CTRP3 Improves Renal Fibrosis via Inhibiting Notch Signaling Pathways

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ABSTRACT

C1q/tumor necrosis factor-related protein-3 (CTRP3) has been extensively reported as an important role involved in anti-fibrosis, anti-apoptosis and anti-inflammation. However, the role of CTRP3 involved in renal fibrosis remains unclear. Our current study explored the role of CTRP3 in renal fibrosis and its underlying mechanisms by using serums and renal biopsy specimens from renal fibrosis patients and control subjects, rats models with the surgery of unilateral ureteral obstruction (UUO) and human renal proximal tubular epithelial cells (HRPTEpiCs). We found that circulating levels of CTRP3 had no significant difference between renal fibrosis patients and healthy subjects; however, renal CTRP3 expression was markedly downregulated in the fibrotic region with an abundant expression of collagen-I. In UUO rat models, circulating levels of CTRP3 has not changed with the prolonged obstruction of the kidney; renal CTRP3 expression was decreased with the severity of renal fibrosis; adenovirus-mediated CTRP3 treatment inhibited renal interstitial fibrosis. In vitro experiments revealed that CTRP3 attenuates TGF-β1 induced tubular epithelial cells fibrotic changes; CTRP3 knockdown facilitates the expression of fibrotic markers in TGF-β1-induced HRPTEpiCs; recombinant CTRP3 or adenovirus-mediated CTRP3 overexpression significantly inhibited Notch signaling pathway-associated factors, and knockdown of CTRP3 increased TGF-β1-mediated activation of Notch

signaling pathways. Collectively, our current study found that CTRP3 could improve renal fibrosis, to some extent, through inhibiting the Notch pathway.

Keywords: C1q/tumor necrosis factor-related protein-3 (CTRP3), renal interstitial fibrosis, tubular epithelial cells, TGF-β1, Notch signaling pathway.

Introduction

The occurrence of chronic kidney disease (CKD) has risen significantly in the past few years, causing a heavy financial burden on public healthcare. It is generally accepted that the development of renal interstitial fibrosis (RIF) plays a critical role in the procession of CKD to end-stage renal disease (Tampe and Zeisberg, 2014). Excessive deposition of extracellular matrix (ECM) initiates RIF, and the inappropriate accumulation of ECM eventually disrupts the functions the functions of renal tubules and glomeruli (Zeisberg and Kalluri, 2013).

With the deepening of studies on renal fibrosis, up-to-date information shows that numerous molecular mediators have been found to contribute to the development of RIF (Lovisa et al., 2015), among which Notch signaling pathway plays a critical role in the activation of renal fibrosis (Kim et al., 2013; Zhao et al., 2017). Previous studies have found that Notch signaling pathway is largely involved in some biological process,

such as differentiation, apoptosis, proliferation, and migration (Bray, 2006). Notch pathway has also been extensively reported to be involved in the fibrotic process (Bielesz et al., 2010; Morrissey et al., 2002). Furthermore, TGF-β signaling pathway induces fibrosis and increases the expression of some key molecules such as Notch-1 and Jagged-1 in the Notch pathway in several systems (Niimi et al., 2007). Inhibition of the Notch signaling pathway by small interfering RNA (siRNA) or an γ-secretase inhibitor to downregulate the expression of Jagged-1 blocks TGF-β1-induced fibrosis (Zavadil et al., 2004). Similarly, Notch signaling pathway activation promotes TGF-β1-induced organ and tissue fibrosis through transcription of Snai1 (Matsuno et al., 2012). Thus, novel treatments focusing on activation of the Notch signaling pathway may ameliorate RIF.

C1q/tumor necrosis factor-related proteins (CTRPs) belong to the adipokine family based on their structures, which all contain a C1q globular domain on the C-terminal (Shapiro and Scherer, 1998). CTRP3 is a newly identified member of this family. In 2001, Maeda et al (Maeda et al., 2001) first found an unknown gene and further studies revealed that the gene encodes a protein of 246 amino acid residues with a molecular weight of approximately 26 kDa and originally named it CORS26 (collagenous repeat-containing sequence of 26-kDa protein). In 2004, Wong et al. classified CORS26 as a CTRP and renamed it CTRP3 (Wong et al., 2004). CTRP3 has been confirmed to be expressed in many organs such as the

