European Review for Medical and Pharmacological Sciences 2019; 23: 289-296

ILK participates in renal interstitial fibrosis by altering the phenotype of renal tubular epithelial cells via TGF-β1/smad pathway

M. LI[1], H. ZHOU[1], J. DI[1], M. YANG[1], F. JIA[2]

[1]Department of Nephrology, The Third Affiliated Hospital of Soochow University, Changzhou, China
[2]Department of Cardiovascular Medicine, The Third Affiliated Hospital of Soochow University, Changzhou, China

Abstract. – OBJECTIVE: To explore the specific role of ILK (integrin-linked kinase) in regulating renal fibrosis and its underlying mechanism.

MATERIALS AND METHODS: NRK-52E cells were induced by transforming growth factor-β1 (TGF-β1) for observing phenotype change. Renal tubular epithelial cell marker, fibrosis marker and expression level of ILK in NRK-52E cells were also detected. After overexpression of ILK, phenotype change of NRK-52E cells was observed. For *in vivo* experiments, we constructed UUO (unilateral ureteral obstruction) model in CD1 mice. Renal tubular epithelial cell marker, fibrosis marker and expression level of ILK in UUO mice were detected. The regulatory effect of ILK on renal fibrosis was detected after injection of ILK overexpression plasmid. Western blot was performed to detect related genes in the TGF-β1/smad pathway.

RESULTS: Accompanied by the TGF-β1-induced phenotype change in NRK-52E cells, both mRNA and protein levels of ILK were upregulated. Overexpression of ILK remarkably stimulated the phenotype change in NRK-52E cells. Similarly, ILK was highly expressed in UUO mice. Renal fibrosis was aggravated after injection of ILK overexpression plasmid in UUO mice. Western blot results showed that expressions of p-smad3 and smad3 were upregulated during the process of renal fibrosis.

CONCLUSIONS: ILK is upregulated during the process of renal fibrosis. ILK participates in the development of renal fibrosis by altering phenotypes of renal tubular epithelial cells via a TGF-β1/smad pathway.

Key Words:
ILK, TGF-β1/smad, Phenotype change, Renal fibrosis.

Introduction

Chronic kidney disease (CKD) severely threatens human health throughout the world. In recent years, the incidence of CKD has increased each year. It is reported that the global incidence of CKD is about 14%[1], and its mortality rate has risen as well[2]. CKD not only affects life quality of affected patients, but also brings a huge economic burden on their families.

Renal interstitial fibrosis is the final pathological performance of CKD caused by various factors, which is a key factor in determining the progression of renal failure[3,4]. Fibroblasts are activated and aggregated in the renal stroma, which in turn produces a large number of extracellular matrices, including collagen I, collagen III, and fibronectin (FN). Fibroblasts are directly related to the progression of CKD[5,6]. Renal interstitial myofibroblasts are originated from many sources, such as kidney-derived fibroblasts, tubular epithelial cells, endothelial cell transdifferentiation, pericyte differentiation, and bone marrow-derived mesenchymal stem cells[7-11]. Among them, epithelial-to-mesenchymal transition (EMT) of renal tubular epithelial cells is of great significance for tubulointerstitial fibrosis[12-15].

Some studies[16-19] have found that activation of transforming growth factor-β1 (TGF-β1)/smad pathway can promote renal fibrosis. ILK (integrin-linked kinase) exerts its role as a co-transcription factor for a variety of transcription factors[20,21]. ILK regulates transcription of target genes *via* TGF-β1/smad pathway[22-25]. Therefore, we hypothesized that ILK is involved in renal interstitial fibrosis. Most studies have been conducted to investigate the role of ILK in tumors,

Corresponding Author: Min Yang, MD; e-mail: horizonminmin@163.com
Fang Jia, MD; e-mail: jiafangsjs@126.com

the specific mechanism of which in renal interstitial fibrosis still remains unclear. We believed that ILK may serve as a novel target in preventing and treating renal fibrosis.

Materials and Methods

Animal Procedure

Male CD-1 mice in clean level weighing 18-20 g (Shanghai laboratory animal center, Shanghai, China) were selected and given to free access to water and diet. Mice were randomly assigned into sham group, Day 1 group, Day 3 group, and Day 7 group, with 4 mice in each group.

For constructing UUO (unilateral ureteral obstruction) mouse model, mice received an intraperitoneal injection of 0.75% pentobarbital sodium at a dose of 70 mg/kg. After anesthesia, the abdominal cavity was exposed for ligation of the left ureter with 4-0 suture twice. Mice in the sham group were only cut open without UUO procedure. Mouse kidney tissue was collected at the postoperative 1st, 3rd, and 7th day, respectively. The Animal Ethics Committee of Soochow University Animal Center and the Institutional Animal Care approved this investigation.

