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FHL2 participates in renal interstitial fibrosis by altering the phenotype of renal tubular epithelial cells via regulating the β-catenin pathway

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Abstract. – OBJECTIVE: To investigate the potential role of FHL2 (four and a half LIM domains protein 2) in the renal interstitial fibrosis and its underlying mechanism.

MATERIALS AND METHODS: NRK-52E, the rat tubular epithelial cell line, was selected for the in vitro experiments. The unilateral ureteral obstruction (UUO) mouse model and phenotype changes of NRK-52E cells were induced by TGF-β (transforming growth factor-β) treatment. Protein and mRNA expressions of biomarkers of tubular cells and renal fibrosis in NRK-52E cells were detected. Meanwhile, phenotype changes of NRK-52E cells were detected after FHL2 overexpression. Furthermore, CD1 mice were selected for constructing the UUO mouse model. Protein and mRNA expressions of biomarkers of tubular cells and renal fibrosis in kidney tissues were detected. CD1 mice with FHL2 overexpression were constructed by tail vein injection of FHL2 plasmid for further observation of renal interstitial fibrosis. Expressions of β-catenin pathway-related genes were detected by Western blot and polymerase chain reaction (PCR), respectively.

RESULTS: The FHL2 expression was increased during the phenotype change of NRK-52E cells induced by TGF-β treatment. Overexpression of FHL2 led to a significant phenotype change. Similarly, the FHL2 expression was elevated in the UUO mouse model. Renal interstitial fibrosis was exaggerated and expression levels of genes related to the β-catenin pathway were increased after injection of FHL2 plasmid.

CONCLUSIONS: FHL2 is involved in renal interstitial fibrosis by altering the phenotype of renal tubular epithelial cells via regulating the β-catenin pathway.

Key Words:
FHL2, β-catenin, Renal interstitial fibrosis.

Introduction

Chronic kidney disease (CKD) is a global public health problem that endangers human health, with an incidence of up to 14% worldwide[1]. CKD is the third most common cause of death, followed by HIV (human immunodeficiency virus) and diabetes[2]. It not only seriously affects the life quality of CKD patients, but also brings tremendous economic burden to their families and society.

Progressive CKD will eventually lead to end-stage renal failure. Renal fibrosis is considered to be the key determinant of renal failure[3,4]. During the process of renal interstitial fibrosis, phenotypic changes of renal innate and interstitial cells are presented. Among them, the epithelial-to-mesenchymal transition (EMT) is of great importance in the renal fibrosis[5-8].

In recent years, accumulating investigations[9-12] have found that the activated β-catenin pathway can promote renal fibrosis. Among them, FHL2 (four and a half LIM domains protein 2) is an essential scaffold protein and adapter protein. FHL2 mediates protein-protein interactions by binding to protein molecules through different LIM domains and exerts a transcriptional function[13,14]. Researches[13-17] have shown that FHL2 can affect the transcriptional activation of β-catenin in different types of cells. Current works have explored the role of FHL2 in tumors and cardiovascular diseases; we, therefore, speculated whether FHL2 could regulate renal interstitial fibrosis via β-catenin pathway. We explored the FHL2 expression in the *in vitro* and *in vivo* models of renal fibrosis, thus exploring its biological function and underlying the potential mechanism in renal fibrosis.

Materials and Methods

Animal Model

12 male CD-1 mice were randomly assigned into four groups, namely sham surgery group, UUO Day 1 group, UUO Day 3 group and UUO Day 7 group, with 3 mice in each group. Briefly, mice

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were anesthetized by intraperitoneal injection of 70 mg/kg sodium pentobarbital (0.75%). Left lower abdominal incision was cut under aseptic conditions. Then, the left ureter was isolated and ligated with 4-0 sutures twice. Abdominal cavity was sutured layer by layer. Mice in the sham surgery group received the same procedure except for ligating the ureter. Mice were sacrificed on the postoperative 3rd, 7th, and 14th day. Kidney tissues of mice in the four groups were collected.