prostate, heart, liver, bone, kidney, and etc. (Akiyama et al., 2006; Hofmann et al., 2011; Hou et al., 2015; Peterson et al., 2010; Schäffler et al., 2003; Wu et al., 2015) Subsequent studies have showed that CTRP3 performs functions in many biological processes such as metabolism, apoptosis, inflammation, and cell proliferation (Huang et al., 2017; Murayama et al., 2014; Petersen et al., 2016; Wolf et al., 2016; Wu et al., 2015). Moreover, CTRP3 has also been reported as an anti-fibrosis molecule. Overexpression of CTRP3 in rats can dramatically inhibit interstitial fibrosis after myocardial infarction. (Yi et al., 2012). Besides, in TGF-β1-treated cardiac fibroblasts, CTRP3 attenuates the expression of some fibrotic markers such as connective tissue growth factor (CTGF) and collagen (Wu et al., 2015). In the kidney, CTRP3 also significantly inhibits expression of CTGF and fibronectin in polymeric IgA-stimulated human mesangial cells (Zhang et al., 2016). However, up to the present, there has been no report on whether CTRP3 can inhibit renal interstitial fibrosis.

Our current study aims to explore whether CTRP3 treatment exerts the anti-fibrosis effects on a unilateral ureteral obstruction (UUO) model and TGF-β1-treated tubular epithelial cells. In addition, the roles of Notch signaling pathway in CTRP3-mediated anti-fibrosis effects was also investigated.

Materials and Methods

Human serum and renal samples. Human serum samples were collected from 16 patients and 20 healthy volunteers at Beijing Friendship Hospital between December 2018 and January 2019. Renal biopsy specimens were collected from 6 patients suffered from CKD stage 5 with severe renal interstitial fibrosis and 6 patients suffered from minimal change nephropathy without renal interstitial fibrosis. Furthermore, 5 randomly selected high-powered fields of each specimen were scored from 1 to 4, 1 means weakest, 4 means strongest, then the average score of each specimen was used for two-group comparison. All scoring was performed by a single operator who knows nothing of this experiment. All the subjects enrolled in this study were diagnosed without diabetes, cardiovascular diseases, infectious diseases, cancer and pregnancy. The demographic characteristics of all the subjects were listed in Supplementary Table 1. The study was carried out in accordance with the Declaration of Helsinki, and the Ethics Committee of Beijing Friendship Hospital has approved the protocol (2018-P2-187-02). Informed consent for the use of serum sample or renal biopsy for research was obtained in writing from all donors or their next of kin.

Animal models. The animal experiments were approved by the Animal Care and Use Committee of Beijing Friendship Hospital (18-1006). Eight-week-old male Sprague-Dawley rats weighing 180–220 g were purchased from the institute of laboratory animal science (Beijing, China). Rats were

randomly divided into four groups: sham + Ad-Null, UUO + Ad-Null, sham + Ad-CTRP3, and UUO+ Ad-CTRP3 (n=6 per group). Each step for UUO operation was depended on an established protocol under anesthesia by pentobarbital (Shokeir, 1995). Sham-operated rats underwent the same surgical procedures but without ureter ligation. Rats in sham + Ad-Null and UUO + Ad-Null groups received a tail vein injection of 5×10^{10} plaque-forming units (PFU) adenovirus-Null (Genechem, Shanghai, China). Rats in sham + Ad-CTRP3 and UUO+ Ad-CTRP3 groups received a tail vein injection of 5×10^{10} PFU adenovirus-CTRP3 (Genechem, Shanghai, China). UUO or sham rats were sacrificed and their serums or kidneys were harvested at indicated times (0, 7 or 14 days) and stored in -80℃ until use. 14-day UUO models and the corresponding sham models were used for further experiments.

Histopathological examination. Kidney specimens were fixed with formalin, embedded in paraffin, and then sectioned at 4 μm thicknesses. Histopathological examination was assessed using Hematoxylin and Eosin staining (HE) and Masson's Trichrome staining (Solarbio, China) according to the manufactures' protocols.

Immunohistochemistry. Paraffin-embedded sections of renal tissues were dewaxed in xylene, dehydrated in alcohol, antigen repaired in citric saline. Then renal sections were treated with 0.3% hydrogen peroxide to block

endogenous peroxidase activity. After blocking by 2% bovine serum albumin, primary antibodies against CTRP3 (1:100, ab36870, Abcam, USA), fibronectin (1:100, ab2413, Abcam, USA) or collagen-I (1:200, ab34710, Abcam, USA) followed by biotinylated secondary antibody were incubated. All the three proteins were visualized by 3,3′-diaminobenzidine tetrahydrochloride (DAB) staining (P0203, Beyotime, China). Nucleus was visualized by hematoxylin.

Serum CTRP3 determination. Serum CTRP3 level was determined using commercial ELISA kit (E01C1243, BlueGene, China) under the manufacturer's protocols.

