European Review for Medical and Pharmacological Sciences 2018; 22: 7621-7627

HGF alleviates renal interstitial fibrosis via inhibiting the TGF-β1/SMAD pathway

J. XU[1], T.-T. YU[2], K. ZHANG[1], M. LI[1], H.-J. SHI[1], X.-J. MENG[1], L.-S. ZHU[1], L.-K. ZHU[3]

[1]Department of Nephrology, Yancheng TCM Hospital Affiliated to Nanjing University of Chinese Medicine, Yancheng, China
[2]Department of Gastroenterology, The First People's Hospital of Yancheng, Yancheng, China
[3]Department of Science and Education, Zhangjiagang TCM Hospital Affiliated to Nanjing University of Chinese Medicine, Zhangjiagang, China

Jing Xu and *Tingting Yu* contributed equally to this work

Abstract. – OBJECTIVE: To study the role of HGF (stem cell growth factor) in renal interstitial fibrosis and to explore its underlying mechanism.

MATERIALS AND METHODS: A unilateral ureteral obstruction (UUO) mouse model was first constructed, and kidney samples of mice were then collected. Fibrosis-related indicators in UUO mice kidney were detected by Western blot. The mRNA and protein levels of HGF in UUO mice were detected by quantitative Real-time-polymerase chain reaction (qRT-PCR) and Western blot, respectively. The HGF overexpression mouse model was established by using UUO mice. For *in vitro* experiments, fibrosis-related indicators and the expression of HGF were detected in transforming growth factor-β1 (TGF-β1)-induced NRK-52E cells. Finally, a p-SMAD3 knockdown mouse model was established to confirm whether p-SMAD3 was involved in HGF-regulated renal interstitial fibrosis.

RESULTS: The expression levels of HGF and α-SMA (α-smooth muscle actin) were both significantly increased in UUO mice, while E-cadherin expression was significantly decreased, which were consistent with results of in vitro experiments. Overexpression of HGF remarkably decreased the protein and mRNA levels of α-SMA in fibrotic NRK-52E cells. After overexpression of HGF in UUO mice, α-SMA was remarkably downregulated, whereas E-cadherin was significantly upregulated. Further, results also demonstrated that HGF was upregulated and α-SMA was downregulated after p-SMAD3 knockdown in UUO mice.

CONCLUSIONS: HGF is highly expressed during renal interstitial fibrosis, which may suppress renal interstitial fibrosis by inhibiting the TGF-β1/SMAD signaling pathway.

Key Words:
HGF, TGF-β1/SMAD, Renal interstitial fibrosis.

Introduction

Chronic kidney disease (CKD) is a public health problem that seriously threatens human health. The global incidence of CKD is about 14%[1], and its mortality ranks only to HIV and diabetes[2]. Meanwhile, CKD not only seriously affects the life quality of patients, but also brings huge economic burden to families and society. Renal interstitial fibrosis is the final pathological outcome of CKD, which is the key factor determining the progression of renal failure[3,4]. It has been elucidated that renal interstitial fibrosis is closely related to epithelial-mesenchymal transition (EMT)[5-8], lipid homeostasis[9,10] and oxidative stress. In recent years, a large number of studies have shown that activation of the transforming growth factor-β1 (TGF-β1)/SMAD signaling pathway can promote renal interstitial fibrosis[11-13]. HGF (stem cell growth factor) exists in the plasma of animals with acute liver injury and can stimulate DNA synthesis of hepatocytes, thus promoting the process of liver regeneration. Reports have shown that HGF not only acts on liver regeneration, but also exerts an important role in the regulation of growth and differentiation of many tissues and cells. Moreover, it has been reported that HGF participates in cell regeneration[14-16], cell movement[17] and tumor necrosis[18-20]. Previous studies[21,22] have indicated that HGF is involved in the occurrence and develop-

Corresponding Author: Like Zhu, MM; e-mail: zjgszyyykjk@126.com

ment of kidney disease. Some researchers have also pointed out that HGF can interfere with the transduction of the TGF-β1/SMAD signaling pathway by inhibiting the nuclear accumulation of SMAD2 and SMAD3.