Cell Culture

Cells were maintained in DMEM/F12 (Dulbecco's Modified Eagle Medium) containing 10% fetal bovine serum, 100 μ/mL penicillin and 100 U/mL streptomycin, and incubated in a 5% CO_2 incubator at 37°C. Serum-free DMEM/F12 medium was replaced after cell confluence was up to 80% for overnight incubation. Cells in the experiment group were treated with different doses of TGF-β1.

Cell Transfection

Cells were seeded in a 6-well plate when the cell confluence was up to 90-95%. 1.5 mL of serum-free and antibiotic-free cell culture were replaced. Cell transfection was conducted according to the instructions of Lipofectamine 2000. Complete Dulbecco's modified eagle Medium (DMEM) was replaced after culturing for 6 h.

Western Blotting

Total proteins were extracted from cells by radioimmunoprecipitation assay (RIPA) method and then quantified using bicinchoninic acid (BCA) based on the instructions. The protocols of Western blot were previously described[18]. Briefly, proteins were separated in a sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and then transferred to a polyvinylidene difluoride (PVDF) membrane. Membranes were blocked with 5% skimmed milk for 1 h, followed by the incubation of primary antibody overnight. Membranes were incubated with the secondary antibody at room temperature for 1 h. Immunoreactive bands were exposed by enhanced chemiluminescence (ECL) method. The primary antibodies used in this study were as follows: fibronectin (FN) (cat: 3648, Sigma-Aldrich, St. Louis, MO, USA), α-smooth muscle actin (α-SMA) (cat: 5691, Sigma-Aldrich, St. Louis, MO, USA), E-cadherin (cat: ab1416, Abcam, Cambridge, MA, USA), actin (sc1616, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-FHL2 (K0055-3, clone 11-134, MBL), anti-phospho-β-Catenin (Ser 675) (9657, Cell Signaling Technology, Danvers, MA, USA), anti-plasminogen activator inhibitor-1 (anti-PAI-1), sc-8979, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-twist (sc-15393, Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Immunohistochemistry

Paraffin-embedded kidney tissue was cut into slices with 3 mm, paraffinized, and rehydrated by xylene, ethanol and purified water. Slices were then blocked with blocking solution for 30 min at room temperature. Primary antibodies were utilized for slice incubation overnight, followed by incubation of the corresponding secondary antibodies for 1 h at room temperature. Finally, slices were sealed, and Nikon Eclipse 80i microscope (Tokyo, Japan) was used for taking pictures.

Statistical Analysis

Each experiment was repeated for three times. Statistical product and service solutions (SPSS 19.0, Armonk, NY, USA) statistical software were utilized for analyzing data. All data were expressed as mean ± standard deviation. Comparison of measurement data was conducted using *t*-test. Comparison of data among groups was conducted using one-way ANOVA followed by Least Significant Difference (LSD). $p < 0.05$ was considered statistically significant.

Results

Phenotype of NRK-52E Cells Was Altered After TGF-β1 Treatment

TGF-β1 exerts a crucial role in renal interstitial fibrosis. In this study, NRK-52E cells were selected as the *in vitro* model. Decreased protein expression of E-cadherin was found after NRK-52E cells were treated with TGF-β1. However, the protein expressions of FN, α-SMA and FHL2 were increased in a time- and dose-dependent manner (Figure 1A-B). The mRNA expression of FHL2 was also increased (Figure 1C-D). The above results suggested that the phenotype of NRK-52E cells is altered and the FHL2 expression is increased after TGF-β1 treatment.

Overexpression of FHL2 in NRK-52E Cells Altered the Cell Phenotype

To further investigate the function of FHL2, FHL2 overexpression vector and blank vector