Cell Culture and Treatments. Human renal proximal tubular epithelial cells (HRPTEpiCs; ScienCell, San Diego, USA) were cultured in epithelial cell medium (ScienCell, USA) supplemented with 5% FBS at 37°C with 5% CO_2 atmosphere. Cells were seeded in 6-well plates and treated with various concentrations of CTRP3 (2, 5 and 10 μg/mL) [E. coli produced human CTRP3 (D46-K246) with 6 His tag on the N-Terminus; 00082-02-100, Aviscera Bioscience, USA] with or without the addition of TGF-β1 (5 ng/ml; 7754-BH-025/CF, R&D Systems, USA) and/or an γ-secretase inhibitor of Notch signaling pathway, DAPT (20 μmol/L; ab120633, Abcam, USA) for 48 hours.

Small interfering RNA (siRNA) transfection. The siRNA specifically targeting human CTRP3 was purchased from Genechem (Shanghai, China). After culturing HRPTEpiCs to 70% confluence, the cells were transfected with 100 nmol/L scrambled siRNA or siCTRP3 using Transfection Reagent (Genechem). After 6 hours, the transfection reagent was replaced with fresh epithelial cell medium. The efficiency of siCTRP3 was evaluated by western blotting.

Quantitative Real-time PCR (qPCR).

RNA was extracted from cells or tissues with Trizol Reagent (Invitrogen, USA), following the manufacturer's protocol. Complementary DNA (cDNA) was reverse-transcribed by a RevertAid cDNA Synthesis Kit (Fermentas, Canada). qPCR was performed using SYBR1 TaqTM Kit (Takara, Japan) based on the ABI PRISM 7000 system. GAPDH expression was used for normalization. Primers were as follows: COL1A1 (collagen-I) forward: 5′-TGCTCGTCGCCGCTGTCCTT-3′, reverse: 5′-TTGGGTCCTACAATATCCTTGATGTCTCC-3′; CDH1 (E-cadherin) forward: 5′-GAGAACGCATTGCCACATACAC-3′, reverse: 5′-GCACCTTCCATGACAGACCC-3′; ACTA2 (α-SMA) forward: 5′-TCCGGGACATCAAGGAGAAAC-3′, reverse: 5′-GCCCATCAGGCAACTCGTAA-3′; GAPDH forward: 5′-

AATGGGCAGCCGTTAGGAAA-3′, reverse: 5′-GCGCCCAATACGACCAAATC-3′.

Western blotting. Total protein form tissues or cells was extracted in ice-cold RIPA lysis buffer (Beyotime, China), sonicated, kept on ice for 30 minutes, and centrifuged with 14000 g for 30 minutes at 4°C. The concentration was determined by BCA kit (Beyotime, China). The same equal amounts of protein lysates (30 μg for cell lysates and 50 μg tissue lysates) were subjected to immunoblotting. The densitometry values of protein lysates were normalized by the expression of GAPDH. The primary antibodies were CTRP3 (1:1000, ab36870, Abcam, USA), collagen-I (1:1000, ab34710, Abcam, USA), α-SMA (1:2000, ab5694, Abcam, USA), E-cadherin (1:1000, ab40772, Abcam, USA), Notch-1 (1:200, ab8925, Abcam, USA), Jagged-1 (1:400, ab7771, Abcam, USA), TGF-β1 (1:1000, ab92486, Abcam, USA), GAPDH (1:5000, ab181602, Abcam, USA), Smad3 (1:500, 9523T, CST, USA) or p-Smad3 (1:500, 9520T, CST, USA). The densitometry values were measured by ImageJ software.

Statistical Analysis. Data are shown as mean ± standard deviation (SD). Significant difference between two groups was determined by Student's t-test and one-way factorial ANOVA followed by LSD test for groups>2. $P<0.05$ was considered significant.

Results

Renal CTRP3 expression decreases in CKD stage 5 patients.

We first used an ELISA kit to detect serum CTRP3 levels in CKD stage 5 patients and healthy subjects. As shown in **Figure 1A**, although serum levels of creatinine were significantly increased in CKD stage 5 patients compared to healthy subjects, the serum levels of CTRP3 exhibited no evident difference between CKD stage 5 patients and healthy subjects. However, in CKD stage 5 patients with severe renal interstitial fibrosis (determined by Masson's Trichrome staining and collagen I visualization), renal CTRP3 expression was notably decreased in tubular epithelial cells and mesangial cells, compared to that in patients with minimal change nephropathy (Figure 1B and C and Supplementary Figure S1). Collectively, renal CTRP3 expression was negatively associated with renal interstitial fibrosis, which might be an anti-fibrosis target.

