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FGF23 promotes renal interstitial fibrosis by activating β-catenin

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Abstract. – OBJECTIVE: To investigate the role and mechanism of fibroblast growth factor 23 (FGF23) in renal interstitial fibrosis.

MATERIALS AND MATERIALS: Rat renal tubular epithelial cell line (NRK-52E) was selected for in vitro experiments. Effect of FGF23 on extracellular matrix was observed. High expression of FGF23 was induced by injecting the plasmid into the caudal vein. The model of unilateral ureteral obstruction (UUO) was established for *in vivo* experiments.

RESULTS: FGF23 increased the expression of extracellular matrix proteins FN, α-SMA and Type 1 collagen of NRK-52E induced by TGFβ1, while FGF23 increased the expression p-β-catenin 675. In UUO model mice, fibrosis the FGF23 high expression group increased significantly compared to that of the control group. Meanwhile, β-catenin signal was activated.

CONCLUSIONS: FGF23 can promote the deposition of extracellular matrix NRK-52E induced by TGFβ1 in vitro. It aggravates the degree of renal interstitial fibrosis in UUO model, which is related to the activation of β-catenin signaling pathway.

Key Words:
FGF23, NRK-52E, renal interstitial fibrosis, β-catenin.

Abbreviations

ADHR = autosomal dominant hypophosphatemic rickets; BALP = bone serum alkaline phosphatase; CKD = chronic kidney disease; FGF23 = fibroblast growth factor 23; HS = heparan sulfate; NaPi = sodium-phosphorus cotransporter IIa; PBS = phosphate buffered saline; UUO = unilateral ureteral obstruction.

Introduction

Chronic kidney disease (CKD) is a worldwide public health problem and its incidence is increasing year by year[1,2]. The prevalence of CKD in China is about 10.8%[3]. Chronic kidney disease is a great burden to society and family. As a consequence, a better understanding of the pathophysiology of chronic kidney disease is crucial for the prevention and treatment of the disease.

Renal interstitial fibrosis is the end result of almost all chronic kidney disease development and is the leading cause of end-stage renal disease[4,5]. Studies have shown that tubulointerstitial lesions are more important than glomerular lesions in the progression of renal disease. Tubulointerstitial fibrosis is characterized by extracellular matrix deposition (ECM). Many cytokines and pathologies have been found to induce the production of ECM[7,8], such as Ang II, PDGF, TNFα, FGF23, among them, fibroblast growth factor 23 is mainly secreted by osteoblasts[9]. FGF23 is the earliest found to be the main pathogenic gene of autosomal dominant hypophosphatemic rickets (ADHR). Due to the decreased binding capacity with heparan sulfate (HS), FGF23 plays a role mainly through the complex of cell surface fibroblast growth factor receptors (FGFRs) and α-Klotho[10]. Physiologically, FGF23 plays a role in maintaining the balance of blood phosphorus and regulating vitamin D metabolism. FGF23 acts on the tubular epithelial cells to displace sodium-phosphorus cotransporter IIa (NaPiIIa), which is located on the brush border of proximal tubule epithelial cells, taking to its degradation. It subsequently decreases NaPiIIa protein and increases urinary phosphate excretion. To accelerate its degradation, FGF23 also induces 1,25-(OH) 2-hydroxyvitamin D3 by reducing the expression of renal α-hydroxylase mRNA and increasing the activity of 25-hydroxyvitamin D3-24 hydroxylase 2-VitD3 destruction. 1,25-(OH) 2-VitD3 reduction can stimulate parathyroid hormone secretion[11,12].

In recent years, the role of FGF23 in CKD has received widespread attention. In patients with CKD, plasma FGF23 level begins to rise early in the disease. As the disease is progressed, FGF23

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level increases sharply. After dialysis, FGF23 may increase to 100-1000 times than that of normal individuals[13,14]. Several clinical studies[15-20] have shown that elevated level of FGF23 is closely related to the occurrence of cardiovascular events in CKD patients. Recent studies showed that FGF23 can participate to the development of CKD by affecting the chemotaxis and migration of neutrophils[21]. However, the pathophysiological significance and mechanism of FGF23 in chronic kidney disease remains unclear. In this experiment, we focus on the involved function of FGF23 in the occurrence of renal interstitial fibrosis and its specific effect. Finally, the possible signaling pathways is further studied.

Materials and Methods

Cell Culture

NRK-52E cells, purchased from Chinese Academy of Sciences Library (Shanghai, China), were cultured in Dulbecco's modified eagle Medium (DMEM) with 10% fetal bovine serum (FBS) in a CO_2 incubator at 37°C with 5% CO_2, 95% air and passaged or plated at 6-well or 12-well. Then, they were treated according to the experimental purpose.

