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Pioglitazone attenuates kidney fibrosis via miR-21-5p modulation

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ABSTRACT

Pioglitazone has been demonstrated to exert anti-fibrotic and renoprotective effects. But the detailed pharmacological mechanisms have not been clearly revealed. The present study aimed to investigate the possible mechanisms of pioglitazone in these two effects. TGF-β1-stimulated HK-2 cells and unilateral ureteral obstruction (UUO) mice were used as in vitro and in vivo models. The results showed that pioglitazone inhibited Smad-2/3 phosphorylation, upregulated Smad-7 expression and downregulated miR-21-5p expression in TGF-β1-exposed HK-2 cells. In addition, miR-21-5p inhibitors replicated the anti-fibrotic effects of pioglitazone, and miR-21-5p mimics inhibited these effects. In in vivo study, pioglitazone attenuated UUO-induced renal fibrosis and significantly decreased the expressions of pro-fibrotic proteins. Whereas, agomir of miR-21-5p inhibited the renoprotective function of pioglitazone in UUO mice. In conclusion, the present data suggest that modulation of miR-21-5p/Smad-7 signal may be involved in the anti-fibrotic effect of pioglitazone in the kidney of UUO mice.

1. Introduction

Chronic kidney disease (CKD) has become a major life-threatening disease worldwide [1,2]. It is a complex process which involves large numbers of independent and overlapping signaling pathways. Renal fibrosis is a final common outcome of CKD [3,4]. The degree of renal fibrosis is closely related with the stage of CKD [5]. Pioglitazone, a type of peroxisome proliferator-activated receptor-γ (PPARγ) agonist, is a widely used anti-diabetic drug. Besides glucose lowering in diabetes treatment, it has been found to exhibit renal protective and anti-fibrotic functions in the diseases that are either associated with diabetes or not [12–15]. However, the pharmacological mechanisms of the renoprotective actions of pioglitazone have not been clearly revealed.

Renal fibrosis is characterized by extensive accumulation of extracellular matrix (ECM) and activation of myofibroblasts. Numerous cytokines, including members of the transforming growth factor-β (TGF-β) superfamily, are involved in this pathological process. TGF-β1 is a key pro-fibrotic cytokine in various organs including kidney [6–8]. It mediates fibrosis by increasing ECM accumulation and inducing epithelial mesenchymal transition [9]. The Smad signaling pathway is the well-known downstream signaling of TGF-β1 and mediates majority of biological effects of TGF-β1 [10]. Smad 2 and Smad 3 are phosphorylated in various human and experimental kidney diseases, and contribute to transcription of pro-fibrotic genes [6]. Smad 7 is another member in the Smad family. In contrast to Smad 2/3, it negatively regulates TGF-β1 induced fibrosis [11].

MicroRNAs (miRNAs) are small, non-coding RNAs of approximately 18–25 nucleotides that participate in physical and pathological processes [16,17]. Numerous miRNAs have been found to be associated with development of acute or chronic kidney diseases [18]. Among those miRNAs, miR-21 is the most abundantly expressed within the kidney and the most strongly associated with renal pathogenesis [19,20]. The expression of miR-21 can be regulated by TGF-β1/Smad2/3 signaling pathway [21]. Interestingly, pioglitazone has been found to modulate miR-21 in rat airway smooth muscle cells [22]. However, whether miR-21 and these signals contribute to the anti-fibrosis actions of pioglitazone in kidney, and is there any association between these signals, have not been revealed yet. In the present study, the TGF-β1-induced HK-2 cells and unilateral ureteral obstructed (UUO) mice were used to investigate the detailed pharmacological mechanisms of anti-fibrotic effects of pioglitazone.

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2. Methods

2.1. Cell culture and procedure

The primary human proximal tubular cell line HK-2 was purchased from Shanghai Zhong Qiao Xin Zhou Biotechnology Co., Ltd. (Shanghai, China). The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FBS; Gibco) and maintained in an incubator with 5% CO_2 at 37 °C.

For experiment 1, cells were incubated with pioglitazone 5 μM with or without 2 ng/mL TGF-β1 (USCN Life Science, Wuhan, China) for 24 h. For experiment 2, miR-21-5p mimics or its negative control were transfected into cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to supplier's instructions. Forty-eight hours after the transfection, the cells were incubated in 2 ng/mL TGF-β1 for 24 h. For experiment 3, the cells transfected with miR-21-5p mimics or negative control were incubated with 5 μM pioglitazone with 2 ng/mL TGF-β1 for 24 h. All the experiments included necessary control cells and three biological repeats were performed.

2.2. Luciferase reporter assay

HEK 293T cells were purchased from Shanghai Zhong Qiao Xin Zhou Biotechnology Co., Ltd. (Shanghai, China). The cells were maintained in DMEM with 10% FBS at 37 °C in a humidified 5% CO_2 incubator. The cells were plated at 1.0×10^5 cells per well in 12-well dishes. miR-21-5p mimics or miR-negative control and wild type smad7 3′UTR or mutant smad7 3′UTR were co-transfected into cells using lipofectamine 2000 (Invitrogen). Luciferase activities were assayed using a Dual-Luciferase Reporter Assay Kit (Promega, Fitchburg, WI, USA) according to the instruction. The absorbance of Firefly and Renilla luciferase activity was detected by a Microplate Luminometer (Berthold Technologies, Bad Wildbad, Germany).