Renal CTRP3 expression decreases in the UUO rats.

In our present study, rat UUO model, a widely used renal fibrotic animal model, was generated to simulate progressive renal fibrosis (Chevalier et al., 2009). In rat UUO model, as HE staining showed, the structure of the obstructed kidney was destroyed; renal interstitial was infiltrated by inflammatory cells together with obvious edema, renal tubular dilation and atrophy, and renal epithelial cell necrosis (**Figure 2A**). As Masson's trichrome staining shown, the interstitial fibrotic area was gradually

enlarged as time elapsed after operation (**Figure 2A**). In agreement with the pathological changes, fibrotic markers such as fibronectin, collagen-I and α-SMA protein expression were markedly increased in a time-dependent manner after operation; while E-cadherin protein expression pattern, on the contrary, was decreased (**Figure 2B** and **C**). We next measured serum levels of CTRP3 in the rat UUO model and the results showed that there was no significant difference in serum CTRP3 levels between sham, UUO 7d and UUO 14d groups (**Supplementary Figure S2A**). Then CTRP3 protein expression in the kidney was measured, as **Figure 2B** and **C**, consistent with the pathological changes, CTRP3 protein in glomerular mesangial areas and renal tubules was gradually and evidently reduced in a time-dependent manner.

Adenoviral CTRP3 delivery improves renal fibrosis in the UUO rats.

Adenovirus Ad-CTRP3 or Ad-Null was injected through the tail vein of rats after UUO surgery. **Figure 3A and B** show the adenoviral delivery efficiency after injection of Ad-CTRP3 in UUO and sham groups. Protein expression of CTRP3 was markedly increased, whereas injection of Ad-Null had no effects on the expression of CTRP3; Ad-CTRP3 injection also increased the serum levels of CTRP3 (**Supplementary Figure S1B**), indicating a high delivery efficiency of Ad-CTRP3. At 14 days after surgery, CTRP3 delivery evidently alleviated renal interstitial fibrosis as

indicated by HE and Masson's trichrome staining (**Figure 3C**). The increased renal fibronectin, collagen-I, and α-SMA protein expressions in rat UUO models were significantly abrogated (**Figure 3D** and **E**). In addition, E-cadherin protein was evidently enhanced after Ad-CTRP3 injection. These results indicated that in vivo Ad-CTRP3 delivery could effectively improve renal fibrosis in the UUO rats.

CTRP3 alleviates TGF-β1-induced fibrosis in HRPTEpiCs.

Next, we tested the effect of CTRP3 treatment on HRPTEpiCs. As shown in **Figure 4A**, without TGF-β1 incubation, CTRP3 treatment had no effect on the expressions of fibrotic markers, since α-SMA, collagen-I and E-cadherin protein expression exhibited no changes after CTRP3 treatment; while under TGF-β1 stimulation, CTRP3 treatment can alleviate TGF-β1 induced fibrotic effects, because α-SMA and collagen-I proteins expression was gradually decreased as the concentration of CTRP3 was increased to 5 μg/ml and E-cadherin protein expression was gradually increased as the concentration of CTRP3 was increased to 10 μg/ml. As there was no statistical difference between the effect of 5 and 10 μg/ml CTRP3 treatments on the expression of collagen-I and α-SMA, we choose 5 μg/ml CTRP3 for subsequent experiments. In agreement with the protein expression, CTRP3 treatment decreased the mRNA levels of collagen-I and α-SMA, but increased the mRNA expression of E-cadherin in TGF-

β1-treated HRPTEpiCs (**Figure 4B**). Our in vitro results highlighted that CTRP3 treatment could alleviate TGF-β1 induced fibrosis in HRPTEpiCs.

CTRP3 silencing facilitates TGF-β1-induced fibrosis in HRPTEpiCs.

To further demonstrate the effect of CTRP3 on TGF-β1-induced fibrosis, siRNA that specifically targeted human CTRP3 was used to knockdown CTRP3 in HRPTEpiCs. As shown in **Figure 5A**, CTRP3 siRNA transfection could significantly inhibit CTRP3 protein expression. Without TGF-β1 treatment, CTRP3 silence could not affect the expression of fibrotic markers such as α-SMA, collagen-I and E-cadherin at both transcriptional and translational levels; with TGF-β1 treatment, CTRP3 silence could enhance the mRNA and protein expression of α-SMA and collagen-I, but reduced the mRNA and protein expression of E-cadherin (**Figure 5B** and **C**). These results further confirmed that CTRP3 expression perturbation could affect TGF-β1-induced fibrosis in HRPTEpiCs.