Due to the reason that the TGF-β1/SMAD signaling pathway was closely related to tissue fibrosis, we hypothesized that HGF participated in renal interstitial fibrosis *via* regulating the TGF-β1/SMAD signaling pathway. Our study aimed to provide a new therapeutic target for the treatment of renal interstitial fibrosis.

Materials and Methods

Experimental Animals

Male CD-1 mice weighing 18-20 g were obtained from Shanghai Laboratory Animal Center. According to the feeding standard of experimental animals, the mice were kept in SFP environment with allowed access to food and water. All mice were maintained in an environment with 23±2°C temperature and 55±10% relative humidity. These experiments were performed in accordance with the Institutional Animal Care. Our study was approved by the Animal Ethics Committee of Yancheng TCM Hospital Affiliated to Nanjing University of Chinese Medicine Animal Center.

Construction of a Unilateral Ureteral Obstruction (UUO) Mouse Model

Experimental mice were randomly assigned into the sham group, the Day-1 group, the Day-3 group and the Day-7 group, with 3 mice in each group. For construction of the UUO mouse model construction, mice were first anesthetized with 70 mg/kg pentobarbital sodium by intraperitoneal injection. Subsequently, kidney tissues were harvested at 3, 7 and 14 days after UUO procedure, respectively.

Cell Culture

All cells were cultured in Dulbecco's Modified Eagle Medium (D-MEM)/F12 (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin. All cells were maintained in a 37°C, 5% CO_2 incubator. The culture medium was replaced by serum-free medium for overnight culture. Cells were then induced with TGF-β1 for subsequent experiments.

Cell Transfection

Cells were seeded in 6-well plates and cultured in complete D-MEM/F12 medium. When cell confluence reached 90-95%, the culture medium was replaced by 1.5 mL serum-free D-MEM/F12. Briefly, 2.5 μg plasmid DNA and 5 μL Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) were added to 250 μL Opti-MEM, respectively, followed by incubation at room temperature for 5 min. Subsequently, the above two transfection solutions were mixed together and maintained at room temperature for 20 min. After 6 h, the culture medium was replaced once.

Construction of a p-SMAD3 Knockdown Mouse Model

The p-SMAD3 knockdown mouse model was constructed by tail vein injection of the p-SMAD3 plasmid. One day prior to the UUO procedure, 1 mg/kg p-SMAD3 plasmid or pcDNA3.1 was diluted into 2 mL normal saline, and immediately injected into the tail vein of mice within 10 s.

Western Blot

Total protein of transfected cells or kidney tissues was extracted. The concentration of each protein sample was detected by a bicinchoninic acid (BCA) kit (Pierce, Rockford, IL, USA). Briefly, 50 μg total protein were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were first blocked with 5% skimmed milk, followed by incubation of specific primary antibodies (α-SMA (α-smooth muscle actin), E-cadherin, anti-HGF, anti-p-SMAD3 and anti-SMAD3) at 4°C overnight. Next, the membranes were incubated with corresponding secondary antibody at room temperature for 1 h. Immunoreactive bands were finally exposed by enhanced chemiluminescence method (Thermo Fisher Scientific, Waltham, MA, USA).

Immunohistochemistry

Kidney tissues were embedded in paraffin wax, cut into 3 um slices, deparaffinized in xylene, and rehydrated in ethanol and pure water. The sections were blocked in the blocking buffer at room temperature for 30 min. Subsequently, the sections were incubated primary antibody at 4°C overnight, followed by the incubation with secondary antibody at room temperature for 1

h. Finally, immunohistochemistry results were captured using a Nikon Eclipse 80i microscope (Tokyo, Japan).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 16.0 Software (SPSS Inc., Chicago, IL, USA) were used for all statistical analysis. Measurement data were expressed as mean ± standard deviation ($\bar{x}\pm s$). Student's *t*-test was performed for comparing the difference between two groups. One-way ANOVA was used for comparison among different groups, followed by Post-Hoc Test (Least Significant Difference). $p < 0.05$ was considered statistically significant.