2.3. Gelatinase zymography

HK2 cells were homogenized in ice-cold RIPA buffer (Beyotime Institute of Biotechnology, Haimen, China) supplemented with 1 mM phenylmethanesulfonyl fluoride for protein extraction. The concentration of protein was determined using a BCA Assay kit (Beyotime). Thereafter, the protein samples were subjected to electrophoresis on 10% SDS–polyacrylamide gel electrophoresis copolymerized with 10 mg/mL gelatin as the substrate. Then, the gel was washed for 40 min twice in a solution contains 2.5% Triton X-100, 50 mmol/L Tris-HCl, 5 mmol/L $CaCl_2$ and 1 μmol/L $ZnCl_2$, pH 7.6, and incubated at 37 °C for 40 h in incubating solution containing 50 mmol/L Tris-HCl , 5 mmol/L $CaCl_2$, 1 μmol/L $ZnCl_2$, 0.02% Brij, 0.2 mol/L NaCl. The gels were stained with 0.05% Coomassie Brilliant Blue G-250 and then destained with a destaining solution 30% methanol and 10% acetic acid. The gelatinolytic activity was visualized as an unstained band against the uniformly stained background. Enzyme activity was assayed by densitometry using analyzing instrument of formation of image of gel (WD-9413B, Beijing Liuyi Biotechnology Co., LTD, Shanghai, China) and ImageJ software.

2.4. Animals and surgery

Male C57BL/6 mice, 8 weeks old, weighing 20–25 g, were obtained from Liaoning Changsheng Biotechnology Co. Ltd. (Benxi, China). The mice were maintained in an environment with temperature of 22 ± 1 °C, relative humidity of 50 ± 1% and a light/dark cycle of 12/12 h. All the studies performed in accordance with the ethical guidelines of the Institutional Animal Ethics Committee of China Medical University.

Thirty mice were randomly divided into five groups: sham, UUO, UUO + pioglitazone (UUO + Pio), UUO + pioglitazone + control agomir (UUO + Pio + NC) and UUO + pioglitazone + mmu-miR-21-5p agomir (UUO + Pio + agomir). After being anesthetized with an intraperitoneal injection of 50 mg/kg pentobarbital sodium, the mice were fixed in left lateral position. Then, the left flank was opened, the left ureter was exposed and ligated with 4–0 silk suture. Mice in the sham group underwent the same procedure without ligation. Seven days after the surgery, mice in the pioglitazone groups received intragastric administration of pioglitazone (10 mg/kg/day, purity ≥ 98%, Aladdin regents Co. Ltd., Shanghai, China) for two weeks. At the same time, mice in the agomir groups received tail vein injection of NC or mmu-miR-21-5p agomir (GenePharma, Shanghai, China) 3 OD/mouse/week (two injections in total). After two-week administration, 24 h urine was collected using metabolic cages. Then, the mice were euthanized and whole blood and kidneys were collected immediately.

2.5. Biochemical indicators measurements

The levels of BUN and creatinine in the serum were determined using Urea Assay Kit and Creatinine Assay Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The 24 h urinary protein was assayed using Urine protein test kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). All the assays were performed according to the manufacturers' instructions.

2.6. Histologic studies

Isolated kidneys were fixed in 4% paraformaldehyde for 24 h at 4 °C, imbedded in paraffin and cut into 6-μm sections. The sections were then stained with Masson's trichrome reagents (Sinopharm Chemical Reagent Beijing Co. Ltd., Beijing, China) for 15 min. For immunohistochemical staining, the sections were blocked in goat serum (Solarbio Science & Technology, Co., Ltd., Beijing, China) and incubated in primary antibodies at 4 °C overnight. Next, the sections were washed with PBS for three times and incubated with biotinylated anti-mouse/rabbit second antibody at 37 °C for 30 min. 3,3′-Diaminobenzidine (DAB) reagent was used to visualize the staining. For each sample, five microscope fields were counted, and the mean was used for analysis. All the evaluations were performed in a blind way. All the samples were observed under an optic microscopy (DP73; Olympus, Tokyo, Japan). The results were analyzed using ImageJ software (NIH, Maryland, USA).

2.7. RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA including miRNA was extracted from the renal tissues or cells using a Total RNA Extraction kit (BioTeke Corporation, Beijing, China) following the manufacturer's instructions. The cDNA was synthesized using oligonucleotide primer and super M-MLV (BioTeke) and qRT-PCR reaction was performed on an Exicycler 96 (Bioneer, Daejeon, Korea) using 2 × Power Taq PCR Master Mix (BioTeke) and SYBR Green (Solarbio). The following primers were used: hsa-miR-21-5p, RT primer: 5′-GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACTCAACA-3′, forward: 5′-GGCAGCCTAGCTTATCAGACT-3′, reverse: 5′-GTGCAGGGTCCGAGGTATTC-3′; mmu-miR-21-5p, RT primer: 5′-GTTGGCTCTGGTGCAGGGTCCGAGGTATTCGCACCAGAGCCAACTCAACA-3′, forward: GGCAGCCTAGCTTATCAGACT, reverse: 5′-GTGCAGGGTCCGAGGTATTC-3′, homo RNU19, forward: 5′-TGGAGTTGATCCTAGTCTGG-3′, reverse: 5′-GTGCAGGGTCCGAGGTATTC-3′, mus RNU19, forward: 5′-TGTGGAGTTGGTCCTGGTCT-3′, reverse: 5′-GTGCAGGGTCCGAGGTATTC-3′ (Sangon Biotech (Shanghai) Co., Ltd., Shanghai, China). The fold change relative to the control or sham samples was calculated by the $2^{-\Delta\Delta Ct}$ method. RNU19 was used as the internal control.