CTRP3 inhibits TGF-β1-induced renal fibrosis by Notch signaling pathway.

As mentioned in the Introduction section, Notch signaling pathway acts an important role in fibrosis. In addition, Notch signaling pathway is also found to have important implications in excessive epithelial injury and

293 inflammation, then leading to subsequently renal fibrosis (Edeling et al.,
294 2016). In our present study, we first measured the expression of two key
295 molecules in Notch signaling pathway, Notch-1 and Jagged-1, in UUO
296 models. We found that both renal Notch-1 and Jagged-1 protein expression
297 was evidently increased after the establishment of the rat UUO models in
298 a time-dependent manner (**Figure 6A**). Ad-CTRP3 delivery significantly
299 inhibited the expression of Notch-1 and Jagged-1 in rat UUO models
300 (**Figure 6B**).

301 TGF-β1 treatment could up-regulate the expression of Notch-1 and Jagged-
302 1 in HRPTEpiCs, which could be partly reversed by CTRP3; in addition,
303 DAPT and CTRP3 co-treatment could completely block TGF-β1 induced
304 increase of Notch-1 and Jagged-1 expression, suggesting that CTRP3 could
305 inhibit TGF-β1 induced fibrotic effect in HRPTEpiCs, to some extent, via
306 inhibiting Notch signaling pathway (**Figure 6C**). To further verify the
307 specificity of CTRP3-Notch axis in the inhibition of fibrosis, we co-treated
308 CTRP3 siRNA and/or DAPT in TGF-β1 stimulated HRPTEpiCs. CTRP3
309 silence enhanced the expression of Notch-1, Jagged-1, α-SMA and
310 collagen-I and reduced the expression of E-cadherin in TGF-β1 stimulated
311 HRPTEpiCs, the effect of which was blocked by co-incubation with the
312 specific inhibitor, DAPT (**Figure 6D** and **E**). Taken together, these
313 findings suggested that CTRP3 inhibited TGF-β1-induced renal fibrosis,
314 partially, by blocking the Notch signaling pathway.

Discussion

Increasing evidence indicates that CTRP3 alleviates the fibrosis of multiple tissues and organs (Hofmann et al., 2011; Hou et al., 2015; Lin et al., 2014; Yi et al., 2012). In the cardiovascular system, CTRP3 reduces the cardiac fibrotic area in the post-MI model and inhibits fibroblast-to-myofibroblast differentiation (Wu et al., 2015); CTRP3 can also attenuate collagen and CTGF expression, and adventitial fibroblasts (AFs) phenotypic conversion, proliferation and migration, thus inhibiting vascular remodeling (Lin et al., 2014). CTRP3 is also found to exert an effective anti-fibrotic effect on colonic lamina propria fibroblasts isolated from Crohn's disease patients by inhibiting TGF-β-induced CTGF secretion and collagen-I expression (Hofmann et al., 2011). Our present study found that renal tubular epithelial cells and mesangial cells are the main cellular resource for CTRP3 production in the kidney, and renal expression of CTRP3 was significantly decreased with the development of renal interstitial fibrosis; CTRP3 overexpression exerted an anti-fibrotic effect on UUO rats by suppressing collagen-I and extracellular matrix deposition.

In the operated kidney, TGF-β1 was increased significantly, which plays a considerable role in triggering renal fibrogenesis by prompting ECM synthesis, inhibiting ECM degradation and activating myofibroblasts (Kaneto et al., 1993; Meng et al., 2016). Treatment of HRPTEpiCs with TGF-β1 obviously contributes to the procession of renal fibrosis

(Grampp and Goppelt-Struebe, 2018). Recombinant CTRP3 treatments or knockdown of CTRP3 were carried out in TGF-β1-treated HRPTEpiCs. The results showed that CTRP3 restored E-cadherin expression and attenuated α-SMA and collagen-I expression. Moreover, the downregulation of CTRP3 facilitated TGF-β1-induced fibrosis. However, there was no obvious effect on sham-operated rats and HRPTEpiCs when treated with CTRP3 alone.