Animal Model

16-18 g male CD-1 mice were chosen for FGF23 high expression model. The over-expression mouse model was established through the tail vein injection of FGF23 plasmid pcDNA3.1(+) was injected into normal littermate mice as comparison. 1 day before the surgery, 1 mg/kg FGF23 high expression plasmid was dissolved in 2 ml normal saline and injected rapidly from the tail vein of the rat for 10 s. The control group was injected with the same concentration of pcDNA3.1 + and the same volume saline. In the past, it was found that the injection began to express after 16 h, and a single injection and a certain expression were detected after 7 days. Therefore, in this experiment, plasmids were injected every 7 days to maintain the concentration of serum FGF23. This study was approved by the Animal Ethics Committee of Chongqing Medical University Animal Center.

Western Blotting

To grind tissue by lysate on ice or to collect cells with cell scraping, cells were centrifuged at 16,000 g for 30 min; then, the supernatant was collected. BCA protein detection kit was used for protein concentration and, then, 4 × SDS sample buffer and deionized water were used to make samples to the same concentration. The cell sample was 10-20 μg/well. After conventional electrophoresis, primary antibodies were incubated at 4°C for 16 h, including FN (F3648, Sigma-Aldrich, St. Louis, MO, USA), α-SMA (ab5694, Abcam, Cambridge, MA, USA), tubulin (T6199, Sigma-Aldrich, St. Louis, MO, USA), GAPDH (G5262, Sigma-Aldrich, St. Louis, MO, USA), Collagen I (ab34710, Abcam, Cambridge, MA, USA), and PAI-1 (sc-27779, Abcam, Cambridge, MA, USA). After washing three times, cells were incubated with secondary antibody at room temperature for 2 hours. Bands were developed and exposed by enhanced chemiluminescence (ECL) (Shanghai Bi Yuntian Biotechnology Co., Ltd., Shanghai, China), with tubulin as the loading control. The relative expression of each protein was calculated by the ratio of IOD to control IOD in each band.

Quantitative Real-time PCR (qRT-PCR)

The cDNA strand synthesis in a 20 μL system was prepared, including 1 μg of total RNA, 10 μL of 2 × RT Buffer, 2 μL of RNA Mix Enzyme, 1 μL of OligDT and RNase-free deionized water. Next, the mixture was incubated at 42°C for 45 min and then at 85°C for 5 min as the reverse transcriptase inactivation. The cDNA obtained by the reaction was diluted 10 times and stored at -20°C. The method used for Real-time PCR amplification was SYBR Green (Invitrogen, Carlsbad, CA, USA). Quantitative PCR reaction system (20 μL) contained 10 μL of SYBR Green qPCR Mix (Hoffmann-La Roche, Basel, Switzerland), 1 μL of upstream and downstream primers, respectively, 1 μL of diluted cDNA and 7 μL of RNAase-free water. Reaction steps included pre-denaturation at 95°C for 5 min, denaturation at 95°C for 15 s, annealing at 60°C for 40 s and extension at 70°C for 30 s. The above reactions were repeated for 40 cycles. The expression of the target gene was calculated by relative quantification.

Immunofluorescent Staining

Fresh tissue was fixed in methanol-acetone solution (1:1) for 10 min at -20°C and, then, washed twice with phosphate buffered saline (PBS) solution containing 0.1% bovine serum albumin (BSA) at room temperature for 15 min.

Tissues were treated with 0.5% Triton X-100 (Dow Chemical Co., Midland, MI, USA) for 10 min and washed twice with phosphate buffered saline (PBS) containing 0.1% BSA for 15 min each. Frozen sections of mouse tissue were incubated for 1 h at room temperature. Then, tissues were incubated with PBS containing 0.1% Triton X-100 and PBS containing 2% BSA; then, they were blocked for 45 min at room temperature. The primary antibody was diluted with 2% BSA in PBS and incubated overnight at 4°C. Tissues were washed three times with the PBS solution at room temperature for 15 min. The secondary antibodies (1:50-1:100) were used to incubate at room temperature for 1 h. Next, tissues were washed with PBS containing 0.1% BSA three times at room temperature for 20 min. Then, nuclei were counterstained and fixed. Finally, fluorescence images were taken with Nikon Eclipse E600 fluorescence microscope (Nikon, Tokyo, Japan).

Statistical Analysis

Statistical analysis was performed by SPSS 22.0 statistical software (Version X; IBM [illegible] monk, NY, USA). All measurement data w[illegible] pressed as mean ± SEM (Mean ± SEM). On[illegible]y ANOVA (LSD or SNK) was used for compar[illegible] among multiple experimental groups. Stud[illegible] *t*-test was used for comparing e[illegible]ental da[illegible] between the two groups. $p < 0$ [illegible]nsidered to be statistically significan[illegible]

[illegible]ults

FGF23 Overe[illegible]on Promot[illegible] TGFβ1-indu[illegible] Ex[illegible]llular Matrix Producti[illegible]n NRK-52[illegible]ells