2.8. Protein extraction and Western blot analysis

The total protein from renal tissues or cells was extracted using RIPA Lysis Buffer (Beyotime) supplemented with phenylmethanesulfonyl fluoride on ice. Protein was separated on 8% or 10% sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gel and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were incubated with antibodies against α-smooth muscle actin (α-SMA), fibronectin, collagen I, smad 7 (Proteintech Group, Inc. Wuhan, China), smad-2, p-smad-2 (Sangon Biotech), Smad-3, and p-Smad-3 (Cell Signaling Technology, Danvers, MA, USA) at 4 °C overnight. Horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse IgG (Beyotime) was incubated with the membranes as the secondary antibody at 37 °C for 45 min. ECL reagent (Beyotime) was used to visualized the protein blots and the grey values were analyzed using Gel-Pro-Analyzer software (Media Cybernetics, Bethesda, MD).

2.9. Statistical analysis

All values are presented as mean ± standard deviation (SD). Data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey post hoc test using SPSS 19.0 software (SPSS Inc., Chicago, IL). $P < 0.05$ was considered statistically significant.

3. Results

3.1. Pioglitazone modulated miR-21-5p and Smad pathway in TGF-β1-stimulated HK-2 cells

TGF-β1 is the most important factor that drives fibrosis in chronic kidney disease [23]. In the experiment 1 of the in vitro study, we investigated the effect of pioglitazone on the expression of miR-21-5p and Smad proteins in TGF-β1-exposed HK-2 cells. As shown in Fig. 1A, after exposed in 2 ng/mL TGF-β1 for 24 h, the expression of miR-21-5p was significantly upregulated in HK-2 cells, while pioglitazone 5 μM reduced this upregulation of miR-21-5p.

TGF-β1 acts through activation of the Smad pathway, a well described pro-fibrotic signaling pathway [24]. Thus, the expression levels of key proteins in this signaling pathway were determined. The results revealed that pioglitazone inhibited TGF-β1-induced phosphorylation of Smad-2 and Smad-3 (Fig. 1B, D and E). In addition, the expression of Smad-7 was downregulated after TGF-β1 stimulation. This downregulation was also reversed by pioglitazone (Fig. 1C). These findings indicate that pioglitazone can modulate TGF-β1-activated Smad pathway and miR-21-5p expression in renal epithelial cells.

3.2. MiR-21-5p inhibitor inhibited TGF-β1-induced expressions of pro-fibrotic proteins

In the experiment 2 of the in vitro study, miR-21-5p mimics and inhibitors were transfected into HK-2 cells to investigate the role of miR-21-5p in TGF-β1-induced fibrogenesis in vitro. As shown in Fig. 2A, miR-21-5p mimics significantly increased the expression level of miR-21-5p in HK-2 cells, and miR-21-5p inhibitor suppressed TGF-β1-induced miR-21-5p expression. In addition, similar as the effect of TGF-β1, miR-21-5p increased the expression levels of α-SMA and fibronectin, activated Smad2/3 phosphorylation, and reduced the expression level of Smad-7. While miR-21-5p inhibitor inhibited the pro-fibrotic effects of TGF-β1 (Fig. 2C). Results of luciferase reporter assay showed that miR-21-5p bound to Smad-7 (Fig. 2D), which indicated that miR-21-5p might regulate TGF-β1/Smad signaling pathway through Smad-7.

3.3. miR-21-5p mimics inhibited the anti-fibrotic effect of pioglitazone

In the experiment 3, we investigated whether miR-21-5p regulation was involved in the anti-fibrotic effect of pioglitazone in TGF-β1-exposed HK-2 cells. The results revealed that pioglitazone inhibited miR-