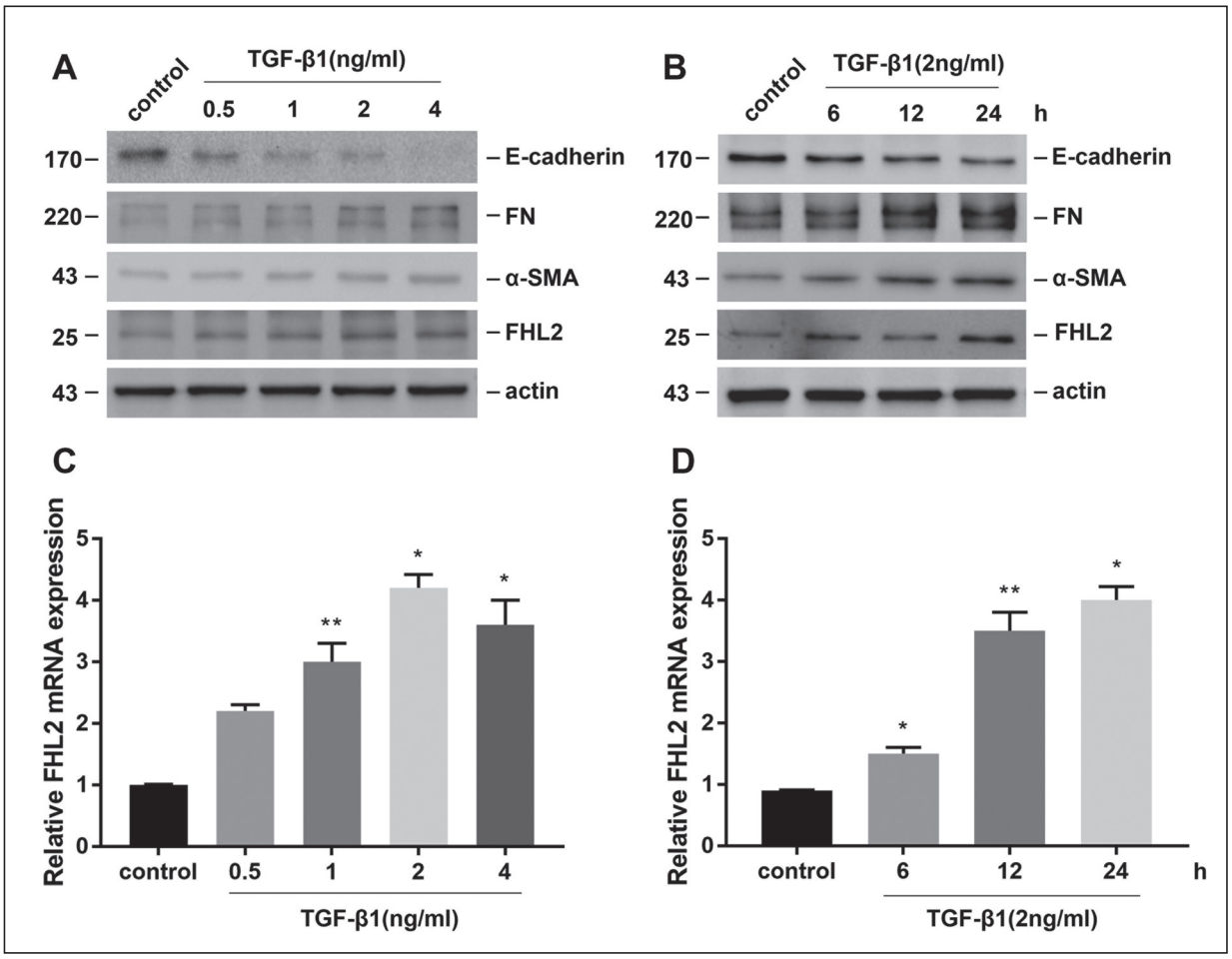

Figure 1. The influence of TGF-β1 in NRK-52E cell line. ***A,*** The protein expressions of E-cadherin, FN, α-SMA and FHL2 in NRK-52E cells stimulated with different doses of TGF-β1 for 48 h. ***B,*** The protein expressions of E-cadherin, FN, α-SMA and FHL2 in NRK-52E cells stimulated with 2 ng/ml TGF-β1 for different time points. ***C,*** The mRNA expression of FHL2 in NRK-52E cells stimulated with different doses of TGF-β1 for 48 h. ***D,*** The mRNA expression of FHL2 in NRK-52E cells stimulated with 2 ng/ml TGF-β1 for different time points (*$p < 0.05$, **$p < 0.01$).

pcDNA3 were transfected in NRK-52E cells. The transfection efficacy was shown in Figure 2A. The effect of up-regulated FHL2 on the phenotype of renal tubular epithelial cells was accessed. Our results suggested that overexpressed FHL2 led to downregulated E-cadherin and upregulated FN and α-SMA, which were similar to the outcome of TGF-β1 stimulation (Figure 2B). These data indicated that upregulated FHL2 in tubular epithelial cells promoted phenotype transdifferentiation of renal tubular cells.