Cell Culture

Rat renal tubular epithelial cell line NRK-52E was cultured in Dulbecco's Modified Eagle Medium/F12 (DMEM/F12; Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin, and maintained in a 5% CO_2 incubator at 37°C. Serum-free medium was replaced for overnight culture when the confluence was up to 80%. Cells were then induced with TGF-β1 for the following experiments.

Cell Transfection

Cells were seeded in a 6-well plate and cultured in DMEM/F12 containing 10% fetal bovine serum. When the cell confluence was up to 90-95%, 1.5 mL of serum-free DMEM/F12 was replaced. In brief, 2.5 μg of plasmid DNA or 5 μL of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was added in 250 μL of Opti-MEM and incubated at room temperature for 5 min, respectively. The two kinds of transfection solution were mixed together and maintained at room temperature for 20 min. Culture medium was replaced 6 h later.

Western Blot

Total protein was extracted from treated cells and quantified using the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). Protein samples were separated by gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The incubation with primary and secondary antibodies was performed following the standard protocols of Western blot. Immunoreactive bands were exposed by enhanced chemiluminescence (ECL) method.

Immunofluorescence

Slides were pre-placed in the 24-well plates. Cells were washed with phosphate buffered saline (PBS) three times and fixed with pre-cooled methanol/acetone (1:1) at -20°C for 20 min. Cells were subsequently incubated with a blocking buffer containing 0.1% TritonX-100 and 2% bovine serum albumin (BSA) for 40 min at room temperature, followed by incubation with primary and secondary antibodies. Cell nuclei were stained with DAPI (4',6-diamidino-2-phenylindole) in the dark, and captured using Nikon Eclipse 80i microscope (Tokyo, Japan).

Immunohistochemistry

Paraffin-embedded kidney tissues were sliced with 3 μm of thickness. Kidney slices were deparaffinized, hydrated in ethyl alcohol and blocked in blocking solution for 30 min. After the incubation with primary antibody at 4°C overnight and secondary antibody at room temperature for 1 h, immunohistochemistry results were captured using Nikon Eclipse 80i microscope (Tokyo, Japan).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 16.0 statistical software (SPSS Inc., Chicago, IL, USA) was used for data analysis. Measurement data were expressed as mean ± standard deviation ($\bar{x} \pm s$). Comparison of measurement data was conducted using the *t*-test. Comparison of differences among each group was conducted using one-way ANOVA, followed by post-hoc analysis. $p < 0.05$ was considered statistically significant.

Results

Phenotype Change of NRK-52E Cells Induced by TGF-β1

Previous studies have shown that TGF-β1 exerts an important role in the renal interstitial. In our

study, NRK-52E cells were induced by TGF-β1. Western blot results indicated that E-cadherin expression was downregulated, whereas expressions of FN, α-SMA, and ILK were upregulated. Moreover, protein expression changes of E-cadherin, FN, α-SMA, and ILK presented time- and dose-dependent manners (Figure 1A and 1B). The mRNA level of ILK was also elevated after TGF-β1 induction in NRK-52E cells (Figure 1C and 1D). The above results suggested that ILK expression is elevated during the process of phenotype changes in the renal tubular epithelial cells.

ILK Overexpression Resulted in Phenotype Change in NRK-52E Cells

To explore the effect of ILK on phenotype alteration of renal tubular epithelial cells, we first constructed ILK overexpression plasmid and pcDNA3 as negative control. Transfection efficacies in NRK-52E cells were verified by qRT-PCR (Figure 2A). Overexpression of ILK remarkably decreased E-cadherin expression, but increased expressions of FN and α-SMA, which were similar to those results of TGF-β1 induction in NRK-52E cells (Figure 2B). Immunofluorescence obtained the similar results (Figure 2C).

ILK Overexpression and Phenotype Change of Renal Tubular Epithelial Cells in UUO Mouse Model

We constructed a model of obstructive nephropathy using unilateral ureteral obstruction (UUO) approach. Western blot results showed that the expression of E-cadherin was decreased in the obstructed renal tissues. However, expressions of FN, α-SMA, and ILK were increased in a time-dependent manner (Figure 3A). The mRNA level of ILK was also markedly upregulated (Figure 3B). Immunohistochemical staining showed that ILK-positive cells were mainly the impaired tubular epithelial cells and renal interstitial cells (Figure 3C and 3D).