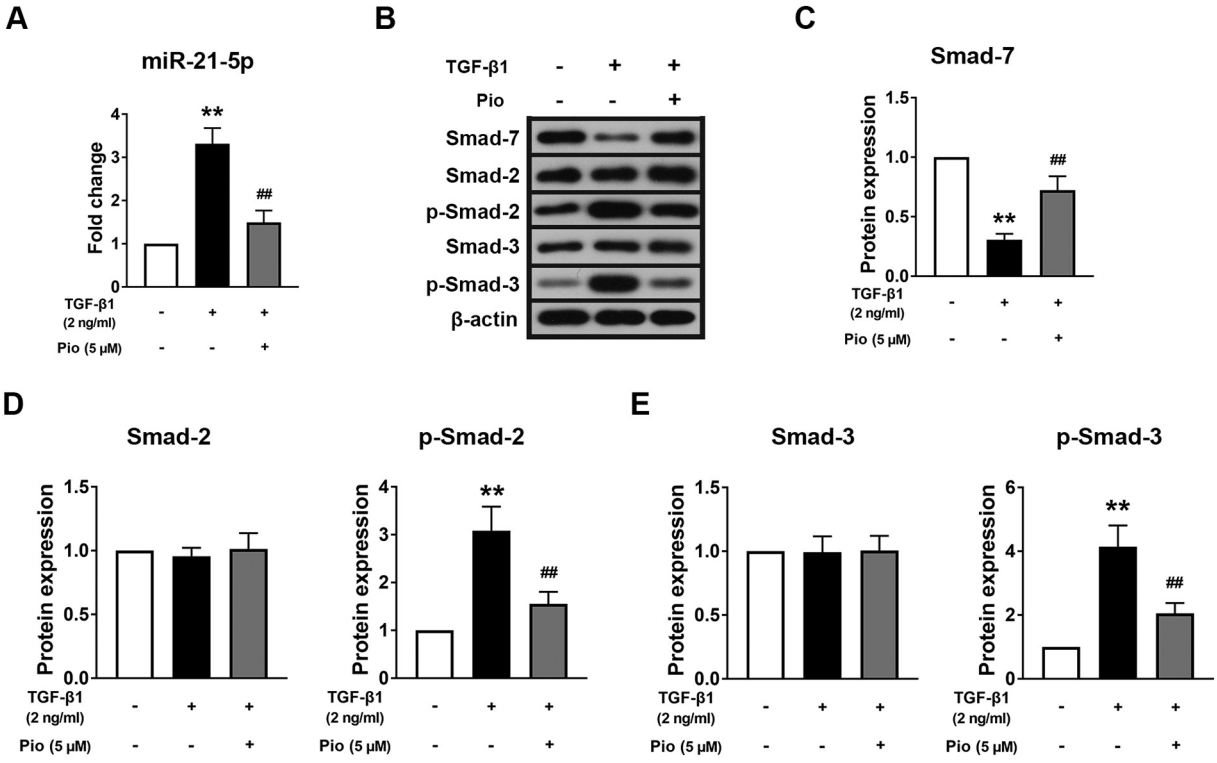

Fig. 1. Pioglitazone inhibited TGF-β1-induced activation of Smad-2/3. Pioglitazone inhibited expression of miR-21-5p (A), upregulated Smad-7 (B and C) and suppressed phosphorylation of Smad-2 (D) and 3 (E) in TGF-β1-exposed HK2 cells. n = 3. $^{**}P < 0.01$ vs. the control cells, $^{\#\#}P < 0.01$ vs. the TGF-β1-stimulated cells.

