European Review for Medical and Pharmacological Sciences 2018; 22: 166-173

Silencing of LncRNA TCONS_00088786 reduces renal fibrosis through miR-132

S.-G. ZHOU, W. ZHANG, H.-J. MA, Z.-Y. GUO, Y. XU

Department of Nephrology, Jining No. 1 People's Hospital, Jining, China

Shengguo Zhou and *Wei Zhang* contributed equally to this work

Abstract. – OBJECTIVE: To examine the role of long non-coding ribonucleic acid (LncRNA) TCONS_00088786 in the development of renal interstitial fibrosis and its potential mechanism in this process.

MATERIALS AND METHODS: Unilateral ureteral obstruction (UUO) was used to induce tubulointerstitial fibrosis. Masson staining showed the degree of renal fibrosis in UUO mice. Immunohistochemistry and immunofluorescence were performed to detect the fibrosis-related proteins, the 24-h urine volume and protein content. The renal functions were reflected via serum creatinine (Scr) and blood urea nitrogen (BUN). Changes in lncRNATCONS_00088786, miR-132 and collagen I and III in the development process of renal fibrosis were detected through reverse transcription-polymerase chain reaction (RT-PCR). Small interfering RNA (siRNA) was transfected into NRK52E cells to mimic the knockdown. Western blot was adopted to detect the changes in miR-132, collagen I and III after the siRNA was transfected by transforming growth factor-β (TGF-β) for 24 h.

RESULTS: With the development of renal fibrosis, lncRNA TCONS_00088786 and miR-132 were increased gradually. After the knockdown of lncRNA TCONS_00088786, miR-132 was decreased and fibrosis-related protein was also decreased.

CONCLUSIONS: Decreased lncRNA TCONS _00088786 inhibits renal interstitial fibrosis by reducing miR-132 and it may be a potential novel molecular target for the treatment of renal interstitial fibrosis.

Key Words:
LncRNA, Renal fibrosis, miR-132, Mice.

Introduction

Chronic kidney disease (CKD) is a progressive disease caused by many reasons[1]. There are no clinical symptoms mostly in the early stage of onset. Due to renal reserve function, early serum creatinine (Scr) often remains normal. Therefore, once the clinical symptoms occur or Scris significantly increased, the disease has often developed into the mid and late stage[2]. If CKD is not diagnosed at early stage, pathogenesis fails to be eliminated or intervention treatment is not provided, it will eventually develop into end-stage renal disease (ERSD), namely the uremia. At this time, only the renal replacement therapy can be performed: peritoneal dialysis (PD), hemodialysis (HD) or renal transplantation[3]. Kidney has many functions, such as endocrine and maintaining the stability of internal environment, so once the kidney disease develops into ERSD, kidney will lose all its functions[4]. What will happen next is that patients will have a series of complications, such as anemia, electrolyte disturbance *in vivo*, retention of water and sodium, hypertension and heart failure. All the above-mentioned complications will result in a lower life quality and shorten the lifespan of patients. Renal interstitial fibrosis is the final pathological outcome of CKD, which is manifested as interstitial deposition of a lot of extracellular matrixes and inflammatory cells, loss of renal tubular cells, accumulation of myofibroblast, thinning of microvasculature around tubules, etc[5]. The process of fibrosis is irreversible. When normal renal tissues are replaced by fibrosis tissues, renal function will be severely impaired[6]. No matter what the cause is, once it develops into the renal interstitial fibrosis stage, the renal function will decline progressively and eventually patients will need dialysis or renal transplantation[7]. Therefore, it is important to fully understand the causes and mechanisms of renal interstitial fibrosis, and to find therapeutic methods to control fibrosis, in order to improve the survival and quality of life of patients with CKD. Micro RNA (miRNA) is a group of small single-stranded non-coding RNA that mediates

Corresponding Author: Yan Xu, MM; e-mail: Xuyan1101396@sohu.com

the post-transcriptional level and regulates the gene expression[7,8]. They do not have open reading frames or encode proteins, but are involved in a variety of important physiological and pathological processes in the body. They can be combined with the complementary sequence of 3'untranslated regions (3'UTR) of target mRNA, and induce the degradation of mRNA or inhibit mRNA expression, resulting in the silencing of specific genes. Long non-coding RNA (lncRNA) is a class of RNA whose length is greater than 200 nt without ability to encode proteins[9,10]. It can play a regulatory role in many development processes of biological events, such as cell cycle, DNA methylation, histone modification, transcription and transcription regulation. In recent years, more and more specifically-expressed lncRNAs have been proved to be closely related to the occurrence and development of many diseases. A number of recent researches have shown that lncRNA is closely related to the renal fibrosis[11,12]. Previous paper indicated that silencing of microRNA-132 reduces renal fibrosis by inhibiting myofibroblast proliferation[13], and expressions of TGF-β-induced Col1a1 and Col3a1 genes were significantly inhibited by TCONS_00088786 siRNA[14]. Therefore, we conducted the study to explore the relationship between miR-132 and TCONS_00088786.