Tubular Epithelial Cell Phenotype Was Altered and FHL2 Expression Was Increased in the UUO Mouse Model

To observe the FHL2 expression in fibrotic renal tissues, *in vivo* UUO model was constructed. The results showed that the E-cadherin expression was decreased. However, the expression levels of FN, α-SMA and FHL2 were elevated in a time-dependent manner (Figure 3A). Meanwhile, the mRNA expression of FHL2 was also remarkably increased (Figure 3B). Immunohistochemical staining results showed that positive expression of FHL2 was mainly observed in lesioned renal tubular epithelial cells and renal interstitial cells. Moreover, FHL2 was expressed in the nucleus of these cells (Figure 3C-D).

Up-regulated FHL2 Aggravated UUO-Induced Renal Interstitial Fibrosis

Mice were injected with the full-length FHL2 plasmid on the first day after the construction of UUO mouse model. The protein expression of

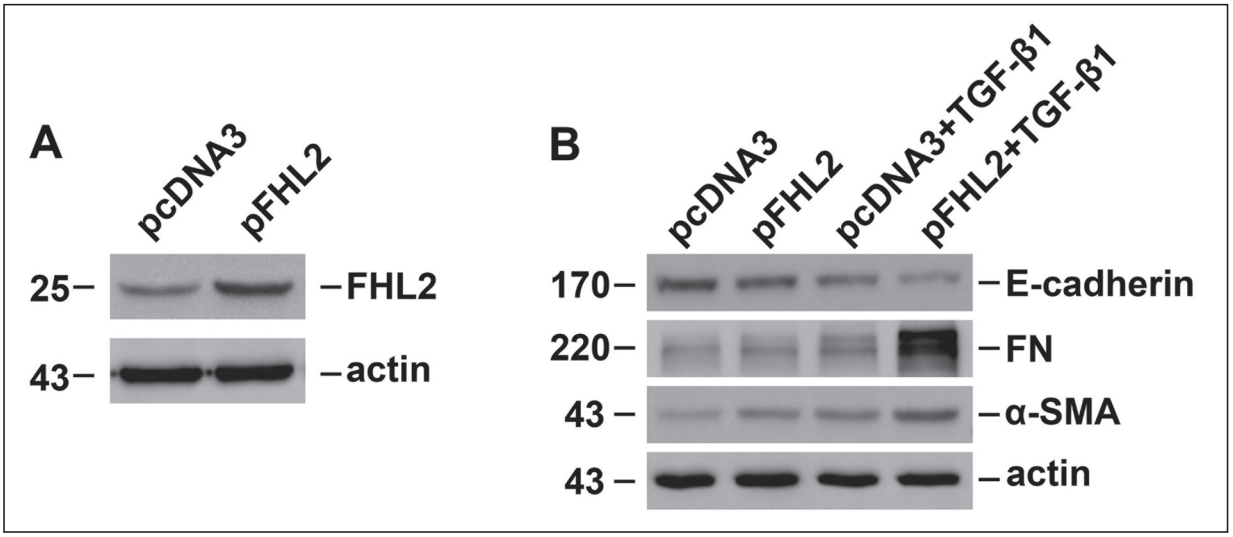

Figure 2. FHL2 enhances the effect of TGF-β1. ***A,*** The protein expression of FHL2 in NRK-52E cells transfected with FHL2 plasmid for 48 h. ***B,*** The protein expressions of E-cadherin, FN and α-SMA in different groups.