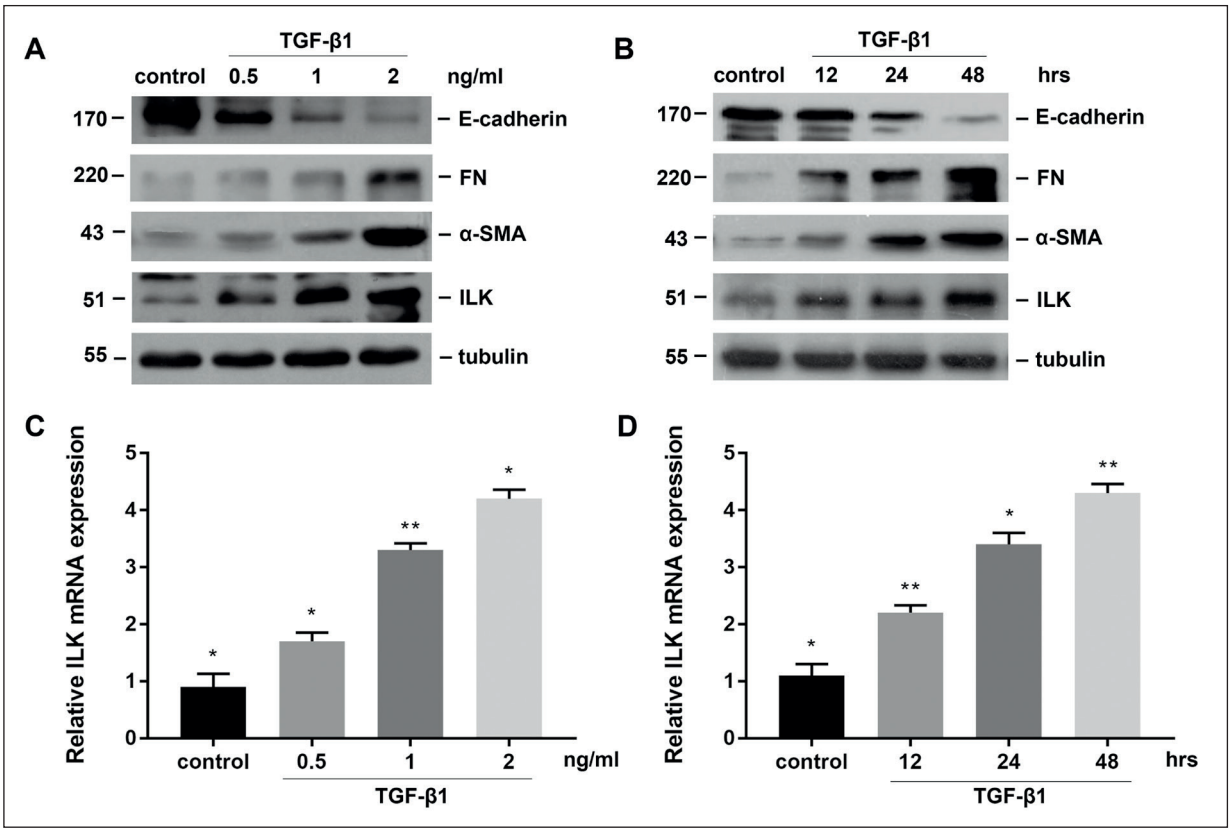

Figure 1. Phenotype change of NRK-52E cells induced by TGF-β1. ***A,*** Protein expressions of E-cadherin, FN, α-SMA, and ILK in NRK-52E cells after TGF-β1 induction for 48 h. ***B,*** Protein expressions of E-cadherin, FN, α-SMA, and ILK in NRK-52E cells after TGF-β1 (2 ng/ml) induction for different time points. ***C,*** The mRNA level of ILK in NRK-52E cells after TGF-β1 induction for 48 h. D, The mRNA level of ILK in NRK-52E cells after TGF-β1 (2 ng/mL) induction for different time points.