Materials and Methods

Establishment of Unilateral Ureteral Obstruction (UUO) Model

Mice were anesthetized via intraperitoneal injection of pentobarbital sodium (50 mg/kg). In UUO group, the ureter was ligated at the proximal hilum of the left kidney, and then the ureter was ligated again away from the hilum of kidney. For mice in the sham operation (Sham) group, the left ureter was separated without ligation and then the abdominal cavity was closed. This work was approved by the Animal Ethics Committee of Jining First People's Hospital Animal Center.

Tissue Harvesting and Cell Culture

Kidney samples were collected at 0, 3, 5, 7, or 14 days after UUO. On the 14th day, the 24-h urine sample was collected using metabolic cages and the urine volume was recorded. Xylene was added for preservation and antisepsis. Next, 5 mL sample were taken to detect the urinary protein content, Scr and BUN under room temperature. Normal rat kidney epithelial (NRK52E) cells were purchased from TongPai Technology (Shanghai, China). TGF-β1 (2 ng/mL) (R&D Systems, Minneapolis, MN, USA) was used to drive NRK52E activation.

Observation of the Degree of Renal Fibrosis Via Masson Staining

The kidney tissues were cut into 3 μm-thick slices after they were fixed, dehydrated and embedded routinely. The slices were dewaxed, hydrated and treated with 5% sodium thiosulfate for 5 min, and then they were stained with Weiger's iron hematoxylin for 5-10 min, followed by differentiation through 1% hydrochloric acid ethanol solution and staining with ponceau acid fuchsin solution for 5-10 min. Slices were treated with 1% ammonium molybdate solution for 2 min and counterstained with aniline blue solution for 5 min. Moreover, they were treated with 1% glacial acetic acid for 1 min and dehydrated using 95% ethanol solution for several times. Finally they were dehydrated with anhydrous alcohol, transparentized *via* xylene and sealed via neutral gum.

Immunohistochemistry

Immunocytochemistry was performed as previously described [34]. Paraffin-embedded hypertrophic scar tissue and normal skin sample slices were immunostained with collagen I and III specific antibodies (Abcam, Cambridge, MA, USA) after deparaffinization and hydration.

Immunofluorescence

UUO kidney tissues were fixed with paraformaldehyde, and sank into the bottom using 20% sucrose and then 30% sucrose, and the operation was repeated with 30% sucrose. The material was taken out and dried. Next, the material was soaked into OTC and its bottom fully touched the liquid nitrogen. When the material was white at the edges for about 15 s, it was placed into the slicing machine at 35°C. The temperature of slicing machine was adjusted (-22°C for the blade and -25°C for the chassis). 8-10 μm-thick slices were cut. Tissues were mounted on slides and dried under room temperature (about 12 h). Slices were treated with 4°C pure acetone for 10 min and treated with acetone after air-drying. They were washed with phosphate buffered saline (PBS) for 10 min for 3 times and treated with 0.3% Trion-100 solution for 10 min for 3 times at 37°C. The slices were rinsed with PBS for 5 min (3 times), sealed with 10% normal rabbit serum for 30 min. Primary rabbit antibodies (specimen

I) and secondary goat antibodies (specimen II) were dropped simultaneously at 4°C overnight. PBS was used to rinse slices for 10 min (3 times). Donkey anti-rabbit-FITC (green) and donkey anti-goat-Tex-Red (red) fluorescence secondary antibodies were dropped simultaneously. After standing at room temperature for 1 h, slices were washed with PBS for 10 min (4 times) and dried. The quenching inhibitor was dropped directly to seal the slices.

RT-PCR

Total RNA was extracted from kidney tissues or cells using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The expressions of lncRNA TCONS_00088786, miR-132, collagen I and III were measured via quantitative RT-PCR (qRT-PCR) with the TaqMan assays reverse transcription kit according to the manufacturer's instructions (Yifeixue Bio Tech, Nanjing, China). GAPDH, U6 or 18s rRNA was used as control.

