 agonist into the tail vein of mice in the UUO model improved renal tubular dilatation, and reduced the degeneration and

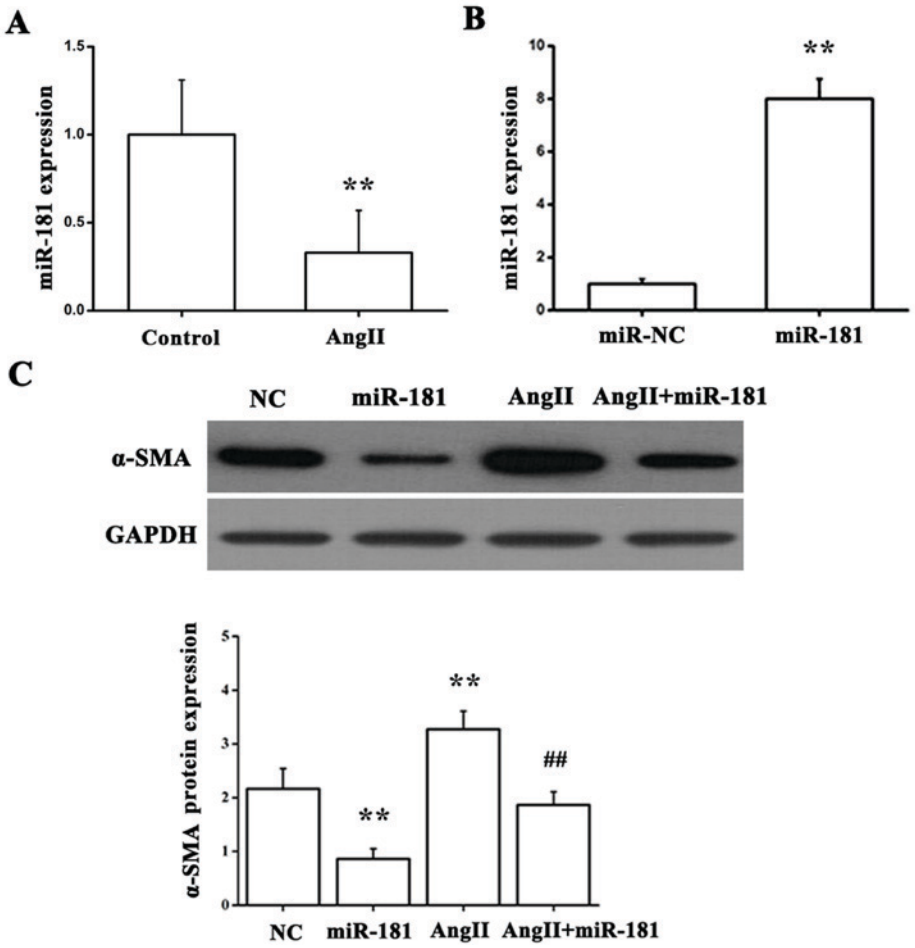

Figure 2. miR-181 downregulates α-SMA expression *in vitro*. (A) miR-181 expression in response to AngII treatment in NRK49F cells was determined by reverse transcription-quantitative polymerase chain reaction assays. The control group cells were treated with PBS. (B) miR-181 overexpression was induced post-transfection with miR-181 mimics. (C) α-SMA protein expression in cells stimulated with AngII, miR-181 and AngII + miR-181 was determined by western blotting. **P<0.01 vs. NC group; $^{\#\#}$P<0.01 vs. AngII group. miR, microRNA; α-SMA, α-smooth muscle actin; AngII, angiotensin II; NC, negative control.