Results

Elevated Expression of HGF in UUO Mice

HGF is a pleiotropic, polypeptide-like cytokine that is widely distributed in human organs. Meanwhile, HGF is highly expressed in kidney, the expression of which is altered during the process of renal damage. To observe the changes of HGF expression in renal interstitial fibrosis, we constructed the most commonly used animal model of renal interstitial fibrosis, namely the UUO mouse model. Western blot results indicated that E-cadherin, a marker of tubular epithelial cell polarity, was remarkably decreased. However, α-SMA, a fibrotic marker, was significantly upregulated in UUO mice (Figure 1A). Meanwhile, we found that the protein level of HGF was increased in a time-dependent manner (Figure 1B). Upregulated mRNA expression level of HGF was also observed in UUO mice (Figure 1C). Immunohistochemistry results showed that positive expression of HGF in renal tubular epithelial cells was significantly increased 7 days after UUO treatment (Figure 1D).

Overexpression of HGF in TGF-β1-Induced Fibrotic NRK-52E Cells

Renal tubular epithelial cells in mice (NRK-52E) were treated with different concentrations of TGF-β1 for 48 h. Western blot results showed downregulated expression of E-cadherin, and upregulated expression of α-SMA and HGF in a dose-dependent manner (Figure 2A). With the increase of TGF-β1 concentration, the mRNA level of HGF increased gradually (Figure 2B). Subsequently, NRK-52E cells were treated with 5 ng/ml TGF-β1 for different time points. Results of