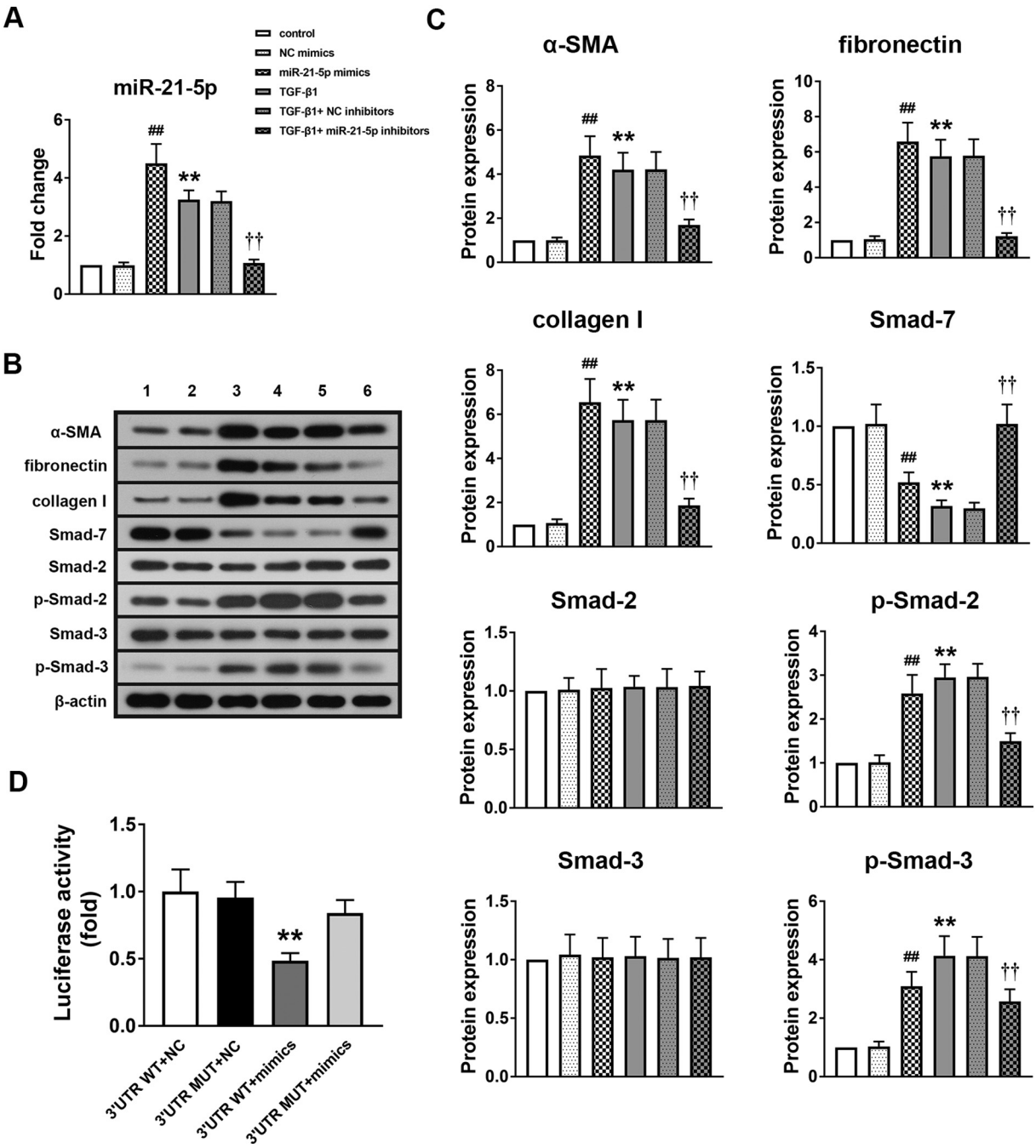

Fig. 2. MiR-21-5p inhibited TGF-β1-induced activation of Smad-2/3. (A) Real-time PCR analysis of the expression of miR-21-5p in HK2 cells. (B) Presentative protein blots of fibrosis-associated proteins. (C) Quantification of the grey values of the protein blots. (D) The binding of has-miR-21-5p and Smad-7 measured using luciferase reporter assay. $^{**}P < 0.01$ vs. the control cells, $^{\#\#}P < 0.01$ vs. the NC mimics-treated cells, $^{\dagger\dagger}P < 0.01$ vs. the TGF-β1 + NC mimics-treated cells. n = 3 $^{**}P < 0.01$ vs. 3′UTR WT + NC-treated cells. 1: control, 2: NC mimics, 3: miR-21-5p mimics, 4: TGF-β1, 5: TGF-β1 + NC inhibitors, 6: TGF-β1 + miR-21-5p inhibitors.

21-5p expression level in TGF-β1-exposed HK-2 cells, while the mimics inhibited the effect of pioglitazone and restored the level of miR-21-5p (Fig. 3A). The results of Western blot analysis showed that pioglitazone inhibited TGF-β1-induced upregulation of α-SMA and fibronectin, downregulation of Smad-7 and phosphorylation of Smad 2 and 3. In addition, the activities of MMP-2 and MMP-9 were also suppressed by pioglitazone. However, this anti-fibrotic effect of pioglitazone was diminished by miR-21-5p mimics (Fig. 3B–D). These findings indicate that miR-21-5p may be involved in the anti-fibrotic effect of pioglitazone.

3.4. Pioglitazone improved renal function in UUO mice

As shown in Table 1, in UUO mice, serum creatinine, BUN and 24 h urinary protein were dramatically increased, which reflected the injury renal function. As expected, pioglitazone significantly decreased the levels of serum creatinine, BUN and 24 h urinary protein compared with the UUO mice. However, miR-21-5p agomir inhibited the effects of pioglitazone, as evidenced by the reduced renal function. In spite of this, compared with the UUO + agomir group, pioglitazone also significantly improved renal function in agomir-injected UUO mice (group 5 vs. group 6, $P < 0.01$). This finding suggests the renoprotective effect