Western Blotting

Tissues and cells were taken to be added with various kinds of proteinase inhibitors such as radio immunoprecipitation assay (RIPA) buffering solution and phenylmethanesulfonyl fluoride (PMSF). Tissues and cells were incubated on ice for 30 min. During this period, they were blown and washed all the time to avoid bubbles and placed into a centrifuge tube at 4°C under 15,000 rpm for 15 min. The detection of sample concentration was performed according to the operation instructions of bicinchoninic acid (BCA) protein quantification kit. Samples were loaded and aligned. An equal volume of sodium dodecyl sulfate-loading (SDS-loading) was added and mixed evenly, followed by boiling at 100°C for 10 min. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE): Sample liquid and standards were loaded. The standards were put into the first well. Proteins were separated by electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membrane using the three-layer sandwich method. The samples were placed on ice under constant voltage of 100 V for 1 h. The membrane was put into boxes with a proper size after transfer. 5% skim milk was added for incubation in a shaking table for 1 h. Primary antibodies were added and directly combined with target protein antigen. Tris-buffered saline and Tween 20 (TBST) was used to wash the membrane for 3 times (5 min each time). The secondary antibodies were added and combined with primary antibodies. Secondary antibodies were coupled with the horseradish peroxidase. When the appropriate number of enzyme substrate was added, the complex was transformed into a chemiluminescent substance by horseradish peroxidase (HRP) oxidation reaction, and the detection was conducted. TBST was used to wash the membrane for 4 times (8 min each time). Exposure: the substrate was added. The signal was collected with membrane, followed by development.

Results

Development of Fibrosis in Kidneys of UUO Mouse

Samples were taken respectively on the 7th day and 14th day to observe the renal pelvis morphology. In Sham group, there were no obvious changes in two kidneys of rats; no enlargement or hydronephrosis occurred on the operation side; no dilatation of renal pelvis was found. In UUO group, hydronephrosis occurred on the operation side; dilatation of renal pelvis was also found, which was larger than the kidney on the other side. It was observed that the renal cortex on the operation side was thinner than that on the contralateral side (Figure 1A); the renal cortex thickness was remarkable decreased on the 7th day and 14th day (Figure 1B).

Collagen I, and Collagen III in Kidneys of UUO Mouse

Next, the collagen I and collagen III in kidneys of UUO mouse at 3 and 7 d were examined using immunohistochemistry, immunofluorescence and RT-RCR. The fibrosis-related proteins were significantly increased in tubulointerstitial space (Figure 2).

The 24-h Urine Volume, Urine Protein, Scr and BUN of UUO Mice Were Significantly Increased

Compared with those in Sham group, the 24-h urine volume, 24-h urine protein, Scr and BUN were all increased (Figure 3).

Increased Expression of lncRNA TCONS_00088786 and miR-132 in UUO Kidneys and Activated NRK52E Cells