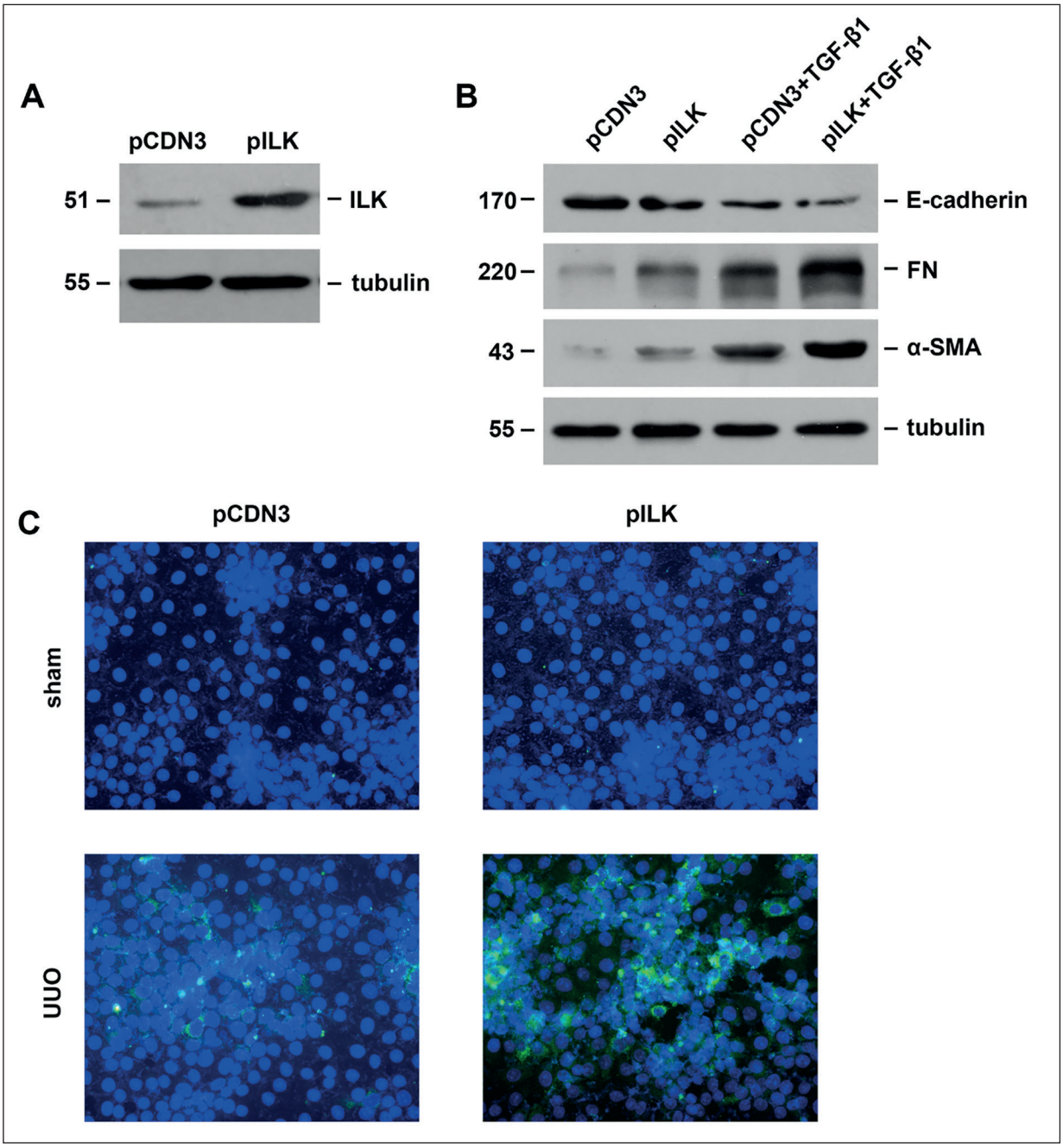

Figure 2. ILK overexpression resulted in phenotype change in NRK-52E cells. ***A,*** Protein expression of ILK after transfection of ILK overexpression plasmid for 48 h. ***B,*** Protein expressions of E-cadherin, FN, and α-SMA in NRK-52E cells. ***C,*** Immunofluorescence results of E-cadherin, FN and α-SMA in NRK-52E cells (magnification 400×).

ILK Overexpression Aggravated Renal Fibrosis

After successful construction of UUO mouse model, we injected the ILK overexpression plasmid into the tail vein on the next day. Both mRNA and protein levels of ILK were elevated in UUO mice (Figure 4A and 4B). Kidney tissues of UUO mice were collected 7 days after UUO procedures. Western blot results showed that protein expressions of FN and α-SMA were markedly elevated in kidney tissues of mice injected with ILK overexpression plasmid, indicating the aggravated renal fibrosis (Figure 4C). Similarly, mRNA levels of FN and α-SMA were increased as well (Figure 4D and 4E).