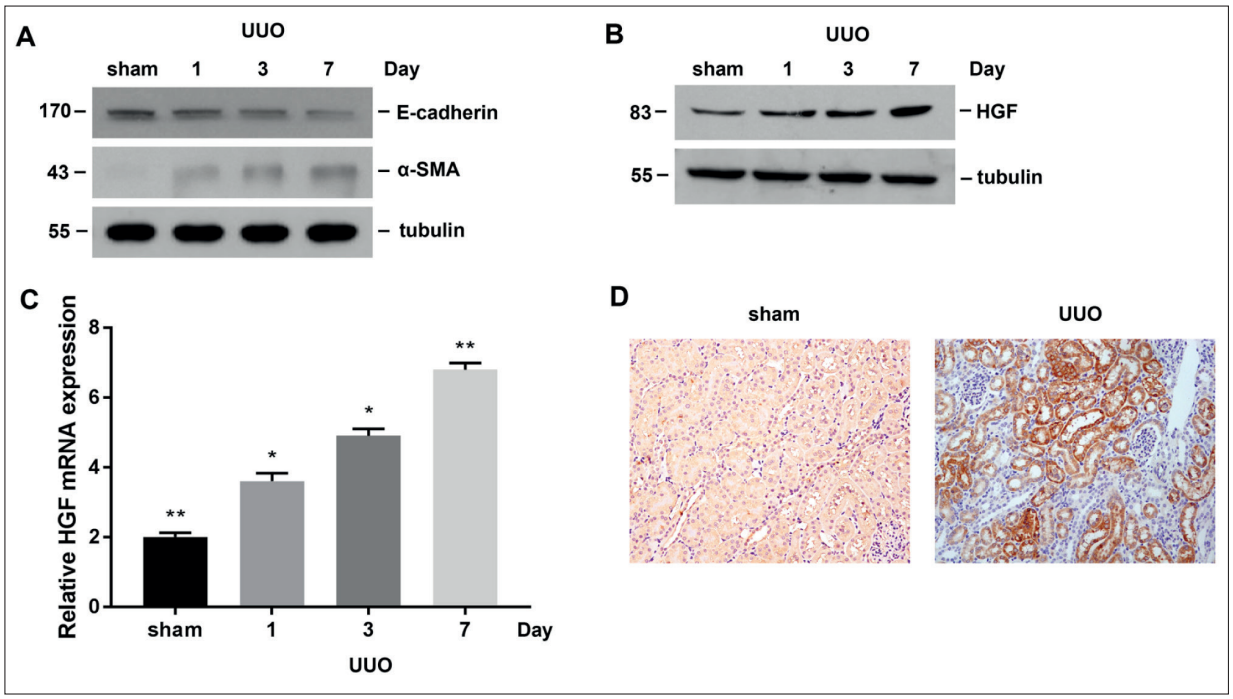

Figure 1. HGF was highly expressed in UUO mice. ***A,*** The protein expression levels of E-cadherin and α-SMA in kidney tissues of UUO mice. ***B,*** The protein expression level of HGF in kidney tissues of UUO mice. ***C,*** The mRNA expression level of HGF in kidney tissues of UUO mice. ***D,*** Immunochemistry results of HGF expression in kidney tissues of UUO mice and controls on the 7th day (400×).