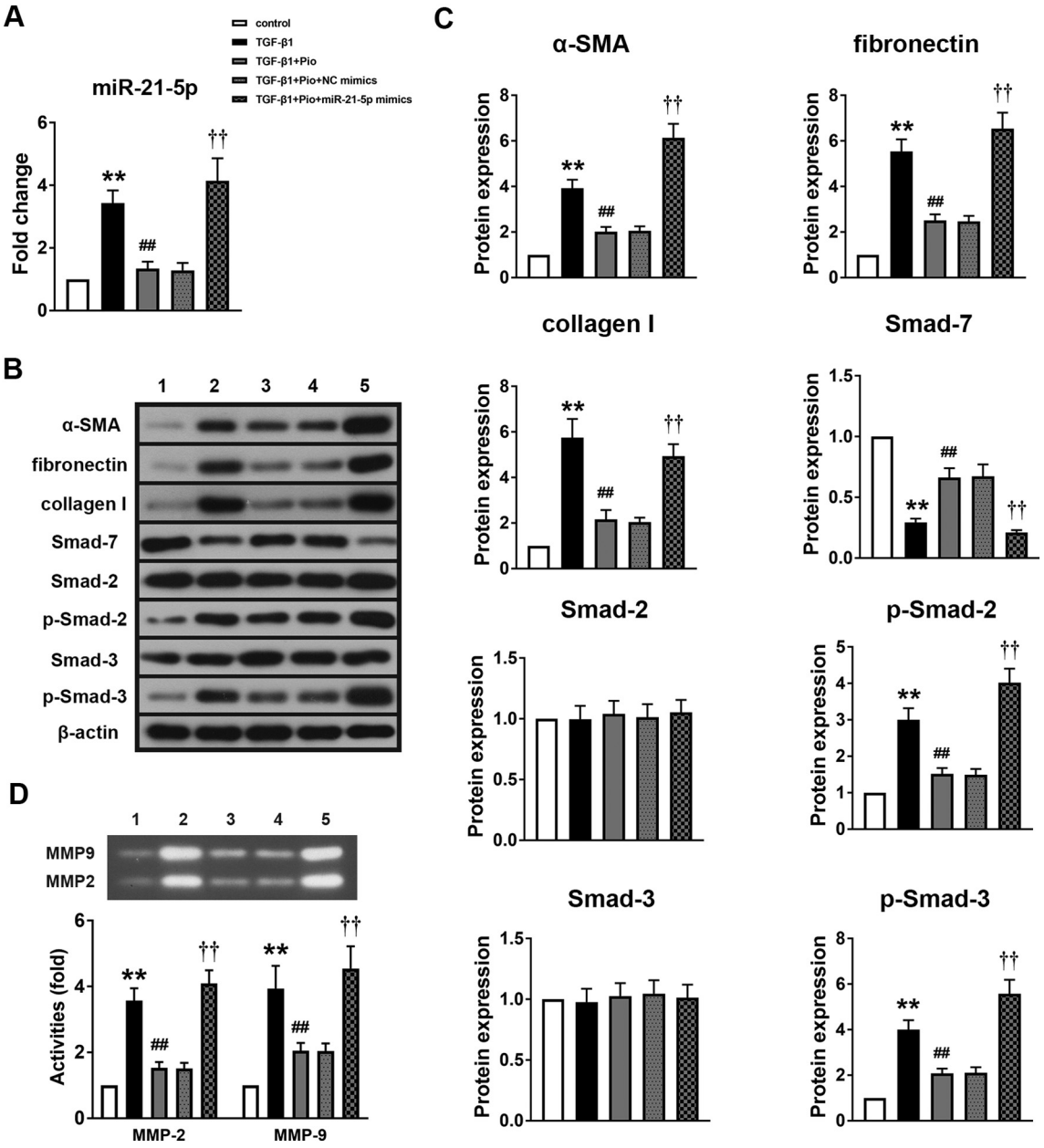

Fig. 3. MiR-21-5p mimics inhibited the anti-fibrotic effect of pioglitazone in HK2 cells. (A) Real-time PCR analysis of the expression of miR-21-5p in HK2 cells. (B) Presentative protein blots of fibrosis-associated proteins. (C) Quantification of the grey values of the protein blots. (D) The activities of MMP2 and MMP9 measured using gelatinolytic zymography. n = 3. $^{**}P < 0.01$ vs. the control cells, $^{\#\#}P < 0.01$ vs. the TGF-β1-treated cells. $^{\dagger\dagger}P < 0.01$ vs. the TGF-β1 + Pio + NC mimics-treated cells. 1: control, 2: TGF-β1, 3: TGF-β1 + Pio, 4: TGF-β1 + Pio + NC mimics, 5: TGF-β1 + Pio + miR-21-5p mimics.

Table 1
Biochemical characters of the mice (mean ± SD).

Characteristic	Group					
	1	2	3	4	5	6
Creatinine (μmol/L)	53.4 ± 14.9	277.6 ± 62.9**	154.3 ± 25.8##	152.8 ± 24.6	243.7 ± 50.9††△△	370.3 ± 54.8##
BUN (mmol/L)	5.22 ± 1.06	15.21 ± 3.32**	8.69 ± 1.83##	9.16 ± 2.36	13.83 ± 2.82††△△	23.0 ± 5.5##
Urinary protein (mg/24 h)	18.7 ± 4.4	86.4 ± 18.2**	43.8 ± 7.9##	43.6 ± 10.7	67.6 ± 15.3††△△	119.8 ± 22.2##

1: sham, 2: UUO, 3: UUO + pioglitazone; 4: UUO + pioglitazone + NC, 5: UUO + pioglitazone + agomir; 6: UUO + agomir. $^{**}P < 0.01$ vs. sham, $^{\#\#}P < 0.01$ vs. UUO mice, $^{\dagger\dagger}P < 0.01$ vs. UUO + pioglitazone + NC, $^{\triangle\triangle}P < 0.01$ vs. UUO + agomir.