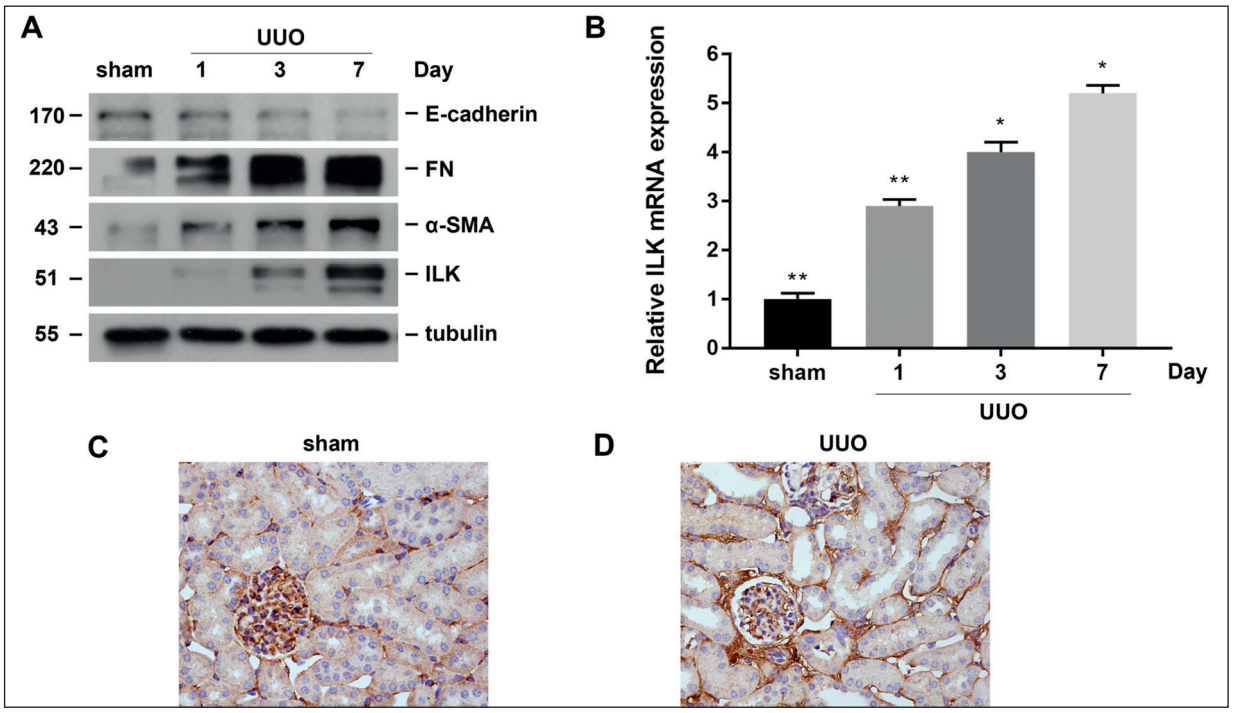

Figure 3. ILK overexpression and phenotype change of renal tubular epithelial cells in UUO mouse model. ***A,*** Protein expressions of E-cadherin, FN, α-SMA and ILK in kidney tissues of UUO mice. ***B,*** The mRNA level of ILK in kidney tissues of UUO mice. ***C,*** Immunohistochemical staining of ILK in kidney tissues of control mice (magnification 400×). ***D,*** Immunohistochemical staining of ILK in kidney tissues of UUO mice (magnification 400×).