Then, RT-PCR was employed to detect the expressions of lncRNA TCONS_00088786 and

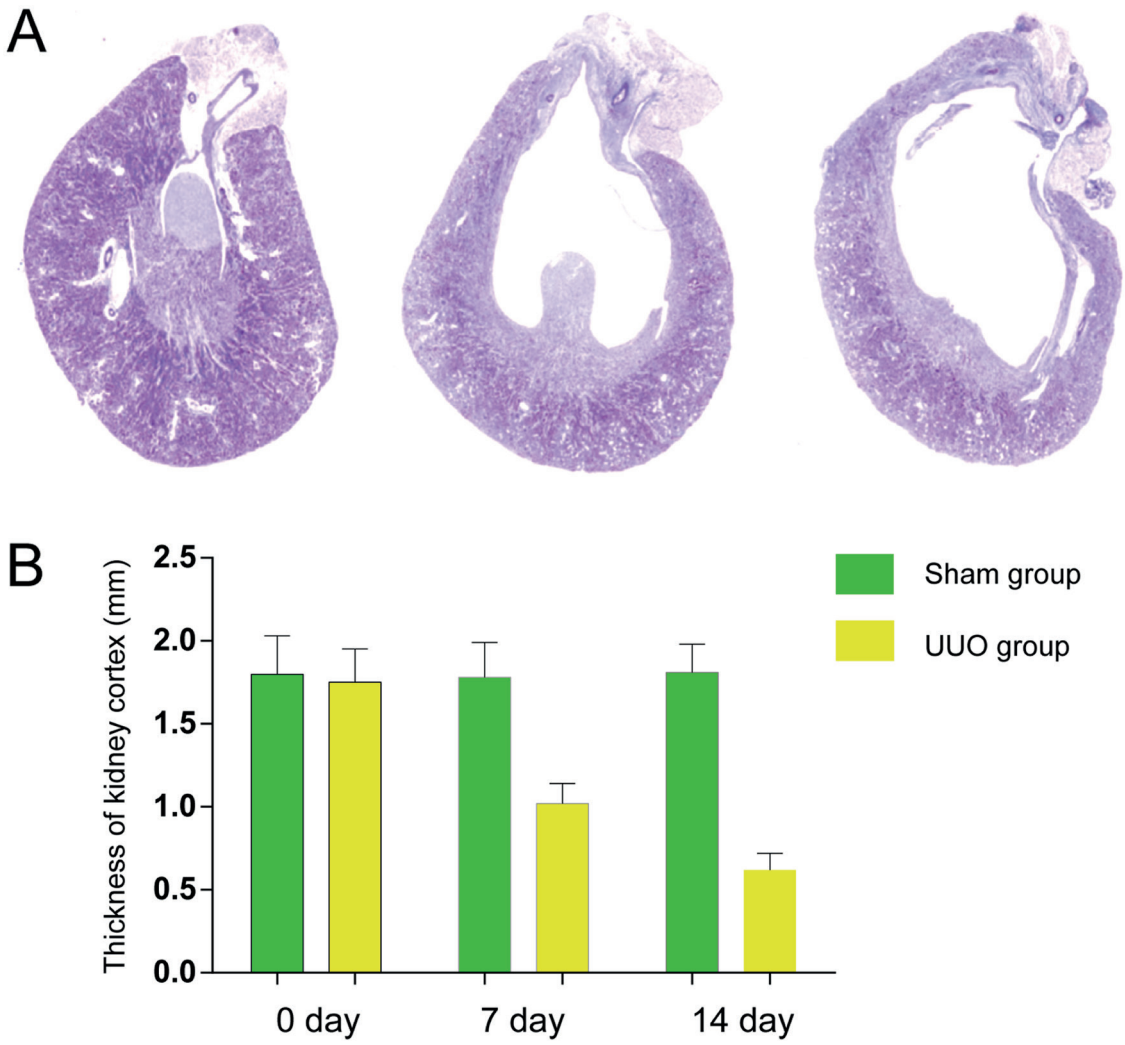

Figure 1. Progress of fibrosis in UUO mouse kidneys. ***A,*** Renal cortex on the operation side was less than that on the contralateral side. ***B,*** The renal cortex thickness was significantly decreased on the 7th day and 14th day.

miR-132. The results demonstrated that lncRNA TCONS_00088786 and miR-132 were significantly increased with the development of renal fibrosis (Figure 4A). When normal kidney epithelial cells (NRK52E) in rats were treated with TGF-β for 24 h, RNA was also increased (Figure 4B).

Silencing of lncRNA TCONS_00088786 Decreased the Expression of miR-132 and Collagen I and III

Si-TCONS_00088786 was transfected into TGF-β-treated NRK52E cells to verify its putative function. The knockdown effect was confirmed *via* RT-PCR (Figure 5A). As shown in Figure 5B, 5C, 5D and 5E, lncRNA TCONS_00088786 decreased miR-132 expression and affected the expression of fibrosis-related genes in inhibiting expressions of TGF-β-induced collagen I and III.

Discussion

The final pathological outcome of CKD is renal interstitial fibrosis, and its histopathological features include the interstitial deposition of a large number of extracellular matrixes and inflammatory cells, loss of renal tubular cells, aggregation of muscle fibers, and thinning of microvasculature around tubules[15,16]. The pro-