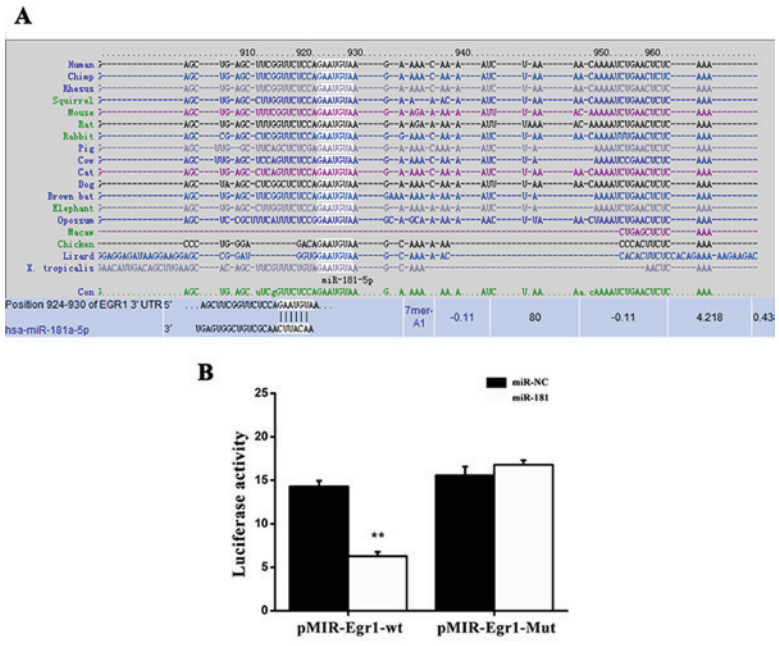

Figure 3. Egr1 is a direct target of miR-181. (A) Predicted target sequence of miR-181 in the 3'-UTR of Egr1. (B) Luciferase activity in cells cotransfected with WT or Mut Egr1 3'-UTR reporter gene and with miR-181 mimics. **P<0.01 vs. pmiR and pmiR-Mut groups. miR, microRNA; UTR, untranslated region; Egr1, early growth response factor-1; WT, wild type; Mut, mutant.

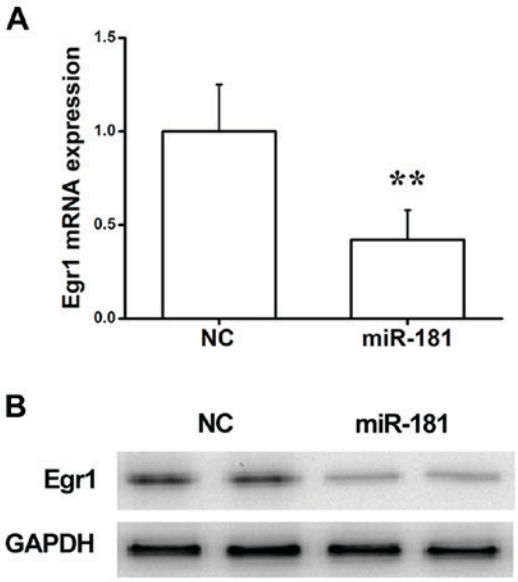

Figure 4. miR-181 regulates Egr1 expression at the mRNA and protein levels. Quantification of (A) Egr1 mRNA levels using reverse transcription-quantitative polymerase chain reaction and (B) Egr1 protein levels using western blotting in NRK49F cells transfected with miR-181 mimics or NC mimics. **P<0.01 vs. NC group. miR, microRNA; Egr1, early growth response factor-1; NC, negative control.

necrosis of epithelial cells, suggesting that miR-181 can alleviate kidney impairment during the fibrotic process *in vivo*. In agreement with previous studies on collagen expression mediated by miR-181, the present study revealed that treatment with miR-181 agonist markedly suppressed COL1A1 and COL3A1 mRNA expression levels in UUO kidneys (Fig. 6B and C), suggesting that miR-181 participates in kidney fibrotic disease via inhibition of collagen deposition.