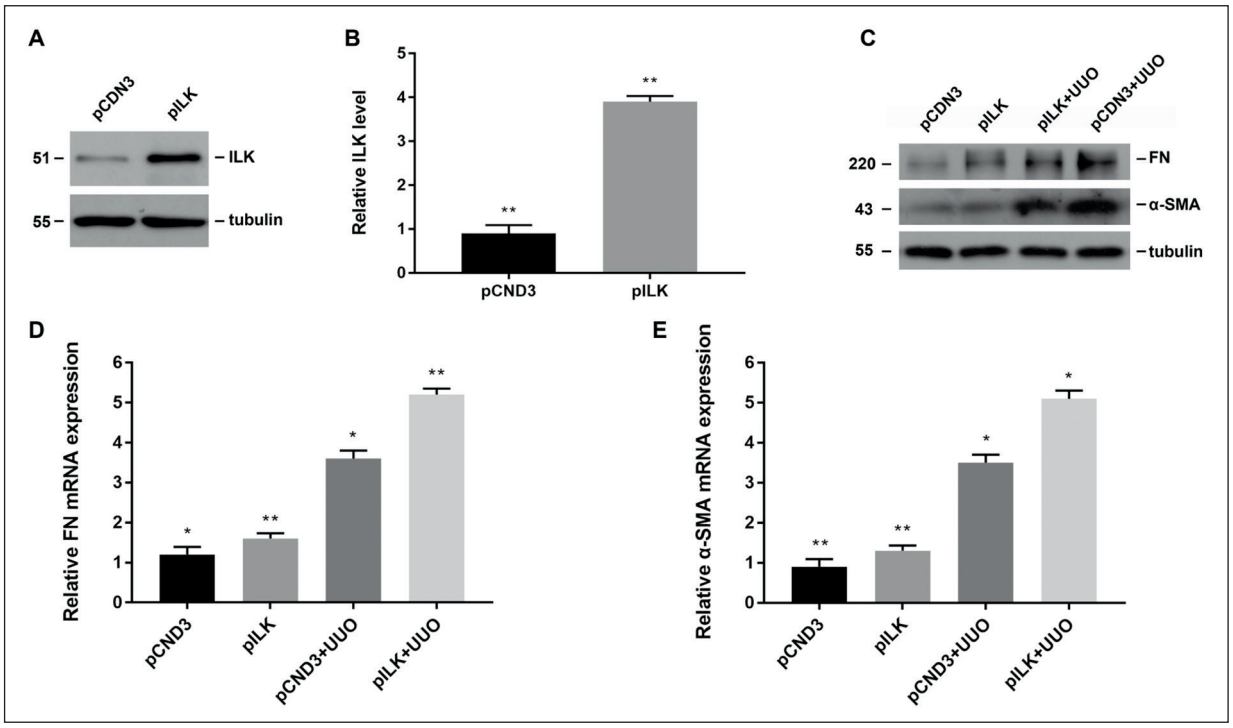

Figure 4. ILK overexpression aggravated renal fibrosis. ***A,*** Protein expression of ILK in kidney tissue after injection of ILK overexpression plasmid for 24 h. ***B,*** The relative level of ILK in kidney tissue after injection of ILK overexpression plasmid for 24 h. ***C,*** Protein expressions of FN and α-SMA in kidney tissues of UUO mice. ***D,*** The mRNA level of FN in kidney tissues of UUO mice. ***E,*** The mRNA level of α-SMA in kidney tissues of UUO mice.

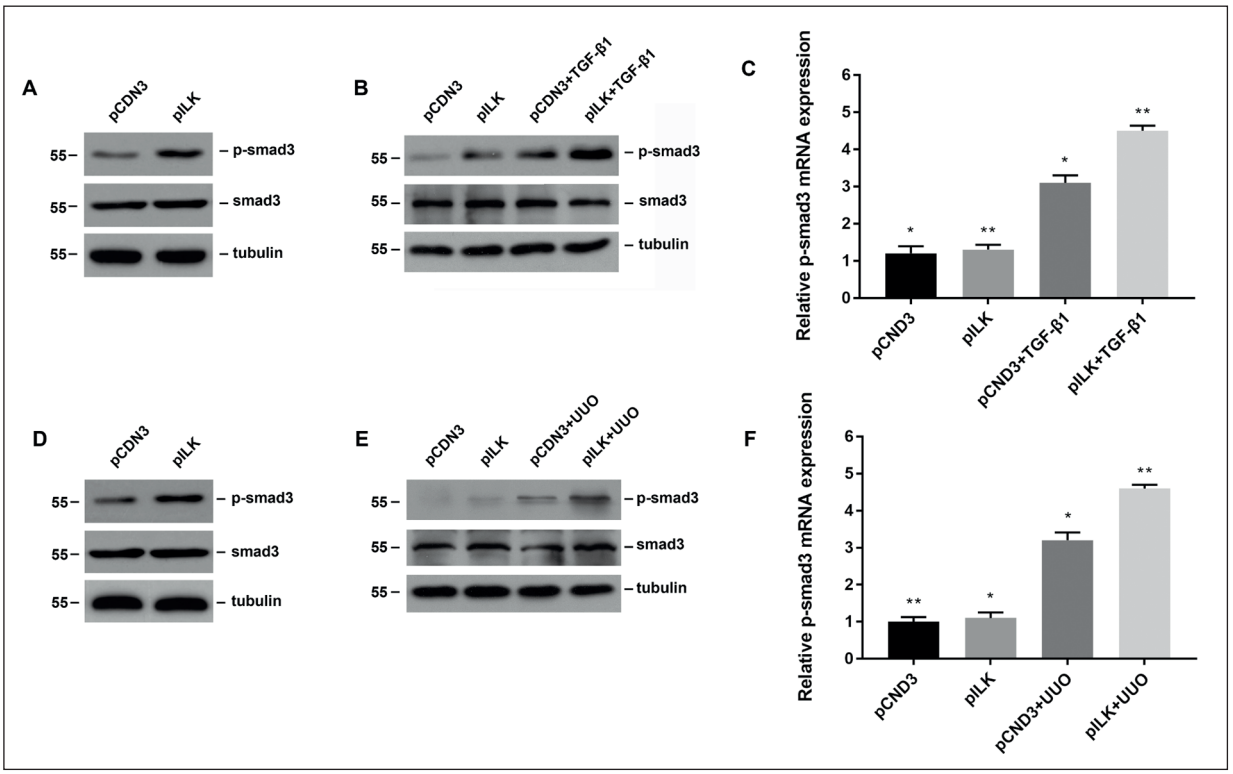

Figure 5. TGF-β1/smad pathway was activated during the process of renal tubular epithelial cell trans-differentiation. ***A,*** Protein expressions of p-smad3 and smad3 in NRK-52E cells after TGF-β1 induction. ***B,*** Protein expressions of p-smad3 and smad3 in NRK-52E cells after ILK overexpression. ***C,*** The mRNA level of p-smad3 in NRK-52E cells. ***D,*** Protein expressions of p-smad3 and smad3 in kidney tissues of UUO mice. ***E,*** Protein expressions of p-smad3 and smad3 in kidney tissues of UUO mice after injection of ILK overexpression plasmid. ***F,*** The mRNA level of p-smad3 in kidney tissues of UUO mice.