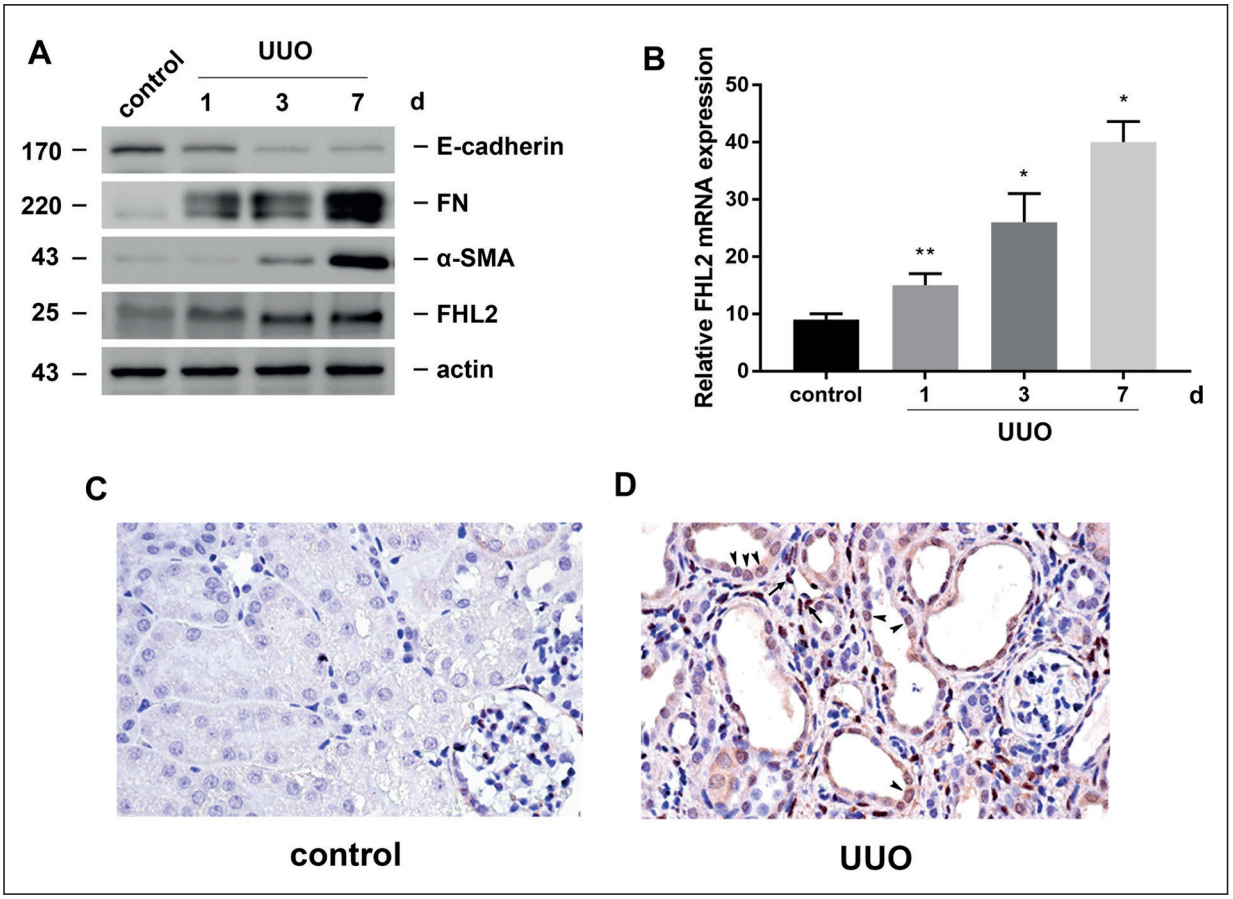

Figure 3. FHL2 is up-regulated in the process of renal interstitial fibrosis. ***A,*** The protein expressions of E-cadherin, FN, α-SMA and FHL2 in kidney tissues on the postoperative 1st, 3rd, and 7th d. ***B,*** The mRNA expression of FHL2 in kidney tissues on the postoperative 1st, 3rd, and 7th d (*$p < 0.05$, **$p < 0.01$). ***C,*** Immunohistochemical staining of FHL2 in kidney tissues of the control group (400×). ***D,*** Immunohistochemical staining of FHL2 in kidney tissues of the UUO 7 d group (400×).