Tubul[illegible]ithelial cells are [illegible]ey components of inn[illegible] cells i[illegible] kidney. Dur[illegible]g the process of ren[illegible]jury, t[illegible]ar epithelial cells induce the exp[illegible] [illegible]rotic m[illegible]cules such as TGFβ1, endoth[illegible]d loss[illegible]egeneration by increas[illegible]e sy[illegible] [illegible]active oxygen species and in[illegible]matory [illegible]ines, which is involved in the [illegible]urrence o[illegible] renal interstitial fibrosis[22,23]. To e[illegible]ct of FGF23 on tubular epithelial [illegible]s, we fi[illegible] overexpressed FGF23 by transient [illegible]nsfection of full-length FGF23 plasmid and, [illegible] induced tubular damage using TGFβ1 (2 ng/[illegible]). According to the different treatments, it was divided into: pcDNA3.1 (+) group, pFGF23 group, pcDNA3.1 (+) + TGFβ1 group, and pFGF23 + TGFβ1 group. The results showed that FGF23 mRNA level increased about 170-fold in NRK-52E cells after being transfected with 1.5 ug FGF23 plasmid for 18 h (Figure 1A). W[illegible] showed that compared with pcDNA3[illegible]) TGFβ cells, the extracellular matrix pr[illegible]s, such as FN and a-SMA, increased sign[illegible]y (Figure 1B-C). Immunofluorescence staining [illegible]lts was consistent with Western bl[illegible]sults (Fi[illegible]D).

Serum FGF23 Leve[illegible]as Sic[illegible]cantly Increased in Ren[illegible]erst[illegible] Fibr[illegible]is Model UUO Mi[illegible]

Epidemiolog[illegible]s show[illegible]t in[illegible]y CKD patients, plas[illegible]GF23 expre[illegible]gins to increase and[illegible]isease progr[illegible]s to the advanced st[illegible] of re[illegible]ease, plasma FGF23 concentration in patients [illegible] 100-1000 times than tho[illegible]mal individu[illegible]owever, expression i[illegible] UUO model is not yet apparently clear. We [illegible]cted CD-1 m[illegible] they were sacrificed at 0, 1, [illegible] and 21 day[illegible]fter UUO, and blood, urine, a[illegible]dney tissu[illegible]mples were collected. Firstly, we [illegible] serum level of FGF23 in the mice. [illegible]ults showed that the serum level [illegible]f FGF23 reached a peak at the first day after [illegible]nd decreased at postoperative day 3 and [illegible]y 7. However, up to 21 days, the level of FGF23 remained at a high level (Figure 2A). Subsequently, we detected the mRNA level of FGF23 in renal tissue. As shown in Figure 3B, the mRNA level of FGF23 in the contralateral kidney tissue after UUO remained unchanged. In the UUO side renal tissue, the mRNA level of FGF23 was not altered at 1 day and 3 days after UUO, and there was a significant increase at postoperative day 7 and day 21 (about 10 times). It suggested that FGF23 level in the early stage of disease has little relationship with the kidney synthesis; so, kidney is not the main source of FGF23.

High Expression of FGF23 May Aggravate Renal Interstitial Fibrosis in UUO Mice

We first established a mouse model overexpressing FGF23. Plasmid injection was performed once a week, and a small amount of blood samples was collected from the orbital vein on the next day. Quantitative PCR showed that the mRNA level of FGF23 in liver tissue reached peak at the first day after injection, and decreased at postoperative day 3, day 7, and day 14, but still remained at a high level (Figure 3A). ELISA results showed that FGF23 in mice was maintained