TGF-β1/Smad Pathway Was Activated During the Process of Renal Tubular Epithelial Cell Transdifferentiation

The TGF-β1/smad pathway is one of the main pathways involved in the regulation of renal tubular phenotype changes. Existing studies have shown that ILK is capable of activating the TGF-β1/smad pathway. In our study, TGF-β1 induction in NRK-52E cells upregulated protein level of p-smad3, which was more pronounced after ILK overexpression (Figure 5A and 5B). The mRNA level of p-smad3 presented the similar elevated trend (Figure 5C). *In vivo* experiments also found p-smad3 upregulation in UUO mice (Figure 5D). Injection of ILK overexpression plasmid remarkably elevated the protein and mRNA levels of p-smad3 in UUO mice (Figure 5E and 5F).

Discussion

Renal interstitial fibrosis is a common pathological manifestation of CKD. A large number of studies[16-19] have shown that TGF-β1/smad pathway is involved in regulating renal fibrosis. TGF-β1/smad signaling pathway also participates in the regulation of many pathophysiological processes, such as organ development, tissue homeostasis, and disease development.

Studies[22-25] have shown that ILK is involved in the regulation of the TGF-β1/smad signaling pathway in a variety of cells. In the present work, we first established a UUO mouse model. ILK expression was upregulated in renal fibrosis mice. Besides, ILK upregulation promoted the transdifferentiation of renal tubular epithelial cells to myofibroblasts. The above researches showed that ILK is a significant factor in regulating renal fibrosis. Subsequently, we observed that ILK can activate the TGF-β1/smad pathway in rat proximal tubular epithelial cells, further upregulating smad3 expression. The above data confirmed that ILK regulates EMT in kidney tissues *via* a TGF-β1/smad pathway.

There are still some. First of all, we didn't construct the tubule-specific $ILK^{-/-}$ mouse to provide

a direct correlation between ILK and renal fibrosis *in vivo*. Secondly, ILK is not only expressed in renal tubular epithelial cells, but also in fibroblasts. Further studies should be carried out to explore whether fibroblast-expressing ILK could regulate renal fibrosis.