Discussion

It is widely accepted that renal fibrosis is the final common stage of numerous forms of progressive kidney disease, which leads to impairment of the renal function (28). At present, a definite therapy for renal fibrosis is missing, which indicates that the precise pathogenesis of this fibrotic disease is not yet fully understood. Notably, a series of miRNAs have emerged as critical regulators of numerous cellular processes, such as proliferation, migration, apoptosis, differentiation and cell cycle progression (29-33). Mounting evidence indicated that several miRNAs are implicated in the development of certain renal disorders, including acute kidney impairment (34), diabetic kidney disease (35) and renal fibrosis (36). However, the association between miR-181 and fibrotic kidney disease remains unclear. In the current study, the functional relevance of miR-181 in the fibrotic response in kidney disease was identified. Additionally, miR-181 was able to halt the progression of renal fibrosis in an established UUO model. Targeting Egr1 may be a mechanism by which miR-181 mediates renal injury. Therefore, miR-181 may contribute to the prognosis and therapy of renal fibrosis.

The present study confirmed an association between miR-181 expression and renal fibrosis. It has been demonstrated that aberrant expression of miRNAs is closely associated with inflammatory responses. Previous studies revealed that miRNA profiling of rodents displayed increased expression of miR-181 in aged kidneys, indicating that epigenetic modulation of renal ageing likely occurred via repressing miR-181-targeted genes (37,38). Accumulation of miRNA-433 can drive renal fibrosis by activation of the TGF-β signaling pathway (39). In addition, miRNA-21 has been demonstrated to exhibit a marked increase in expression in UUO kidneys, suggesting that miRNA-21 may exert an important activity during renal fibrosis (40). The present study further revealed that miR-181 is critical for mediating the fibrotic process in experimental models and in human kidney diseases. Using RT-qPCR, the expression of miR-181 was determined in the serum of renal fibrosis patients and in renal tissues of UUO mice. Downregulation of miR-181 expression occurred in patients with renal fibrosis. Consistently, in UUO kidneys, miR-181 displayed a reduced expression, suggesting that miR-181 may be involved in the progression of renal fibrosis. Thus, miR-181 was subjected to further investigation due to its expression signature.

AngII, a key regulator of proteinuria and fibrosis, can contribute to the release of inflammatory and fibrogenic substances, which leads to the fibrotic response. Zanchi *et al* (41) reported that AngII failed to trigger miR-181 expression, since AngII does not exert a direct effect on miR-181. To validate the functional implications of AngII in miR-181 expression, NRK49F cells were exposed to AngII in the current study. The data revealed that miR-181 exhibited a significantly reduced expression in response to AngII, suggesting that AngII is an effective agent for modulating miR-181 expression in rat kidney fibroblasts. Furthermore, western blotting revealed that miR-181 caused a sustained downregulation of α-SMA, which is a characteristic of activation of fibrogenesis (42). Coinciding with the signature of α-SMA, other fibrotic markers, such as collagens (43-45), can be induced to activate a cascade signaling pathway to drive fibrosis. Previous studies have reported that the accumulation of α-SMA, COL1A1, COL3A1 and CTGF promoted renal fibrosis, which was mediated by a miRNA-433-regulated feedback loop of TGF-β signaling (46,47). During the fibrotic process, elevated COL3A1 has been verified in early myocardial remodeling, whereas COL1A1 deposition has been observed at a later stage (48). In the present study, RT-qPCR assay revealed that miR-181 exerted an inhibitory effect on the mRNA expression levels of α-SMA, CTGF, COL1A1 and COL3A1. To support this notion, an established UUO mouse model was used to conduct relevant functional experiments *in vivo*. Masson's trichrome staining indicated that miR-181 rescued the renal impairment induced by ureteral occlusion, as demonstrated by the reduced COL1A1 and COL3A1 levels detected using RT-qPCR analysis. These results emphasize the significance of miR-181 in halting renal fibrosis by downregulation of profibrotic markers.