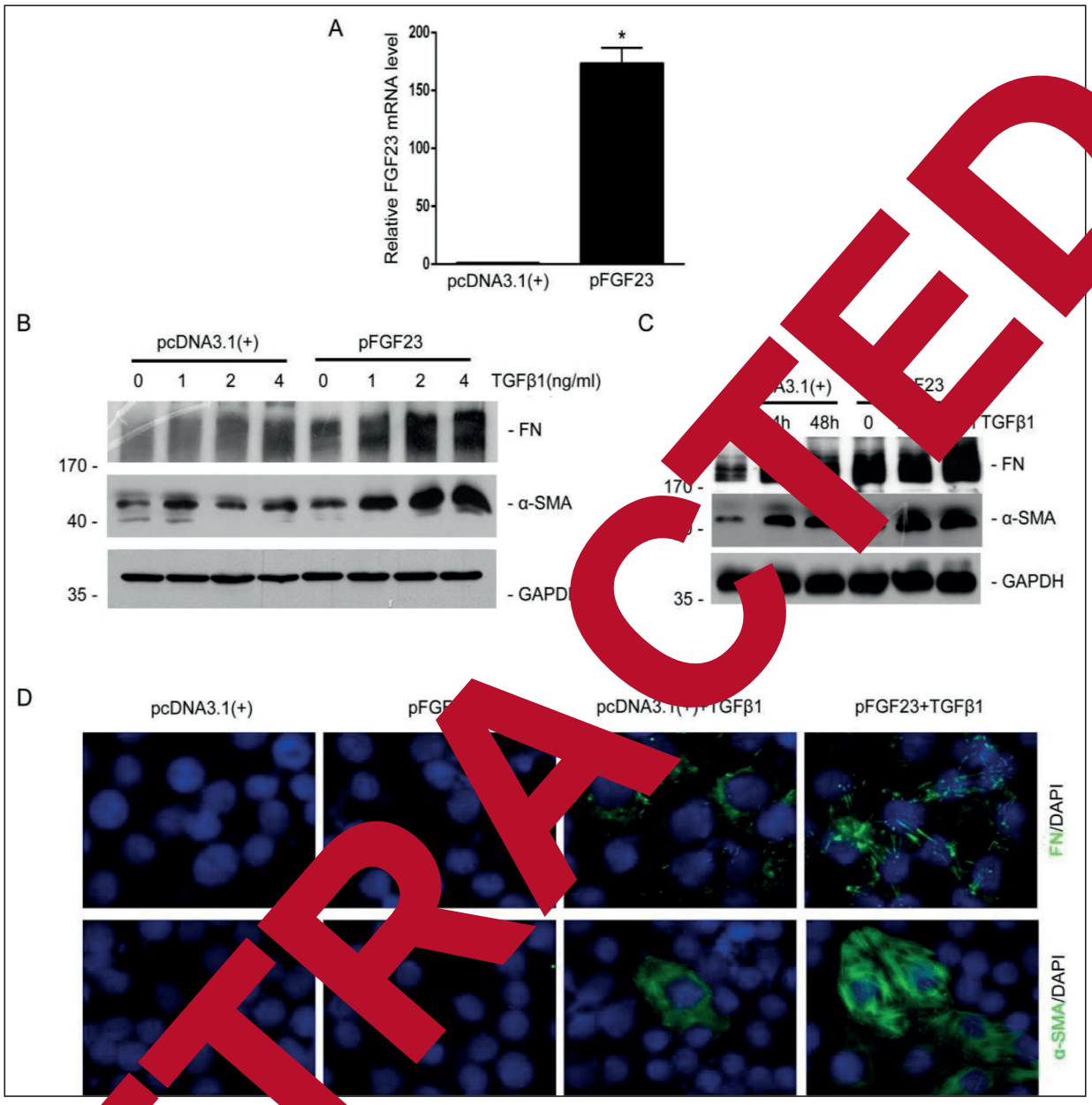

Figure GF23 overexpressio motes TGFβ1-induced extracellular matrix production in NRK-52E cells. ***A,*** Quanti on of m A level of FGF 3 in NRK-52E cells after transfected with full-length pFGF23 plasmid (*$p < 0.05$, n = 3). ***B*** F23 inc s the extracellular matrix production induced by TGFβ1 in a dose-dependent manner in NRK-52E cells. ***C,*** p inc s TGFβ1 duced extracellular matrix production in a time-dependent manner in NRK-52E cells. ***D,*** 48 h after tr mmun escence detection of the expressions of FN, α -SMA and other molecules.

at a h level (F gure 3B). Therefore, for *in vivo* e e injected the plasmids weekly at first day after the success of the UUO model mice. Then, the full-length FGF23 plasmid was d. 7 days after the operation, PAS, Masson, and rius Red stains were performed on mouse kidney tissues (including the contralateral and UUO side renal tissues). We found that there was no evident pathological change in the pFGF23 group compared with the pcDNA3.1 (+) group in the contralateral kidney tissue. However, in the UUO side kidney tissue, collagen deposition in pFGF23 group was significantly enhanced compared with the pcDNA3.1 (+) group (Figure 3C). In the same group, contralateral kidney tissue, extracellular matrix protein in pFGF23 group

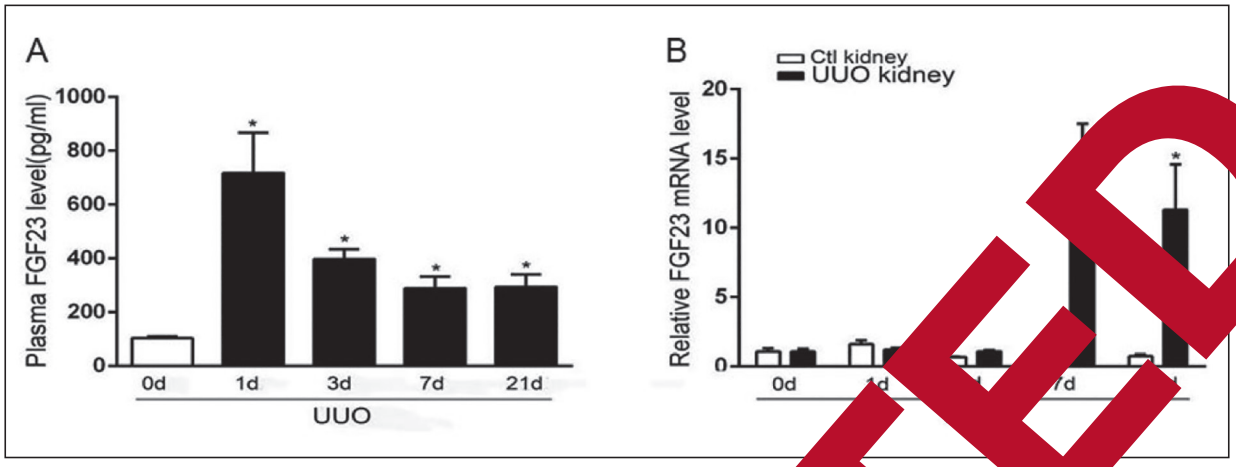

Figure 2. Serum level of FGF23 is significantly increased in the UUO mouse [illegible] interstitial [illegible]s. ***A,*** After the mice were modeled by UUO, their serum levels of FGF23 were measured at [illegible]3, 7 a[illegible]ays postoperatively (*$p < 0.05$, n = 4-5). ***B,*** Q-PCR showed the mRNA level of FGF23 in the renal tissues of the contralater[illegible]UUO sides at different time points after UUO (*$p < 0.05$, n = 4-5).