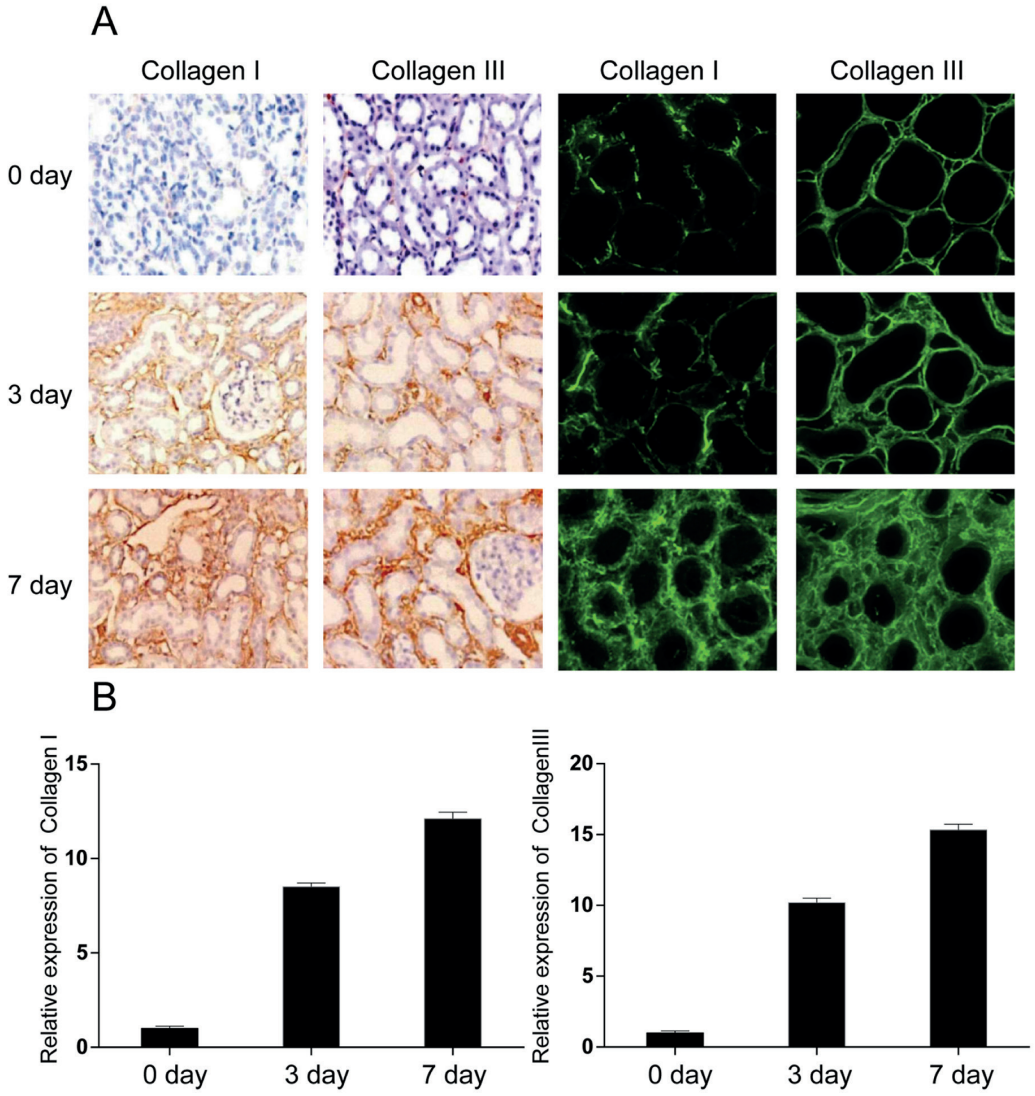

Figure 2. Collagen I and collagen III levels in kidneys of UUO mouse. Collagen I and collagen III significantly raised in tubulointerstitial space.

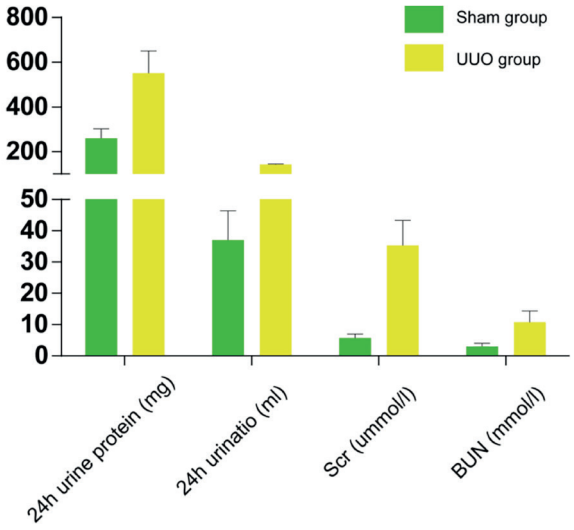

Figure 3. The 24-h urine volume, urine protein, Scr and BUN of UUO mice were significantly increased.