One important finding of the present study is that miR-181 serves a crucial role in the fibrotic response in kidney disease via directly targeting the Egr1 gene. A recent study reported that Egr1, which is regarded as an intracellular TGF-β target, can induce the expression of type III and IV collagens (49,50). In addition, TGF-β can induce the transcription of genes such as collagens and Egr1 to promote the biological processes that are responsible for fibrosis (51). Notably, miRNAs are able to suppress their targeted genes though binding to their 3'-UTR to induce inhibition of protein translation or

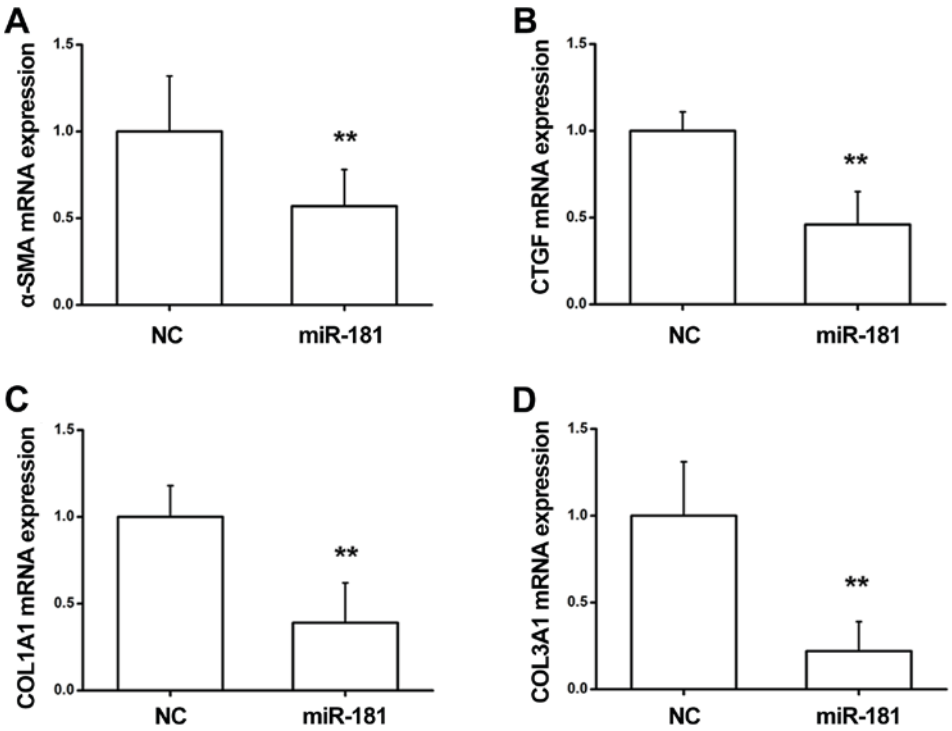

Figure 5. miR-181 inhibits the progression of renal fibrosis via downregulation of profibrotic markers. mRNA expression levels of (A) α-SMA, (B) CTGF, (C) COL1A1 and (D) COL3A1 in NRK49F cells transfected with miR-181 mimics and NC miRNA mimics. **P<0.01 vs. NC group. miR, microRNA; NC, negative control; α-SMA, α-smooth muscle; CTGF, connective tissue growth factor; COL1A1, collagen type I α1; COL3A1, collagen type III α1.