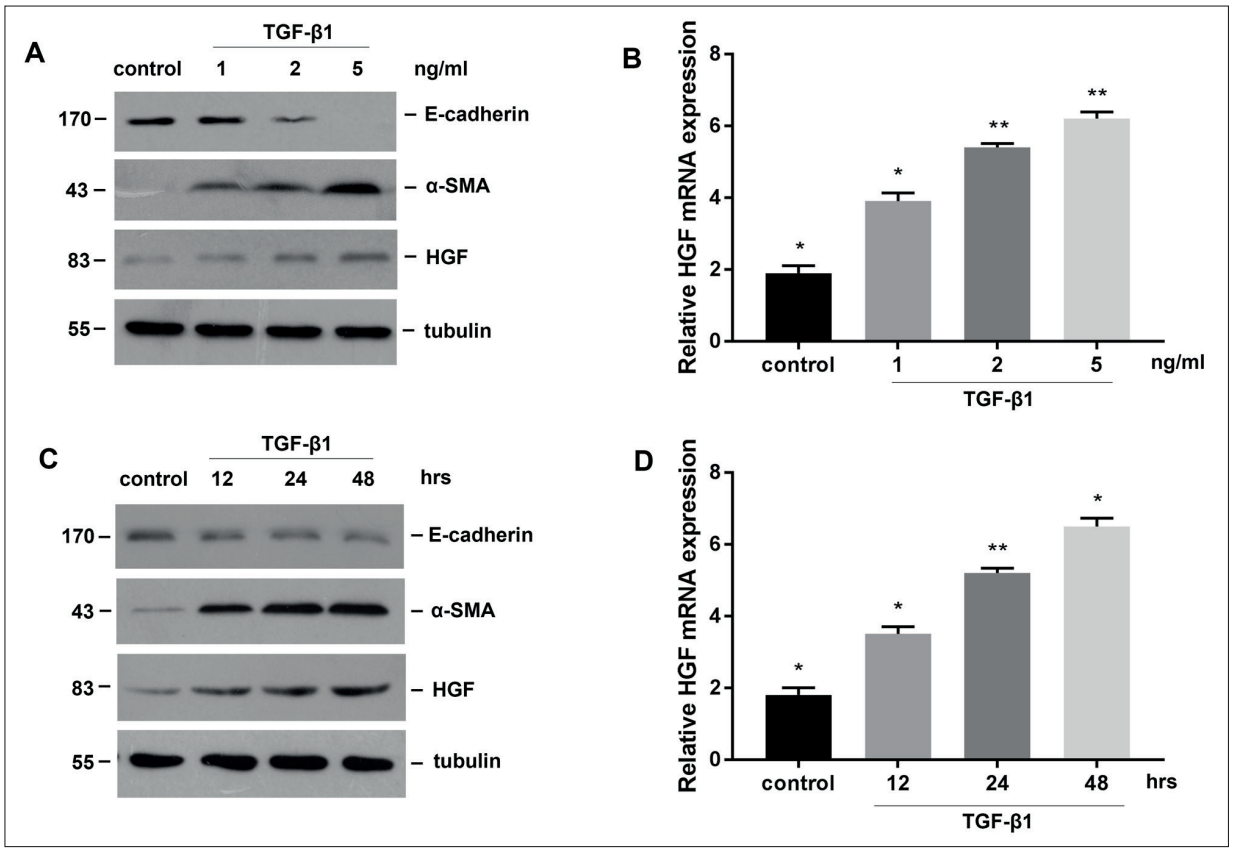

Figure 2. HGF was overexpressed in TGF-β1-induced fibrotic NRK-52E cells. ***A,*** The protein expression levels of E-cadherin, α-SMA and HGF in NRK-52E cells treated with different doses of TGF-β1. ***B,*** The mRNA expression level of HGF in NRK-52E cells treated with different doses of TGF-β1. ***C,*** The protein expression levels of E-cadherin, α-SMA and HGF in NRK-52E cells treated with 5 ng/mL TGF-β1. ***D,*** The mRNA expression level of HGF in NRK-52E cells treated with 5 ng/mL TGF-β1.

Western blot demonstrated that E-cadherin was downregulated, whereas α-SMA and HGF were increased in a time-dependent manner (Figure 2C). Similar results were observed in the changes of mRNA expression of E-cadherin, α-SMA and HGF (Figure 2D).

HGF Alleviated Renal Interstitial Fibrosis in NRK-52E Cells

We next explored the effect of HGF on renal interstitial fibrosis. Transfection efficiency of the HGF overexpression plasmid in NRK-52E cells was first verified by qRT-PCR (Figure 3A). Results demonstrated that overexpression of HGF significantly decreased the protein level of α-SMA in fibrotic NRK-52E cells (Figure 3B). Moreover, the mRNA expression level of α-SMA was also downregulated after HGF overexpression (Figure 3C). Tail vein injection of the HGF overexpression plasmid in UUO mice significantly increased the expression of *in vivo* (Figure 3D). On the 7th postoperative day, we found that α-SMA was remarkably downregulated in UUO mice with HGF overexpression, indicating that renal interstitial fibrosis was alleviated by HGF overexpression in UUO mice (Figure 3E).

HGF Alleviated Renal Interstitial Fibrosis via Inhibiting the TGF-B1/SMAD Pathway

Previous studies have shown that HGF affects liver fibrosis by inhibiting nuclear translocation and accumulation of SMAD2/3 *via* the TGF-β1/SMAD signaling pathway. Therefore, we hypothesized that HGF might also affect renal interstitial fibrosis *via* the TGF-β1/SMAD signaling pathway. Results showed that p-SMAD3 was significantly downregulated in UUO mice with higher level of HGF than that of controls (Figure 4A). Subsequently, we constructed a p-SMAD3 knockdown mouse model (Figure 4B). Western blot results demonstrated that the level of HGF