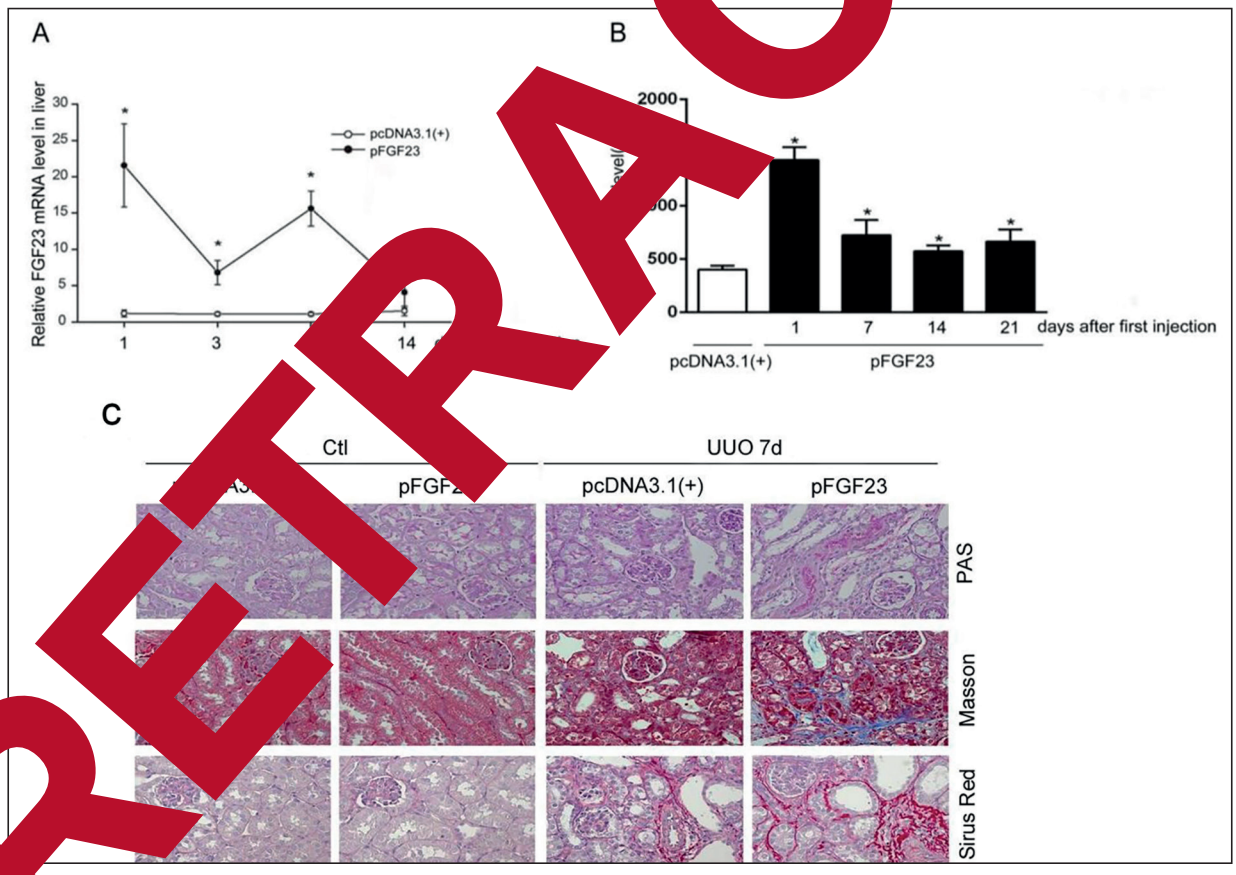

Figure 3. The mRNA and plasma level of FGF23 after injection FGF23 plasmid and pcDNA3.1+ in UUO mice and relevant pa[illegible]ical changes. ***A,*** Quantitative PCR showed that mRNA level of FGF23 in mouse liver at different time points after singl[illegible]njection of FGF23 plasmid (*$p < 0.05$, n = 4-5). ***B,*** ELISA detection for the serum level of FGF23, results showed that FGF23 was maintained at a high level in mice (*$p < 0.05$, n = 4-5). ***C,*** 7 d after UUO, mice were sacrificed, PAS, Masson and Sirus Red staining were performed on the entire kidney tissue including contralateral and UUO side renal tissues (×400).

was slightly increased compared with pcDNA3.1 (+) group, which was more evident in renal tissues of UUO side (Figure 4A). Then, the UUO side renal tissue lysates of the same group and different mice were separated and subjected to Western blot analysis. As a result, FGF, Type I collagen, α-SMA, PAI, and other extracellular matrix proteins were abundantly expressed (Figure 4B, 4C).