Notch-1 and Jagged-1 expression have been found to be enhanced in the kidney of UUO models (Morrissey et al., 2002). Also, Notch signaling pathway activation in tubular epithelial cells results in interstitial fibrosis development, and Jagged-1 silencing or DAPT treatment attenuates renal fibrosis (Bielesz et al., 2010). In addition, Notch signaling pathway activation significantly promotes Snai1 expression, which is the main driver of in the progression of renal fibrosis (Matsuno et al., 2012). These findings indicate that Notch signaling pathway has important implications in organ fibrosis. Our current study found that Notch-1 and Jagged-1 expression is upregulated in the rat UUO models and TGF-β1 stimulated tubular epithelial cells; CTRP3 delivery inhibits Notch pathway in rat UUO models; in vitro CTRP3 treatment inhibits TGF-β1-induced fibrosis though downregulating Notch-1 and Jagged-1 expression; in addition, CTRP3 silence elevates Notch-1, Jagged-1 and pro-fibrotic proteins expression in TGF-β1-stimulated HRPTEpiCs, which is blocked by co-treatment with

359 the DAPT. These results indicated that CRTP3 has an inhibitive effect on
360 TGF-β1-induced renal fibrosis, to some extent, by blocking the Notch
361 signaling pathway.
362 Except for Notch signaling pathway, TGF-β1 and its downstream Smad
363 pathway have been widely found play important roles in the development
364 of renal fibrosis. Generally, TGF-β interacts with TGF-β receptor type I
365 and II to phosphorylate Smad2/3 with subsequent oligomerization with
366 Smad4, which then translocates to the nucleus to activate the transcription
367 of fibrogenesis genes (Samarakoon et al., 2013). Blocking TGF-β-
368 SMAD2/3 signaling pathway has been confirmed to improve renal fibrosis.
369 For example, in Smad3 knockout mice, renal fibrosis, inflammation, and
370 apoptosis are significantly attenuated after UUO (Inazaki et al., 2004). In
371 our study, we found that adenovirus-mediated overexpression of CTRP3
372 in UUO rats downregulated TGF-β1 expression and Smad3
373 phosphorylation (Supplementary Figure S3), which is in agreement with
374 previous studies; namely, CTRP3 can suppress Smad3 phosphorylation
375 and subsequent nuclear translocation and TGF-β1 expression (Wu et al.,
376 2015). In human primary colonic lamina propria fibroblasts which isolated
377 from Crohn's disease patients, TGF-β1 expression was also significantly
378 diminished by CTRP3 treatment (Hofmann et al., 2011). Our current study
379 mainly found another important pathway, Notch signaling pathway, was
380 involved in CTRP3 mediated anti-fibrosis. However, the major pathway

involved in CTRP3 medicated renal fibrosis improvement and the crosstalk across TGF-β and Notch signaling pathways under CTRP3 treatment should be further explored.

It has long been established that inflammation and apoptosis have a close relationship with the extent of renal fibrosis apart from TGF- β and Notch signaling pathways under CTRP3 treatment (Mao et al., 2008; Sun et al., 2015). Previous studies have revealed the central effect of CTRP3 in the regulation of inflammation and apoptosis processes (Hou et al., 2014; Hou et al., 2015; Li et al., 2014; Yoo et al., 2013). Therefore, anti-inflammation and anti-apoptosis effects of CTRP3 may also contribute to alleviating renal fibrosis, which should be investigated in a further study. Furthermore, CTRP3 significantly enhanced HIF-1α expression in an intracerebral hemorrhage model of rats and exerted protective effects such as reduced brain edema, improved neurological functions, and promoted angiogenesis (Wang et al., 2016). HIF-1α serves as a considerable mediator of oxygen homeostasis, which has been reported to stimulate the Notch signaling pathway (Gustafsson et al., 2005; Main et al., 2010). As aforementioned, Notch signaling pathway is involved in excessive epithelial injury and inflammation; therefore, in renal fibrosis, the role of CTRP3-Notch signaling pathway axis in inflammation should also be further elucidated.

In summary, CTRP3 can improve renal fibrosis in UUO rats and inhibit TGF-β1-induced fibrotic changes of renal tubular epithelial cells, to some

extent, via antagonism of the Notch signaling pathway. Our findings can provide a new therapeutic target for renal fibrosis.

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Conflict of interest

The authors declare no conflicts of interest.

Author Contributions

Author contributions: Liu WH and Chen XP provided the concept and designed the study; Chen XP performed experiments; Chen XP and Wu YR interpreted the results; Chen XP prepared figures; Chen XP drafted the manuscript; Chen XP, Han X, Li DS, Diao ZL, Ruan XZ and Liu WH edited and revised the manuscript; Chen XP, Wu YR, Han X, Li DS, Diao ZL, Ruan XZ and Liu WH approved the final version of the manuscript.