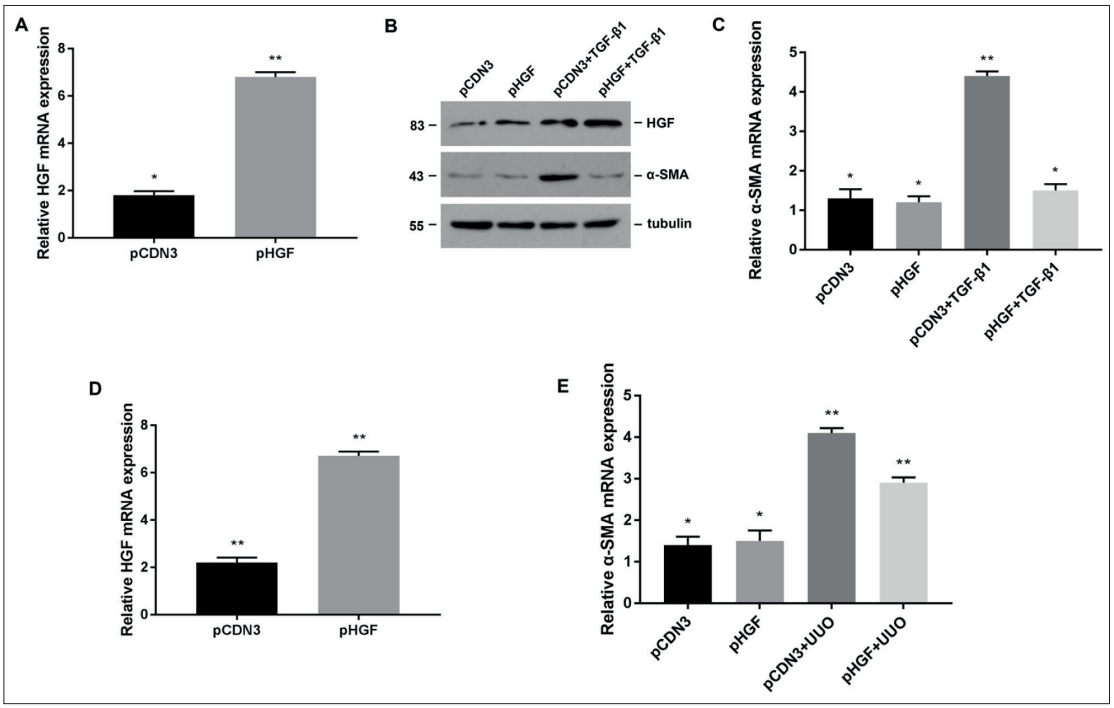

Figure 3. HGF alleviated renal interstitial fibrosis in NRK-52E cells. ***A,*** Transfection efficiency of the HGF overexpression plasmid. ***B,*** The protein expression levels of HGF and α-SMA after HGF overexpression. ***C,*** The mRNA expression level of α-SMA after HGF overexpression. ***D,*** HGF expression in UUO mice injected with overexpression plasmid and control mice. ***E,*** The mRNA expression level of α-SMA in UUO mice injected with overexpression plasmid and control mice.