β-catenin Signaling in UUO Kidney Tissues of FGF23-overexpressing Mice Was Further Activated

A large amount of literature[24-27] suggested that β-catenin signaling plays an important role in renal interstitial fibrosis. Klotho protein, a type I transmembrane protein, is mainly expressed in the proximal tubules of the kidney, parathyroid glands, and the choroid plexus of the brain[28,29]. Studies have confirmed that Klotho protein binds to FGFR, and then can specifically enhance the binding of FGFR and FGF23[30]. It has been reported that the expression of Klotho protein in the kidney tissue of CKD patients shows a decreasing trend during the course and the Klotho protein may act agai... fibrosis through the β-catenin signali... athway[31,32]. Therefore, we do not know whe... GF23 produces extracellular matrix in NRK-... lls and aggravates the degree of re... nterstiti... sis after UUO is achieved ... p-catenin sig... In UUO7d mice, the ... ease o... catenin ... p-β-catenin (Ser675 ... FGF... group ... d the increase of β-cat... n a... 5 in I... side renal tissue we... more o... (Fi... 5A-C); similar result... e also foun... 14d mice (Figure 5D... nohistochem... staining of UUO14d ... ney t... revealed that the number of β-catenin nuclei wa... eased in renal tubular epit... ells of UUO..., which was more e... nt in pFGF23 group than in pcDNA3.1 (+) ... p (Figure 5C...

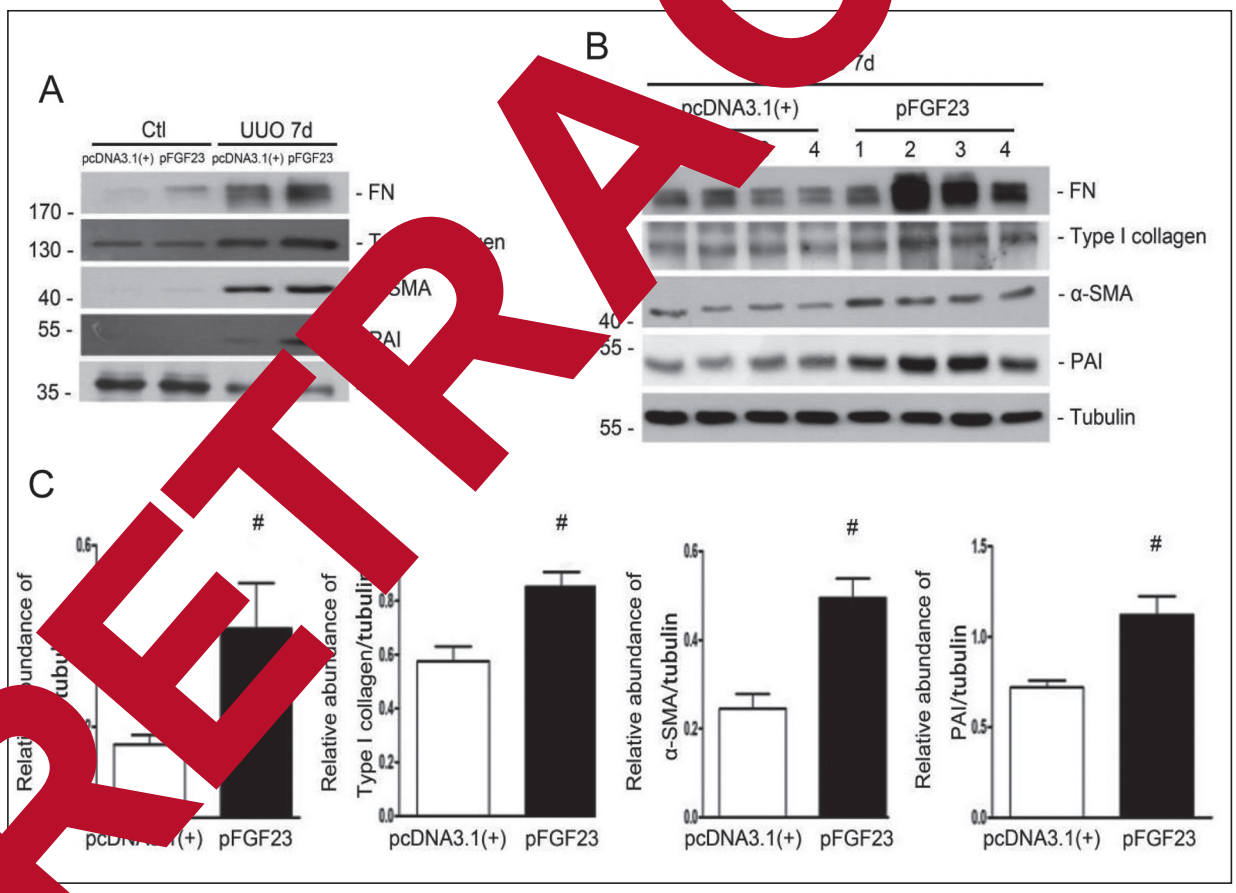

Figure 4. Overexpressed FGF23 can exacerbate renal interstitial fibrosis in UUO mice. ***A-B,*** 7 d after UUO, Western blot result... of renal tissues. ***C,*** 7 d after UUO, quantitative analysis of histological extracellular matrix proteins FN, Type I collagen, α-SMA, PAI in UUO side renal tissues ($^{\#}p < 0.05$, n = 4, $^{\#}$represents the contrast between the two groups in the UUO side kidney).