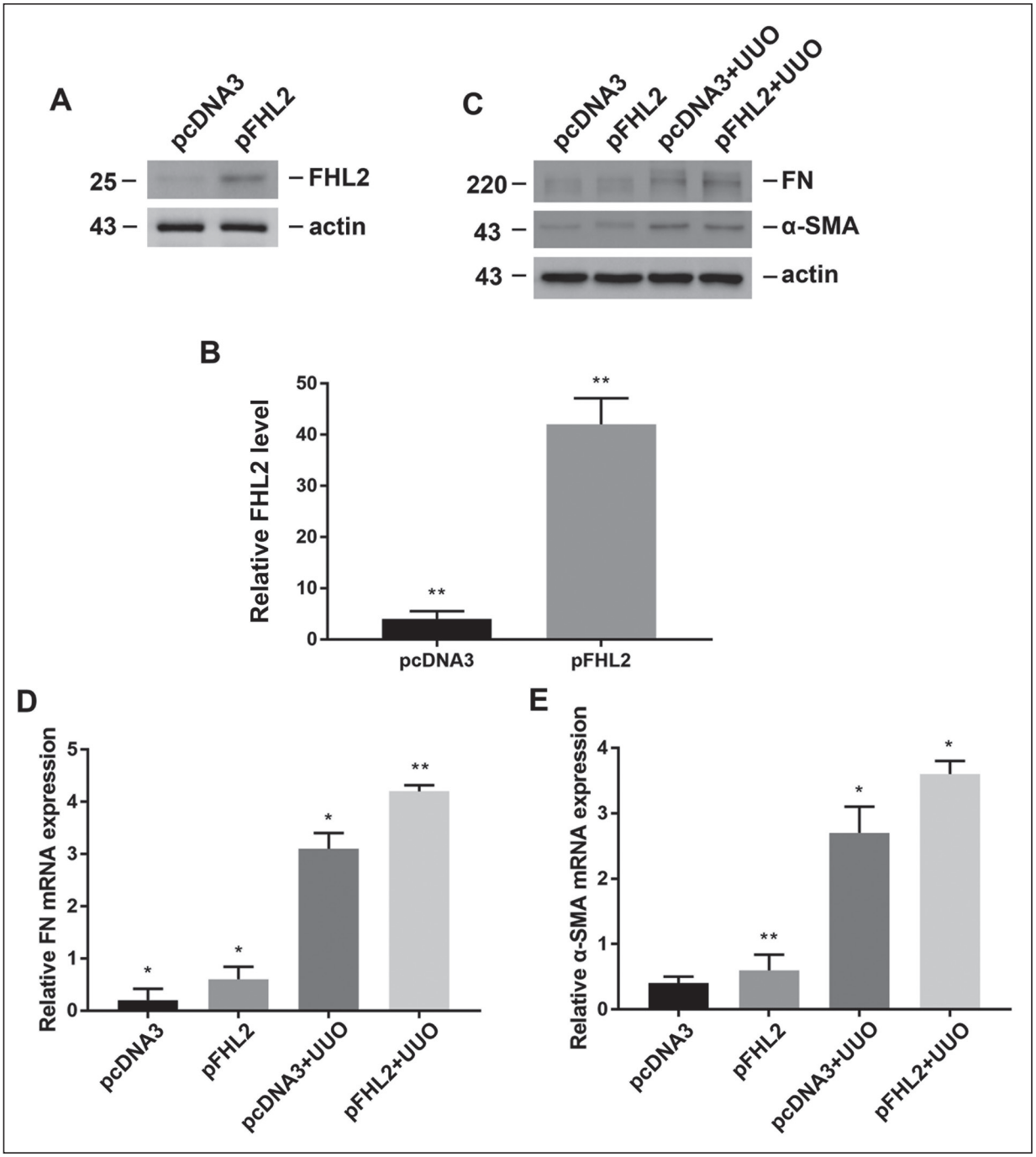

Figure 4. Up-regulated FHL2 accelerates the process of renal interstitial fibrosis. ***A,*** The protein expression of FHL2 in kidney tissues after tail vein injection of FHL2 plasmid for 24 h. ***B,*** The relative level of FHL2 in kidney tissues after tail vein injection of FHL2 plasmid for 24 h. ***C,*** The protein expressions of FN and α-SMA in kidney tissues of each group. ***D,*** The mRNA expression of FN in kidney tissues of each group. ***E,*** The mRNA level of α-SMA in kidney tissues of each group (*$p < 0.05$, **$p < 0.01$)