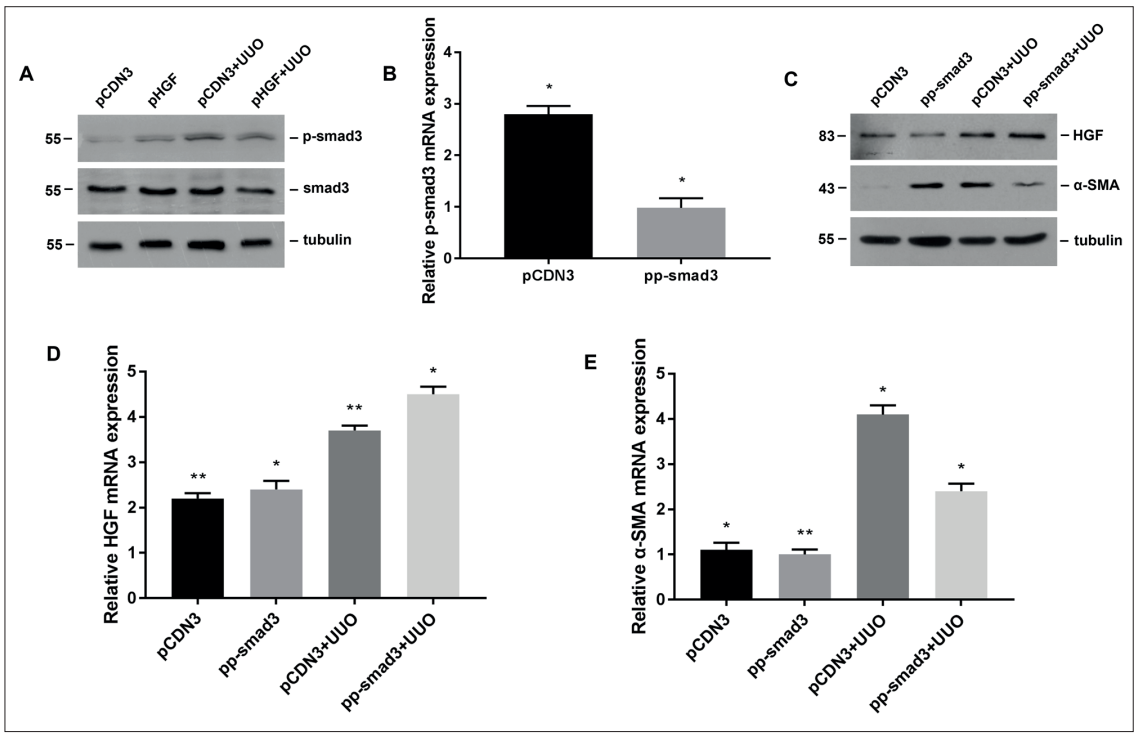

Figure 4. HGF alleviated renal interstitial fibrosis in UUO mice *via* inhibiting the TGF-β1/SMAD pathway. ***A,*** The protein expression levels of p-SMAD3 and SMAD3 in NRK-52E cells. ***B,*** The expression of p-SMAD3 in p-SMAD3 knockdown UUO mice and control mice. ***C,*** The protein expression levels of HGF and α-SMA in kidney tissues. ***D,*** The mRNA expression level of HGF in kidney tissues. ***E,*** The mRNA expression level of α-SMA in kidney tissues.

was increased while the level of α-SMA was decreased in p-SMAD3 knockdown mouse after UUO procedure (Figure 4C). Similar results were also observed in the changes of HGF and α-SMA mRNA expression (Figure 4D, 4E).

Discussion

Renal interstitial fibrosis is a common pathological manifestation of CKD. Multiple studies [11-13] have shown that abnormal activation of the TGF-β1/SMAD signaling pathway can cause renal fibrosis. The TGF-β1/SMAD signaling pathway is highly conserved and participates in the regulation of numerous pathophysiological processes, such as organ development, tissue homeostasis and disease development.

To explore the interaction between the TGF-β1/SMAD signaling pathway and renal interstitial fibrosis, we established a UUO mouse model. Results showed that HGF was highly expressed in UUO mice. Immunohistochemistry indicated that HGF was mainly distributed in renal tubular epithelial cells and fibroblasts. Meanwhile, HGF overexpression in UUO mice could significantly alleviate renal interstitial fibrosis. Relative studies have shown that HGF attenuates the TGF-β1/SMAD signaling pathway by reducing nuclear accumulation of SMAD2 and SMAD3. Hence, we suggested that HGF alleviated renal interstitial fibrosis by inhibiting the TGF-β1/SMAD signaling pathway. In the present study, a p-SMAD3 knockdown mouse model was established by injection of corresponding plasmids into the tail vein of UUO mice. Subsequent experiments showed that p-SMAD3 knockdown resulted in upregulated HGF and downregulated α-SMA expression in UUO mice. Our results demonstrated that HGF alleviated renal interstitial fibrosis by inhibiting the TGF-β1/SMAD pathway. There are still some limitations in this study: (1) since we failed to construct $HGF^{-/-}$ mice, we lack *in vivo* evidence to prove the direct interaction between HGF and renal interstitial fibrosis; (2) HGF was also expressed in fibroblasts in addition to tubular epithelial cells; (3) the lack of in-depth experiments on exploring the role of fibroblasts in renal interstitial fibrosis.

Conclusions

We showed that HGF is highly expressed during renal interstitial fibrosis, which may reduce renal interstitial fibrosis by inhibiting the TGF-β1/SMAD signaling pathway.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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