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Figure legends

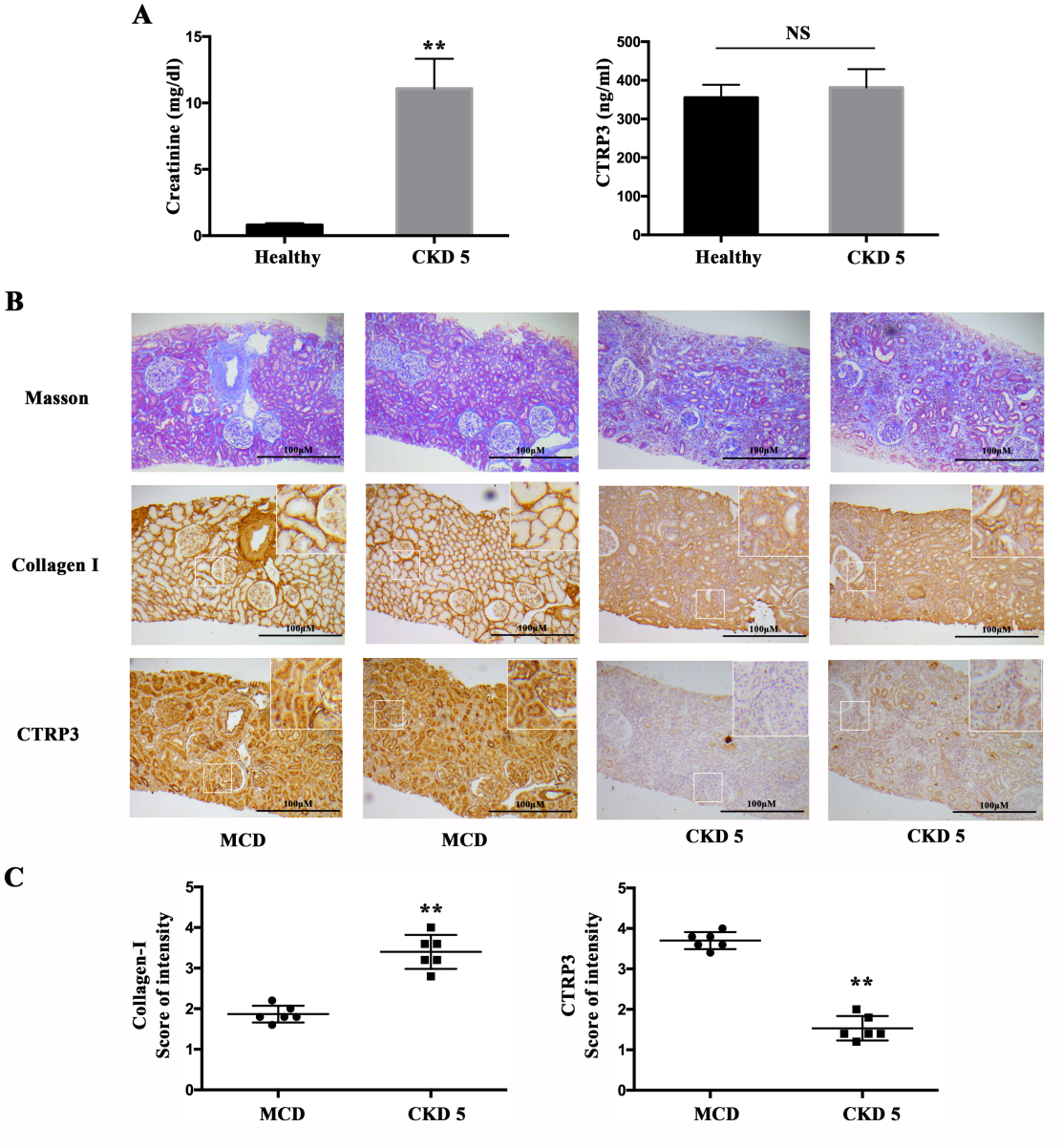
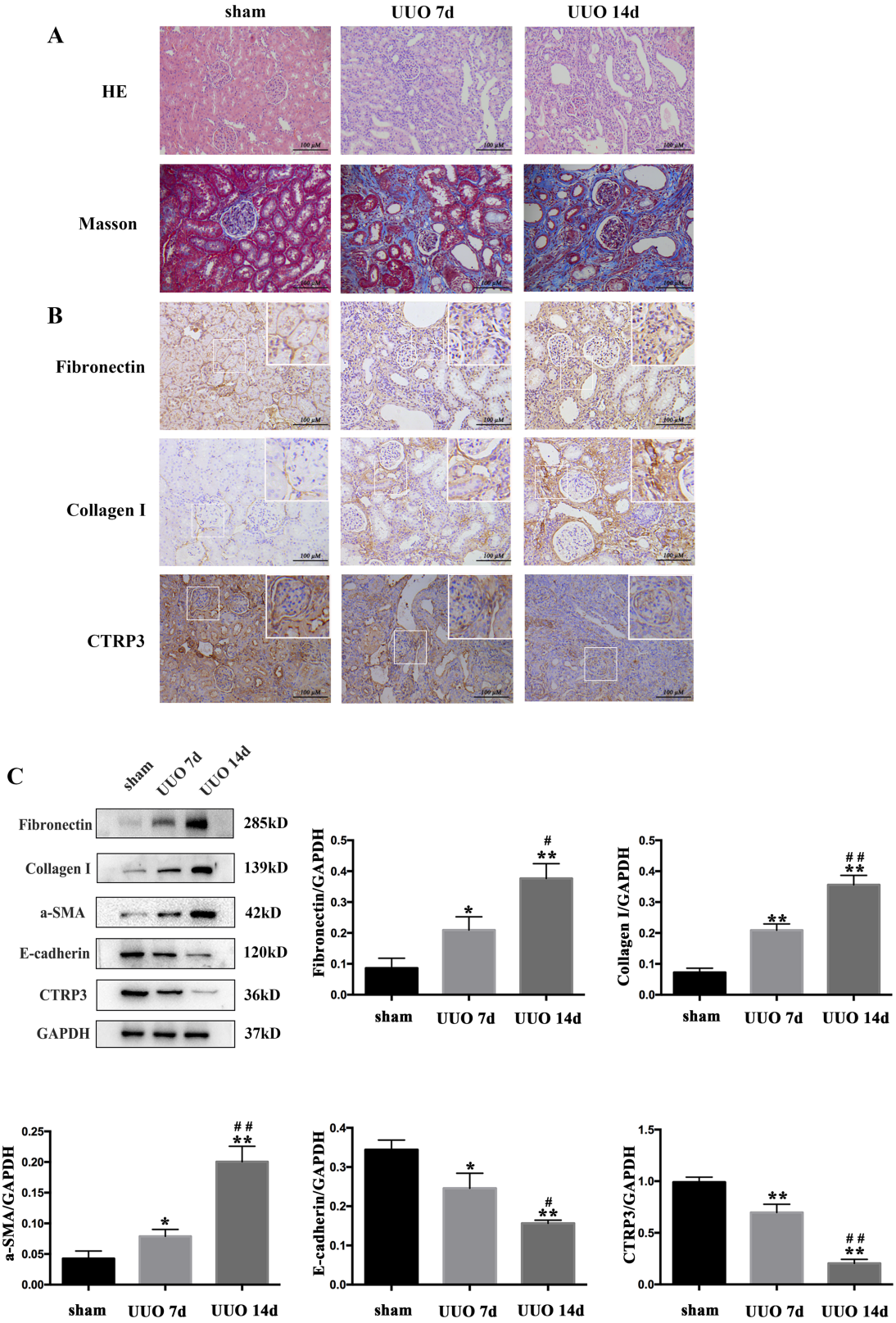