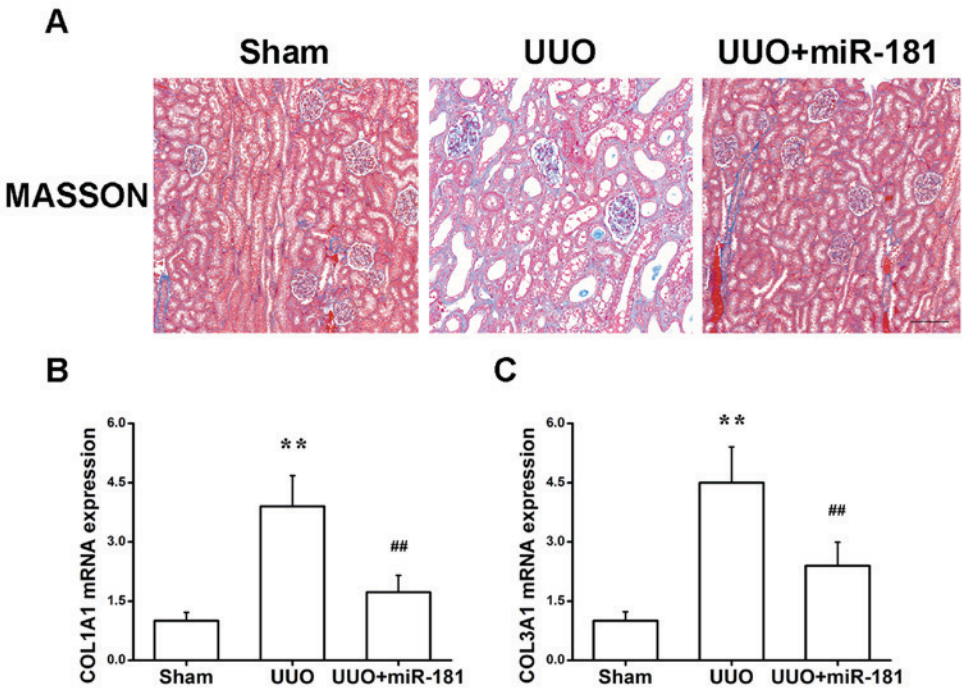

Figure 6. Renal fibrosis morphology in UUO mice is associated with miR-181 regulation. An UUO mouse model was established, and an miR-181 agonist was injected into the tail vein of the mice. (A) Masson's trichrome staining of the kidney in different groups of mice. Scale bar, 50 μm; magnification, x20. (B) COL1A1 and (C) COL3A1 mRNA expression levels in mice. **P<0.01 vs. Sham group; $^{\#\#}$P<0.01 vs. UUO group. miRNA, microRNA; UUO, unilateral ureteral obstruction.

mRNA degradation (52). To further investigate the molecular mechanism by which miR-181 exerted an inhibitory activity during renal fibrosis, the two putative miR-181 target sites in the 3'-UTR of Egr1 were screened, and the results of luciferase reporter assay indicated that miR-181 directly targeted the 3'-UTR of Egr1. Furthermore, the mRNA and protein levels of Egr1 were attenuated by miR-181 compared with the control group, suggesting that miR-181 negatively

regulated Egr1 by directly binding to the Egr1 gene. These *in vitro* and *in vivo* data clearly demonstrated that miR-181 downregulated profibrotic indicators during renal fibrosis by targeting Egr1.

Egr1 is a generally expressed member of the zinc-finger family of transcription factors, which has been reported to be a potential regulator of the connective tissue factor CCN family (including the cysteine-rich 61, CTGF and nephroblastoma overexpressed members) (53). In addition, $Egr1^{-/-}$ mice exhibited a deficiency in the expression of tendon genes, including scleraxis, COL1A1 and COL1A2, where Egr1 was recruited to the COL1A1 and COL2A1 promoters, and affected their expression (54). In the current study, miR-181 negatively regulated Egr1 by directly binding to the Egr1 gene, and the mRNA expression levels of α-SMA, CTGF, COL1A1 and COL3A1 were significantly reduced, suggesting that Egr1 may serve a regulatory role in the transcription of α-SMA, CTGF, COL1A1 and COL3A1. However, future experiments are required to verify this hypothesis.

In conclusion, the present study has provided direct evidence to demonstrate that miR-181 serves an important role during renal fibrosis by targeting Egr1. By combining *in vitro* and *in vivo* data, the present study demonstrated that miR-181 serves a role as a downstream regulator of profibrotic markers to alleviate fibrotic kidney processes. These observations indicate that targeting miR-181 may exert an antifibrotic effect in kidney disease. However, whether miR-181 is a biomarker or serves an additional role in the progression of renal fibrosis requires further investigation.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XZ designed and supervised the study, performed experiments, analyzed the data, and wrote and edited the manuscript. CM, ZY and YH performed the experiments. CM and XZ analyzed the data and drafted the manuscript.

Ethics approval and consent to participate

Written informed consent was obtained from all patients and the experiment was approved by the Ethics Committee of Heping Hospital Affiliated to Changzhi Medical College. All animal experiments were performed in accordance with national and international laws and policies, and were approved by the Animal Experimental Ethics Committee, Heping Hospital Affiliated to Changzhi Medical College.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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