FHL2 in kidney tissues of the experimental group was significantly elevated (Figure 4A), as well as the relative amount of FHL2 (Figure 4B). Kidney tissues were collected 7 days after UUO and more severe renal tissue fibrosis was observed in FHL2-overexpressing mice (Figure 4C). Likewise, mRNA expressions of FN and α-SMA were also increased (Figure 4D).

β-Catenin Pathway Was Activated During the Transdifferentiation of Renal Tubular Epithelial Cells

β-catenin is considered as one of the major intracellular signals involved in the regulation of tubular phenotype. Previous investigations have shown that FHL2 may participate in the β-catenin pathway. Therefore, we observed expression levels of downstream factors of β-catenin pathway. The results showed that protein expressions of downstream factors in β-catenin pathway were significantly increased after TGF-β1 treatment, which was further improved by FHL2 overexpression (Figure 5A).

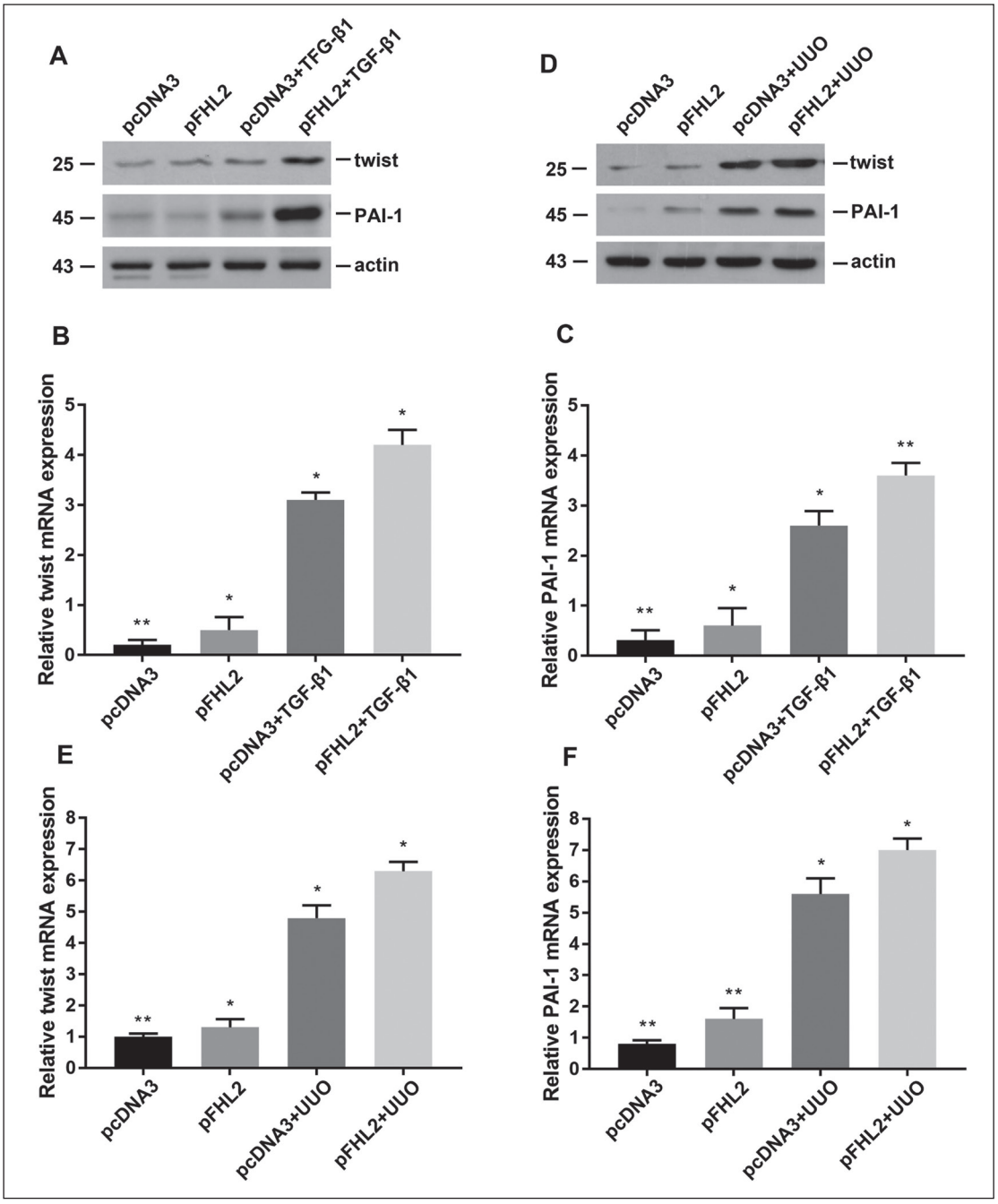

Figure 5. FHL2 is up-regulated and activate the β-catenin pathway. ***A,*** The protein expressions of Twist and PAI-1 in NRK-52E cells of each group. ***B,*** The mRNA expression of twist in NRK-52E cells of each group. ***C,*** The mRNA expression of PAI-1 in NRK-52E cells of each group. ***D,*** The protein expressions of Twist and PAI-1 in kidney tissues of each group. ***E,*** The mRNA expression of twist in kidney tissues of each group. ***F,*** The mRNA expression of PAI-1 in kidney tissues of each group (*$p < 0.05$, **$p < 0.01$).

The mRNA expressions of these factors were increased as well (Figure 5B-C). Similar results in the UUO mouse model were obtained (Figure 5D-F).

Discussion

Renal interstitial fibrosis is a common pathological manifestation of chronic renal insufficiency. Numerous experiments have shown that the activated β-catenin pathway can lead to renal fibrosis[11]. β-catenin pathway is a highly conserved and complex pathway. It participates in the regulatory of many pathophysiological processes, such as organ development, tissue homeostasis, and the development of human disease. β-catenin pathway is relatively quiescent in adult kidneys. However, growing evidence[19-23] has indicated that renal damage activates the β-catenin pathway.