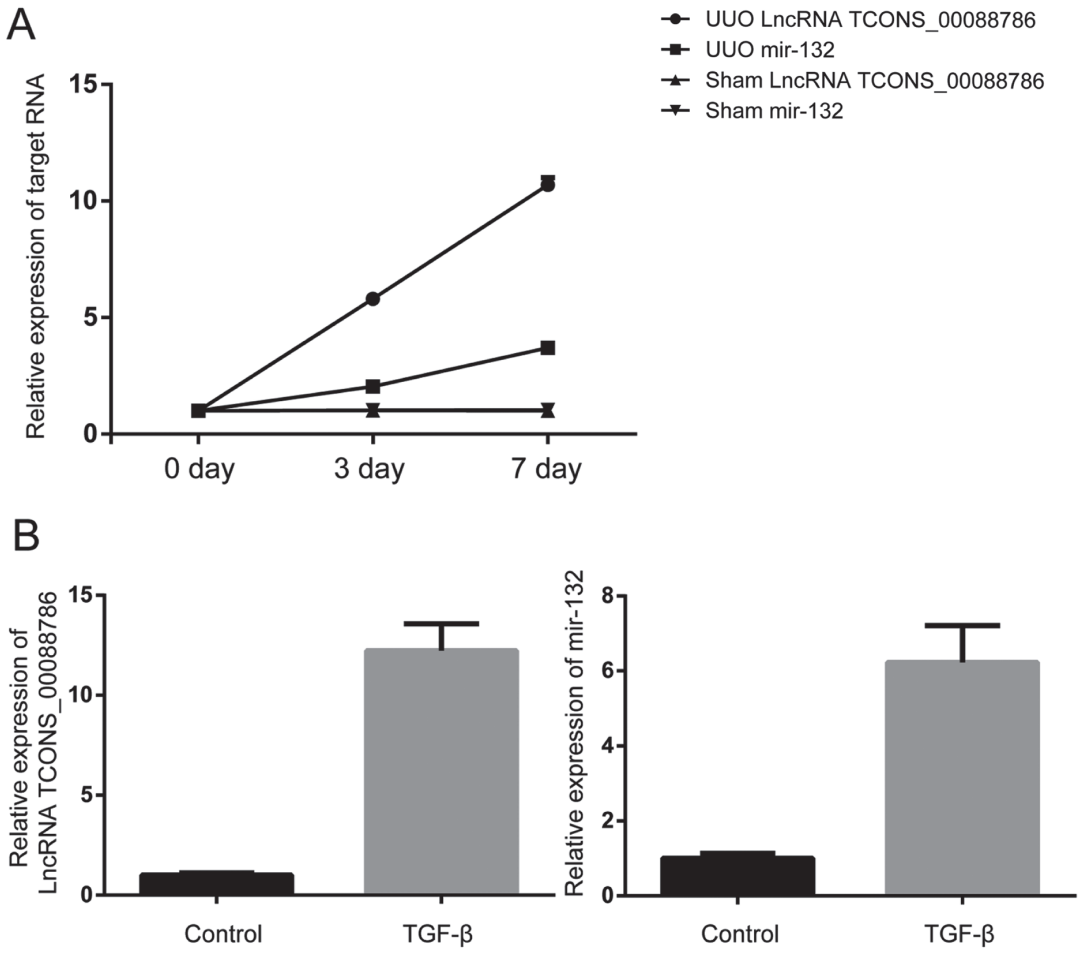

Figure 4. The expression of lncRNA TCONS_00088786 and miR-132 enhanced both in UUO kidneys ***(A)*** and activated NRK52E cells ***(B)***.

cess of fibrosis is irreversible. When normal renal tissues are replaced with fibrosis tissues, renal function will be severely damaged. Regardless of the causes, once it develops into the renal interstitial fibrosis stage, the renal function of patients will decline progressively and patients will need dialysis or renal transplantation eventually. Therefore, it is important to fully understand the causes and mechanisms of renal interstitial fibrosis, and to find therapeutic methods to control fibrosis, in order to improve the survival and quality of life of patients with CKD[17]. The UUO model is now recognized as a mature renal interstitial fibrosis model, and the pathological and physiological characteristics are renal interstitial fibrosis[18-20]. It is simple to establish a UUO model, which has a high successful operation rate and fewer errors due to human factors. Therefore, it can guarantee the success of follow-up experiments and the reliability of data. The operation success rate of UUO model established in this experiment was 100%. Masson staining, immunohistochemistry, immunofluorescence, related fibrosis index determination, and detection of 24h urine volume, urine protein, Scr and BUN showed that the kidney had progressive fibrosis and renal dysfunction after UUO.

Previous study indicated that the decreased microRNA-132 reduces renal fibrosis by inhibiting myofibroblast proliferation, and expression of TGF-β-induced Col1a1 and Col3a1 genes is significantly inhibited by TCONS_00088786 siRNA. Therefore, the relationship between miR-132 and TCONS_00088786 was explored in this study.