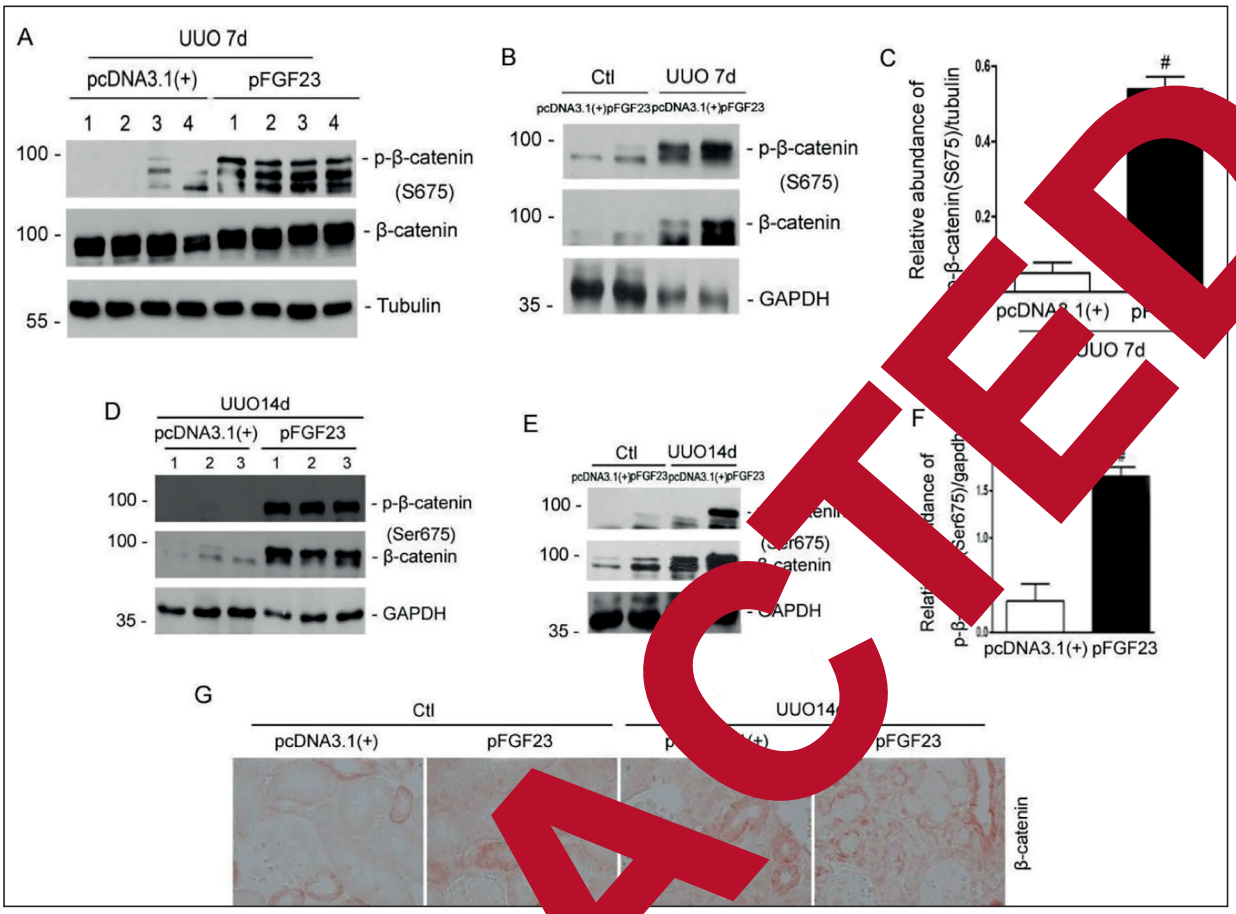

Figure 5. Further activation of β-cat naling in kidney tissue in FGF23-overexpressing mice. ***A-B,*** 7 d after UUO, Western blot results of β-cate ***C,*** Quan e analysis of p-β-catenin (S675) in UUO side of renal tissues ($^{\#}p < 0.05$, n = 4 $^{\#}$represents the U e). ***D-*** d after U estern blot results of β-catenin activation in renal tissues; ***F,*** 14 d after UUO, quantitative an of p-β-c n (S675) in UO side renal tissues, ($^{\#}p < 0.05$, n = 3 $^{\#}$represents the contrast between the two groups in U kidne UO, immunohistochemical analysis of β-catenin in renal tissues (×400).