Conclusions

We found that ILK is upregulated during the process of renal fibrosis. ILK participates in the development of renal fibrosis by altering phenotypes of renal tubular epithelial cells *via* a TGF-β1/smad pathway. We consider that ILK may serve as a potential target for alleviating CKD.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) McGrath MJ, Binge LC, Sriratana A, Wang H, Robinson PA, Pook D, Fedele CG, Brown S, Dyson JM, Cottle DL, Cowling BS, Niranjan B, Risbridger GP, Mitchell CA. Regulation of the transcriptional coactivator FHL2 licenses activation of the androgen receptor in castrate-resistant prostate cancer. Cancer Res 2013; 73: 5066-5079.
2) Lin J, Qin X, Zhu Z, Mu J, Zhu L, Wu K, Jiao H, Xu X, Ye Q. FHL family members suppress vascular endothelial growth factor expression through blockade of dimerization of HIF1alpha and HIF1beta. Iubmb Life 2012; 64: 921-930.
3) Cao CY, Mok SW, Cheng VW, Tsui SK. The FHL2 regulation in the transcriptional circuitry of human cancers. Gene 2015; 572: 1-7.
4) Genini M, Schwalbe P, Scholl FA, Remppis A, Mattei MG, Schafer BW. Subtractive cloning and characterization of DRAL, a novel LIM-domain protein down-regulated in rhabdomyosarcoma. DNA Cell Biol 1997; 16: 433-442.
5) Barnes JL, Gorin Y. Myofibroblast differentiation during fibrosis: role of NAD(P)H oxidases. Kidney Int 2011; 79: 944-956.
6) Fujigaki Y, Muranaka Y, Sun D, Goto T, Zhou H, Sakakima M, Fukasawa H, Yonemura K, Yamamoto T, Hishida A. Transient myofibroblast differentiation of interstitial fibroblastic cells relevant to tubular dilatation in uranyl acetate-induced acute renal failure in rats. Virchows Arch 2005; 446: 164-176.
7) Kriz W, Kaissling B, Le Hir M. Epithelial-mesenchymal transition (EMT) in kidney fibrosis: fact or fantasy? J Clin Invest 2011; 121: 468-474.
8) Le Douarin NM, Teillet MA. Experimental analysis of the migration and differentiation of neuroblasts of the autonomic nervous system and of neurectodermal mesenchymal derivatives, using a biological cell marking technique. Dev Biol 1974; 41: 162-184.
9) Zhou SG, Zhang W, Ma HJ, Guo ZY, Xu Y. Silencing of LncRNA TCONS_00088786 reduces renal fibrosis through miR-132. Eur Rev Med Pharmacol Sci 2018; 22: 166-173.
10) Kramann R, DiRocco DP, Humphreys BD. Understanding the origin, activation and regulation of matrix-producing myofibroblasts for treatment of fibrotic disease. J Pathol 2013; 231: 273-289.
11) Campanholle G, Ligresti G, Gharib SA, Duffield JS. Cellular mechanisms of tissue fibrosis. 3. Novel mechanisms of kidney fibrosis. Am J Physiol Cell Physiol 2013; 304: C591-C603.
12) Yu H, Ma Q, Lin J, Sun YF, Zheng F. Expression and purification of GST-FHL2 fusion protein. Genet Mol Res 2013; 12: 6372-6378.
13) Dahan J, Nouet Y, Jouvion G, Levillayer F, Adib-Conquy M, Cassard-Doulcier AM, Tebbi A, Blanc F, Remy L, Chen J, Cairo S, Werts C, Si-Tahar M, Tordjmann T, Buendia MA, Wei Y. LIM-only protein FHL2 activates NF-kappaB signaling in the control of liver regeneration and hepatocarcinogenesis. Mol Cell Biol 2013; 33: 3299-3308.
14) Friedrich FW, Reischmann S, Schwalm A, Unger A, Ramanujam D, Munch J, Muller OJ, Hengstenberg C, Galve E, Charron P, Linke WA, Engelhardt S, Patten M, Richard P, van der Velden J, Eschenhagen T, Isnard R, Carrier L. FHL2 expression and variants in hypertrophic cardiomyopathy. Basic Res Cardiol 2014; 109: 451.
15) Chu PH, Chen J. The novel roles of four and a half LIM proteins 1 and 2 in the cardiovascular system. Chang Gung Med J 2011; 34: 127-134.
16) Wang S, Zhao X, Yang S, Chen B, Shi J. Knockdown of NLRC5 inhibits renal fibroblast activation via modulating TGF-beta1/Smad signaling pathway. Eur J Pharmacol 2018; 829: 38-43.
17) Li SS, He AL, Deng ZY, Liu QF. Ginsenoside-Rg1 protects against renal fibrosis by regulating the Klotho/TGF-beta1/Smad signaling pathway in rats with obstructive nephropathy. Biol Pharm Bull 2018; 41: 585-591.
18) Zhang YF, Wang Q, Luo J, Yang S, Wang JL, Li HY. Knockdown of eIF3a inhibits collagen synthesis in renal fibroblasts via Inhibition of transforming growth factor-beta1/Smad signaling pathway. Int J Clin Exp Pathol 2015; 8: 8983-8989.
19) Li R, Wang Y, Liu Y, Chen Q, Fu W, Wang H, Cai H, Peng W, Zhang X. Curcumin inhibits transforming growth factor-beta1-induced EMT via PPARgamma pathway, not Smad pathway in renal tubular epithelial cells. PLoS One 2013; 8: e58848.
20) de la Puente P, Weisberg E, Muz B, Nonami A, Luderer M, Stone RM, Melo JV, Griffin JD, Azab AK. Identification of ILK as a novel therapeutic target for acute and chronic myeloid leukemia. Leuk Res 2015:

21) Stallings AM, Velez MW, Fiessinger LA, Piliang MP, Mesinkovska NA, Kyei A, Bergfeld WF. ILK index and regrowth in Alopecia areata. J Investig Dermatol Symp Proc 2015; 17: 47-49.

22) Pang M, Wang H, Rao P, Zhao Y, Xie J, Cao Q, Wang Y, Wang YM, Lee VW, Alexander SI, Harris DC, Zheng G. Autophagy links beta-catenin and Smad signaling to promote epithelial-mesenchymal transition via upregulation of integrin linked kinase. Int J Biochem Cell Biol 2016; 76: 123-134.

23) Qi F, Cai P, Liu X, Peng M, Si G. Adenovirus-mediated P311 inhibits TGF-beta1-induced epithelial-mesenchymal transition in NRK-52E cells via TGF-beta1-Smad-ILK pathway. Biosci Trends 2015; 9: 299-306.

24) Vi L, de Lasa C, DiGuglielmo GM, Dagnino L. Integrin-linked kinase is required for TGF-beta1 induction of dermal myofibroblast differentiation. J Invest Dermatol 2011; 131: 586-593.

25) Li Y, Yang J, Dai C, Wu C, Liu Y. Role for integrin-linked kinase in mediating tubular epithelial to mesenchymal transition and renal interstitial fibrogenesis. J Clin Invest 2003; 112: 503-516.