Scholars[17,24] have shown that FHL2 interacts with β-catenin in several mammalian cells, including HKC293 (human embryonic kidney cells) and human foot cells. Therefore, the UUO mouse model was established for the following experiments. FHL2 is overexpressed in renal fibrosis, suggesting the predominant expression of FHL2 in renal tubular epithelial cells and fibroblasts. Up-regulated FHL2 by transfection of overexpression plasmids promoted the transdifferentiation of renal tubular epithelial cells into myofibroblasts. Meanwhile, overexpression of FHL2 also increased the production of extracellular matrix, such as FN. The above experiments indicated that FHL2 is a crucial factor in the regulation of renal fibrosis.

In this work, FHL2 enhanced transcription of target genes in rat proximal tubular epithelial cells, such as snail, twist, vimentin, PAI-1 and MMP-7 (matrix metalloproteinases-7). These target genes are closely associated with renal fibrosis regulated by over-activated β-catenin pathway[25,26]. The above data further confirmed that FHL2 is involved in EMT (epithelial-mesenchymal transition) and β-catenin pathway.

There were still some limitations in this work. Firstly, FHL2 knockdown mice was failed to be constructed. *In vivo* experiments could not be carried out to verify that FHL2 is closely related to the progression of renal fibrosis. Moreover, FHL2 is also expressed in fibroblasts, and the potential role of FHL2 in fibroblasts needs to be further explored.

Conclusions

We first showed that FHL2 regulates transdifferentiation of renal tubular cells and renal fibrosis in CKD via the β-catenin pathway, providing a potential therapeutic target for further treatment.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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