FGF23 Purifi in Promo he Activation -cat in NRK-52E Cells

We hyp sized that 23 may cause the accumul of extracellula rix proteins by affect ne acti tion of β-cat nin. *In vitro* cultur RK-52 ls were treated with different conc ti FGF2 urified eggs (mFGF23) at diffe ne poi Effect of β-catenin pro- activa as erved. The results showed tha FGF2 oted β-catenin signaling in a t -dependen and dose-dependent manner (F

Discussion

This study demonstrates that FGF23 increases TGFβ1-induced aggregation of NRK-52E extracellular matrix. Also, the mouse model of FGF23 overexpression was established by plasmid injection of tail veins. FGF23 was found to increase the renal interstitial fibrosis after UUO model. Western blot and immunohistochemistry showed that the effect of FGF23 may be related to the activation of β-catenin signaling.

In this experiment, we first proposed that serum level of FGF23 was significantly increased in mice from UUO-induced renal interstitial fibrosis model, which achieved the peak one day after UUO. Quantitative PCR showed that mRNA level of FGF23 in renal tissue from early stage was not significantly altered, which was increased significantly 7 days and 21 days after UUO. It was suggested that dramatic change of FGF23 level in early stage has little relationship with the renal synthesis; osteoblast secretion is still its

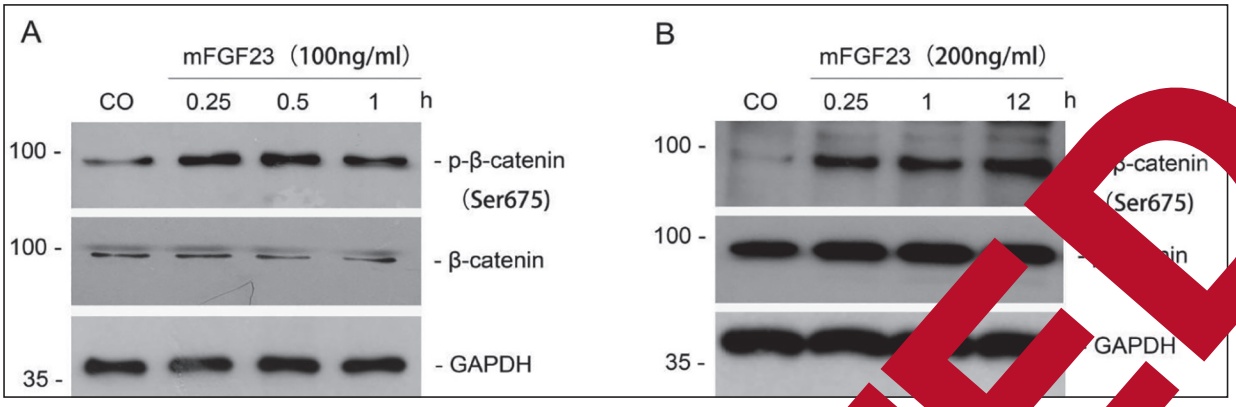

Figure 6. FGF23 promotes β-catenin signaling in NRK-52E cells. ***A,*** NRK-52E cells were treated with 100 ng/mL mFGF23 for 0.25 h, 0.5 h and 1 h, respectively. Western blot was used to detect the expression of p-β-catenin (Ser675). ***B,*** 200 ng/mL mFGF23 were used to treat NRK- 52E cells at 0.25 h, 1 h and 12 h. Western blot was used to detect the phosphorylation of β-catenin (Ser675) expression.

main source. As the course of disease progressed, the synthesis of FGF23 in the kidney tissue of the UUO increased, but it is unclear which cells of the kidney are involved in the synthesis of FGF23. Effect of the secreted FGF23 involved in the pathological damage of the renal tissue is also unknown.

Wnt/β-catenin signaling plays a very important role in the process of renal interstitial fibrosis. As early as 2010, studies have reported that *in vivo* injection of FGF23 purified protein into mouse marks the co-localization of β-catenin and klotho protein[33]. Subsequently, reports indicated that Klotho protein is involved in the regulation of Wnt pathway. Current specific studies are focused on FGF23 and p-β-catenin signaling. In this investigation, UUO nephropathy was used as a disease model and found that after overexpression of FGF23, the activation of p-β-catenin (Ser675) in the UUO side of the experimental group was significantly increased. This result was further confirmed in the *in vitro* culture of NRK-52E cells. TGFβ1 was a common stimulator of β-catenin signaling. TGFβ1 induced fibroblast activation through the Rictor/mTORC2 signaling pathway and involved in the development of renal fibrosis. However, the correlation between FGF23 induced fibrosis and TGF-β1/β-catenin signaling is still unclear and needs to be further explored.

Conclusions

FGF23 was first discovered by Japanese scholars in 2000. With further research, a large number of epidemiological studies have shown that serum level of FGF23 is significantly associated with disease progression in patients with CKD. FGF23 can be used as an important biological indicator to assess the occurrence, development and prognosis of disease. However, the specific pathophysiological role of FGF23 in CKD remains unclear. We found that high level FGF23 can directly increase renal interstitial fibrosis caused by UUO, which also provides a new basis for clinical treatment.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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