After the transfection with TCONS_00088786 siRNA, it was also found in this study that ln-

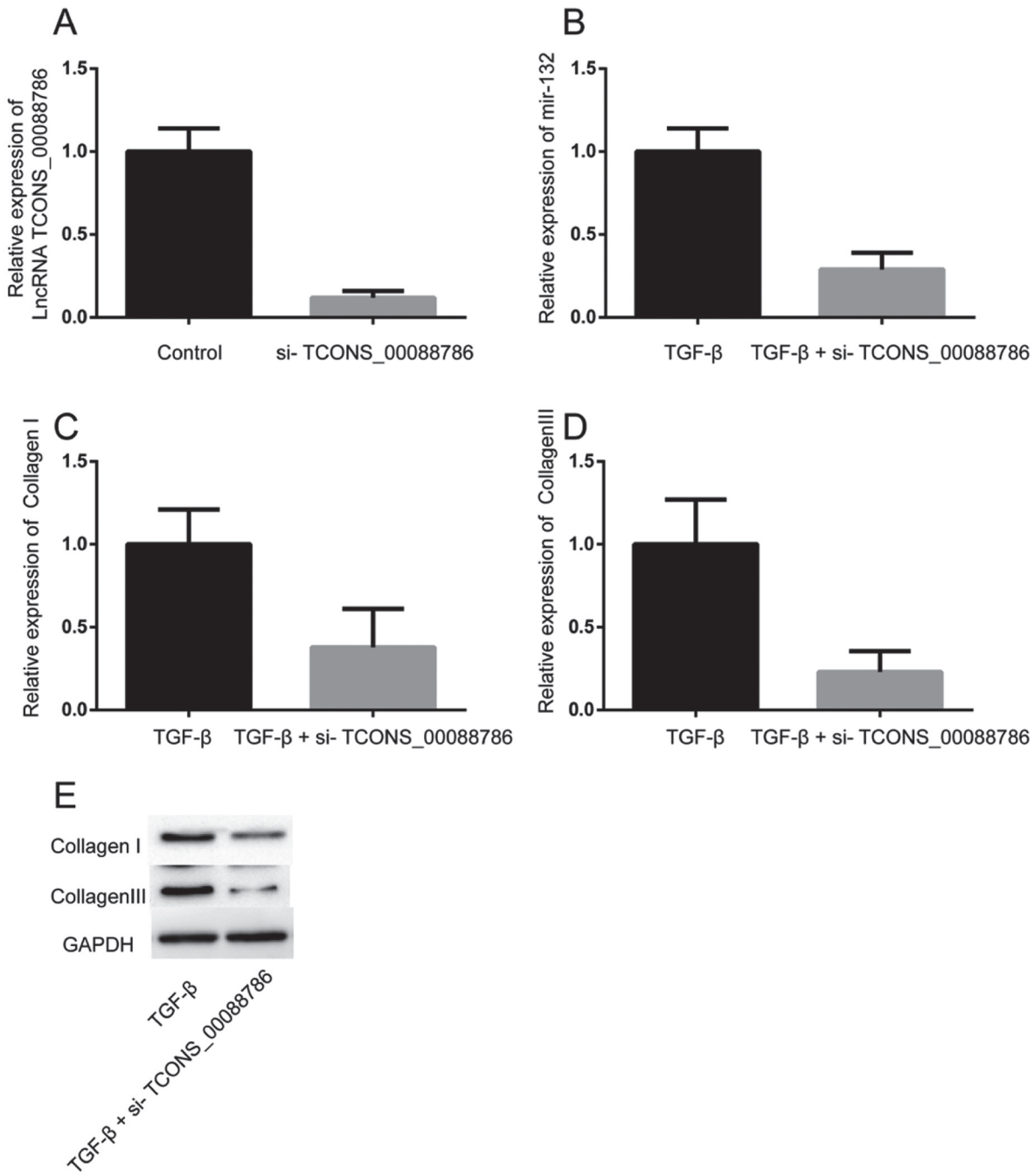

Figure 5. Silencing of lncRNA TCONS_00088786 inhibited the level of miR-132 and collagen I and III.

cRNA TCONS_00088786 was decreased, and the expressions of miR-132 and TGF-β-induced Collagen I and III were also decreased, proving that lncRNA TCONS_00088786 may influence the occurrence and development of fibrosis by regulating the expression of miR-132.