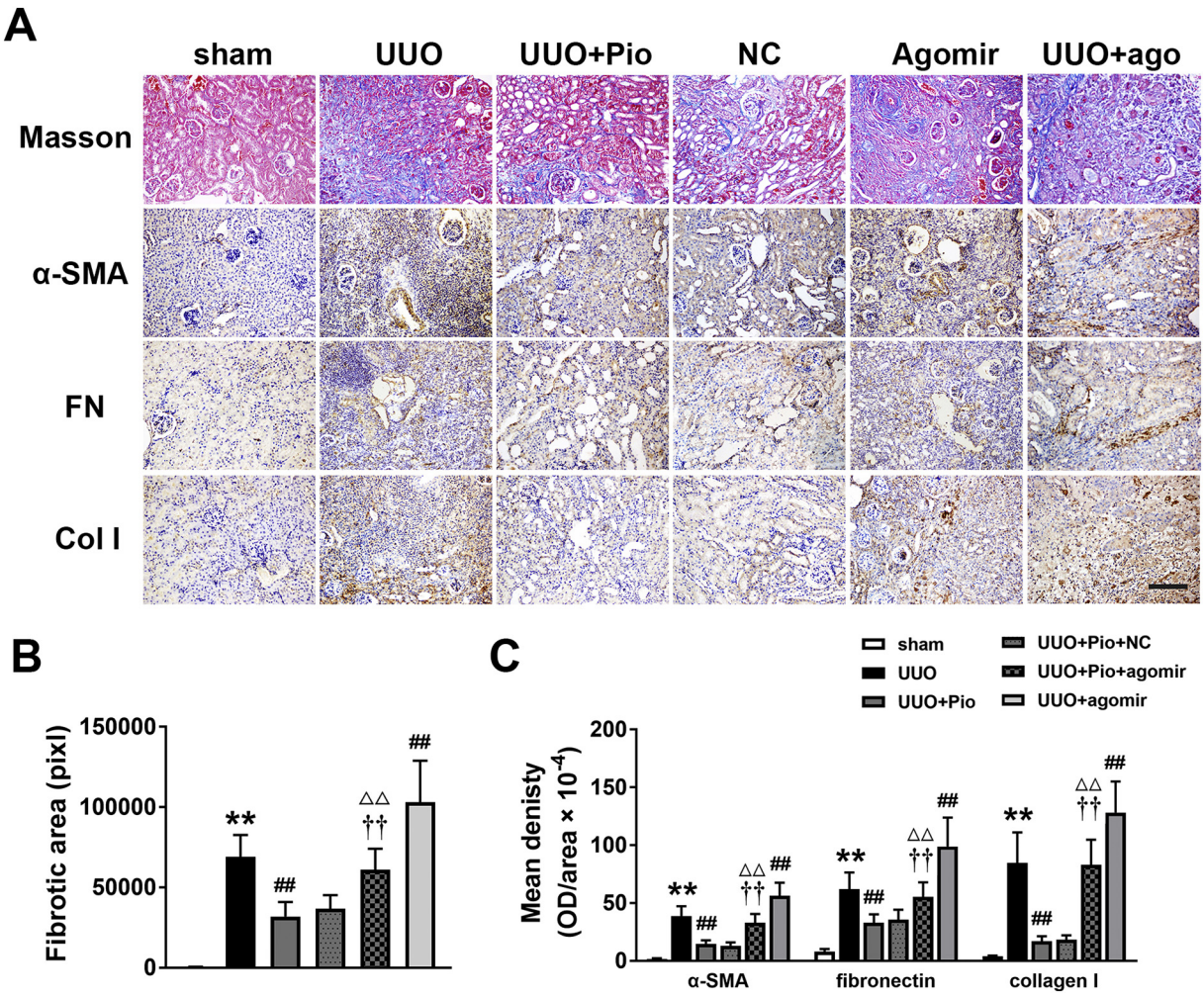

Fig. 4. MiR-21-5p inhibited renoprotective effects of pioglitazone. (A) Masson's trichrome staining and immunohistochemical staining of α-SMA, fibronectin, and collagen I in the kidney. (B) Quantification of fibrotic areas based on Masson's trichrome staining. (C) Quantification of immunohistochemical staining. Scale bar: 200 μm. n = 6. $^{**}P < 0.01$ vs. sham, $^{\#\#}P < 0.01$ vs. UUO, $^{\dagger\dagger}P < 0.01$ vs. UUO + Pio + NC, $^{\triangle\triangle}P < 0.01$ vs. UUO + agomir. UUO + ago: UUO + agomir.

of pioglitazone may be associated with miR-21-5p.

3.5. Effect of miR-21-5p on the renoprotective action of pioglitazone on UUO mice

In the in vivo study, the results demonstrated that UUO induced increased Masson-positive areas and expressions of α-SMA, fibronectin and collagen I in the kidney, and pioglitazone partly, but significantly restored these changes. Compared with the UUO + Piog group, miR-21-5p agomir markedly abolished the anti-fibrotic effect of pioglitazone. The Masson-positive fibrotic area was enlarged and those pro-fibrotic proteins were re-upregulated. However, compared with the UUO + agomir group, pioglitazone also reduced fibrotic areas and the expressions of the pro-fibrotic proteins in the kidney (UUO + Pio + agomir group vs. UUO + agomir group) (Fig. 4).

In accordance with the finding in the in vitro study, the expression of miR-21-5p was upregulated and Smad-7 was downregulated in the kidney of UUO mice, and pioglitazone restored the changes. MiR-21-5p agomir inhibited the effect of pioglitazone. It increased the miR-21-5p and reduced Smad-7 expression (Fig. 5A and B). Importantly, the expression level of miR-21-5p was also decreased in the UUO + Pio + agomir group compared with the UUO + agomir group, which indicated that pioglitazone regulated miR-21-5p in the kidney of UUO mice. In addition, like hsa-miR-21-5p, rno-miR-21-5p was also found to bind to Smad-7 (Fig. 5C). Our findings indicate that modulation of miR-21-5p may be involved in the anti-fibrotic function of pioglitazone in UUO mice.

4. Discussion

Pioglitazone is a widely used insulin sensitizer in type 2 diabetes treatment [25]. Since the discovery of this kind of compounds, as research progressed, more and more functions of pioglitazone have been constantly revealed. Thus, the underlying mechanisms, either associated with PPARγ or not, need to be investigated.

TGF-β1 is the most important fibrosis mediator. It persistently overexpressed in fibrosis of various organs, including kidney, lung, liver, heart and muscle [26]. Given that TGF-β1 activates Smad-2/3, and the latter induce transcription of profibrotic genes, factors that can interference this signaling pathway may affect fibrosis. In other words, all the key proteins in this signaling pathway have the potential to become therapeutic targets of fibrosis. In the present study, we found that pioglitazone inhibited TGF-β1-induced phosphorylation Smad-2 and 3, and upregulated the expression of Smad-7. Based on these findings, the mechanism underlying the antifibrotic effects of pioglitazone in kidney is associated with the inhibition of TGF-β1/Smad-2/3 signaling pathways. Notably, in some previous studies, the findings demonstrated that pioglitazone failed to affect TGF-β1-induced Smad-2 phosphorylation in vitro [27–29]. However, the results of an in vivo study, pioglitazone inhibited phosphorylation of Smad-2 in the kidney