Conclusions

Silencing of lncRNA TCONS_00088786 can inhibit the development of interstitial fibrosis via reducing expression of miR-132, which may be a target for treating renal interstitial fibrosis.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Gouveia MC, Vella JP, Cafeo FR, Affonso FF, Bacci MR. Association between irisin and major chronic diseases: a review. Eur Rev Med Pharmacol Sci 2016; 20: 4072-4077.
2) Gigante A, Di Mario F, Barbano B, Rosato E, Di Lazzaro GG, Pofi R, Gasperini ML, Amoroso D, Cianci R, Laviano A. Nutritional status and intrarenal arterial stiffness in cardiorenal syndrome: a pilot study. Eur Rev Med Pharmacol Sci 2017; 21: 313-316.
3) Rietveld A, Berden JH. Renal replacement therapy in lupus nephritis. Nephrol Dial Transplant 2008; 23: 3056-3060.
4) Ma DQ, Li Y, Han ZG, Zheng M, Xu N, Fan XJ. Analysis on kidney injury-related clinical risk factors and evaluation on the therapeutic effects of hemoperfusion in children with Henoch-Schonlein purpura. Eur Rev Med Pharmacol Sci 2017; 21: 3894-3899.
5) Yang G, Zeng G, Wu JP, Jiang O, Zeng YB, Huang SJ, Huang JJ, Wu DQ. Glipizide blocks renal interstitial fibrosis by inhibiting AKT signaling pathway. Eur Rev Med Pharmacol Sci 2017; 21: 867-872.
6) Markovic-Lipkovski J, Zivotic M, Muller CA, Tampe B, Cirovic S, Vjestica J, Tomanovic N, Zeisberg M, Muller GA. Variable expression of neural cell adhesion molecule isoforms in renal tissue: possible role in incipient renal fibrosis. PLoS One 2015; 10: e137028.
7) Mulder GM, Melenhorst WB, Celie JW, Kloosterhuis NJ, Hillebrands JL, Ploeg RJ, Seelen MA, Visser L, van Dijk MC, van Goor H. ADAM17 up-regulation in renal transplant dysfunction and non-transplant-related renal fibrosis. Nephrol Dial Transplant 2012; 27: 2114-2122.
8) Guo ML, Guo LL, Weng YQ. Implication of peripheral blood miRNA-124 in predicting acute myocardial infarction. Eur Rev Med Pharmacol Sci 2017; 21: 1054-1059.
9) Fu XM, Guo W, Li N, Liu HZ, Liu J, Qiu SQ, Zhang Q, Wang LC, Li F, Li CL. The expression and function of long noncoding RNA lncRNA-ATB in papillary thyroid cancer. Eur Rev Med Pharmacol Sci 2017; 21: 3239-3246.
10) Mercer TR, Dinger ME, Mattick JS. Long non-coding RNAs: insights into functions. Nat Rev Genet 2009; 10: 155-159.
11) Xie H, Xue JD, Chao F, Jin YF, Fu Q. Long non-coding RNA-H19 antagonism protects against renal fibrosis. Oncotarget 2016; 7: 51473-51481.
12) Zhou Q, Huang XR, Yu J, Yu X, Lan HY. Long noncoding RNA Arid2-IR is a novel therapeutic target for renal inflammation. Mol Ther 2015; 23: 1034-1043.
13) Bijkerk R, de Bruin RG, van Solingen C, van Gils JM, Duijs JM, van der Veer EP, Rabelink TJ, Humphreys BD, van Zonneveld AJ. Silencing of microRNA-132 reduces renal fibrosis by selectively inhibiting myofibroblast proliferation. Kidney Int 2016; 89: 1268-1280.
14) Sun J, Zhang S, Shi B, Zheng D, Shi J. Transcriptome identified lncRNAs associated with renal fibrosis in UUO rat model. Front Physiol 2017; 8: 658.
15) Pergola PE, Raskin P, Toto RD, Meyer CJ, Huff JW, Grossman EB, Krauth M, Ruiz S, Audhya P, Christ-Schmidt H, Wittes J, Warnock DG. Bardoxolone methyl and kidney function in CKD with type 2 diabetes. N Engl J Med 2011; 365: 327-336.
16) Hallan SI, Ritz E, Lydersen S, Romundstad S, Kvenild K, Orth SR. Combining GFR and albuminuria to classify CKD improves prediction of ESRD. J Am Soc Nephrol 2009; 20: 1069-1077.
17) Chevalier RL, Forbes MS, Thornhill BA. Ureteral obstruction as a model of renal interstitial fibrosis and obstructive nephropathy. Kidney Int 2009; 75: 1145-1152.
18) Leelahavanichkul A, Yan Q, Hu X, Eisner C, Huang Y, Chen R, Mizel D, Zhou H, Wright EC, Kopp JB, Schnermann J, Yuen PS, Star RA. Angiotensin II overcomes strain-dependent resistance of rapid CKD progression in a new remnant kidney mouse model. Kidney Int 2010; 78: 1136-1153.
19) Hasegawa S, Nakano T, Torisu K, Tsuchimoto A, Eriguchi M, Haruyama N, Masutani K, Tsuruya K, Kitazono T. Vascular endothelial growth factor-C ameliorates renal interstitial fibrosis through lymphangiogenesis in mouse unilateral ureteral obstruction. Lab Invest 2017: 1439-1452.
20) Lee S, Kang KP, Park SK. COMP-angiopoietin-1 ameliorates renal fibrosis in a unilateral ureteral obstruction model. J Am Soc Nephrol 2006; 17: 2474-2483.