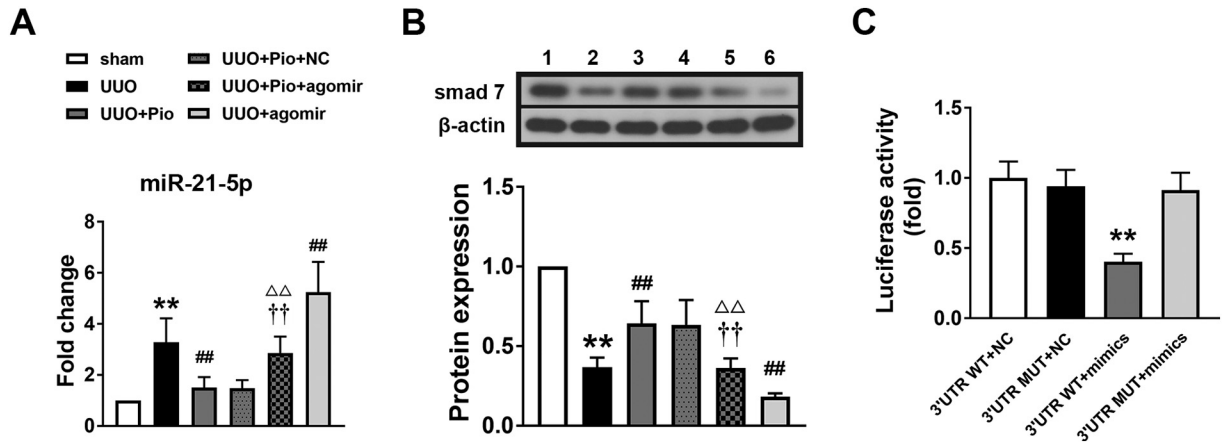

Fig. 5. rno-miR-21-5p binds to Smad-7. (A) Real-time PCR analysis of the expression of miR-21-5p in kidneys of mice. (B) Western blot analysis of the expression of Smad-7. (C) The binding of rno-miR-21-5p and Smad-7 measured using luciferase reporter assay. n = 6. $^{**}P < 0.01$ vs. sham, $^{\#\#}P < 0.01$ vs. UUO, $^{\dagger\dagger}P < 0.01$ vs. UUO + Pio + NC, $^{\triangle\triangle}P < 0.01$ vs. UUO + agomir. 1: sham, 2: UUO, 3: UUO + Pio, 4: UUO + Pio + NC, 5: UUO + Pio + agomir, 6: UUO + agomir.

of UUO mice, support our findings [30]. The discrepancy between our and others' findings may due to the dosage of TGF-β1 and the cell types. The dosage of TGF-β1 we used was lower than that in Wei's study [28]. In Guo and Jin's studies, they used mesangial cells and endothelial cells. The responses of these cells to pioglitazone may be different. The clearly mechanisms between these discrepancies should be detailed investigated in future.

In the results, we found that pioglitazone inhibited miR-21-5p expression in TGF-β1-exposed HK-2 cells and UUO kidney. In addition, miR-21 inhibitors inhibited TGF-β1-induced activation of Smad-2/3, and miR-21-5p mimics/agomirs abolished the anti-fibrotic effects of pioglitazone in TGF-β1-exposed HK-2 cells or in the kidney of UUO mice. Furthermore, the results of luciferase reporter assays demonstrated that both has-miR-21-5p and rno-miR-21-5p bound to Smad-7. Activation of Smad 3 is believed to be the key downstream mediator of TGF-β1 in renal fibrosis activation. It directly promotes the expression of profibrotic genes, including collagens, fibronectin, and α-SMA [31]. Previous study suggests that miR-21-5p is upregulated by TGF-β1 via Smad-3 activation, not Smad-2 [32]. Smad-7 is a negative mediator which inhibits activation of Smad-2 and 3 [11]. In normal state, Smad-3 activation can induce expression of Smad-7, which forms a negative feedback mechanism [33]. However, in pathological situations, we found that the expression of Smad-7 was suppressed and the negative feedback was damaged, which may due to the upregulated expression of miR-21-5p. Pioglitazone treatment inhibited expression of miR-21-5p and increased the expression of Smad-7 in both UUO mice and agomir-injected UUO mice. These findings indicate that modulation of miR-21-5p may be involved in the anti-fibrotic effect of pioglitazone in the kidney of UUO mice. However, it should be noted that there are limitations in this study. In the present study, we did not prove the specific binding or interaction between pioglitazone and miR-21-5p. But we observed the downregulated miR-21-5p in pioglitazone-treated mice and HK-2 cell models, which at least proved that pioglitazone could modulate miR-21-5p. In addition, it has been demonstrated that miR-21-5p can be regulated by Smad-2/3 activation [34]. MiR-21-5p may be directly modulated by pioglitazone, or indirectly via Smad2/3. These detailed mechanisms need to be investigated in our following study.

In conclusion, modulation of miR-21-5p/Smad-7 signaling pathway is involved in the anti-fibrotic effect of pioglitazone in kidney. These signals finally upregulate Smad-7 expression and suppress activation of Smad2/3, inhibit the transcription of pro-fibrotic genes. The more detailed crosstalk between pioglitazone, miR-21-5p, and these signaling pathways still need to be further studied.

Declaration of Competing Interest

There are no conflicts of interest, financial or otherwise, declared by the authors.

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