Figure 1. Serum and renal levels of CTRP3 in CKD patients.

(**A**) Circulating levels of CTRP3 in CKD stage 5 patients and healthy subjects. Serums from CKD patients (n = 16) and healthy subjects (n = 20) were collected and the CTRP3 levels were determined by an ELISA kit. ** $p < 0.01$, compared with the healthy subjects; NS represents no significant changes. (**B**) Masson's trichrome staining and immunostaining of CTRP3 and collagen-I (CKD 5, chronic kidney disease stage 5; MCD, minimal change nephropathy). Scale bar = 100 μm (×100). (**C**) Results of the average score of each specimen (1=weakest, 4=strongest). Each dot represents a unique specimen. ** $p < 0.01$, compared to the minimal change nephropathy.



583

584 **Figure 2. CTRP3 expression decreases in the UUO rats.**

(**A**) Renal histological changes assessed by HE staining. Scale bar = 100 μm (×200). Renal fibrosis was determined by Masson's trichrome staining. Blue indicates collagen fibers; red represents muscle fibers. Scale bar = 100 μm (×200). (**B)** Locations and expressions of renal fibronectin, collagen I and CTRP3 determined by immunostaining. Scale bar = 100 μm (×200). (**C**) Fibronectin, collagen-I, α-SMA, E-cadherin and CTRP3 expressions in UUO models detected by western blotting. Results were normalized to GAPDH expression. **P*<0.05 compared with the sham groups, ***P*<0.01 compared with the sham groups; $^{\#}P$<0.05 compared with the UUO 7d groups, $^{\#\#}P$<0.01 compared with the UUO 7d groups; n = 3 for each group.

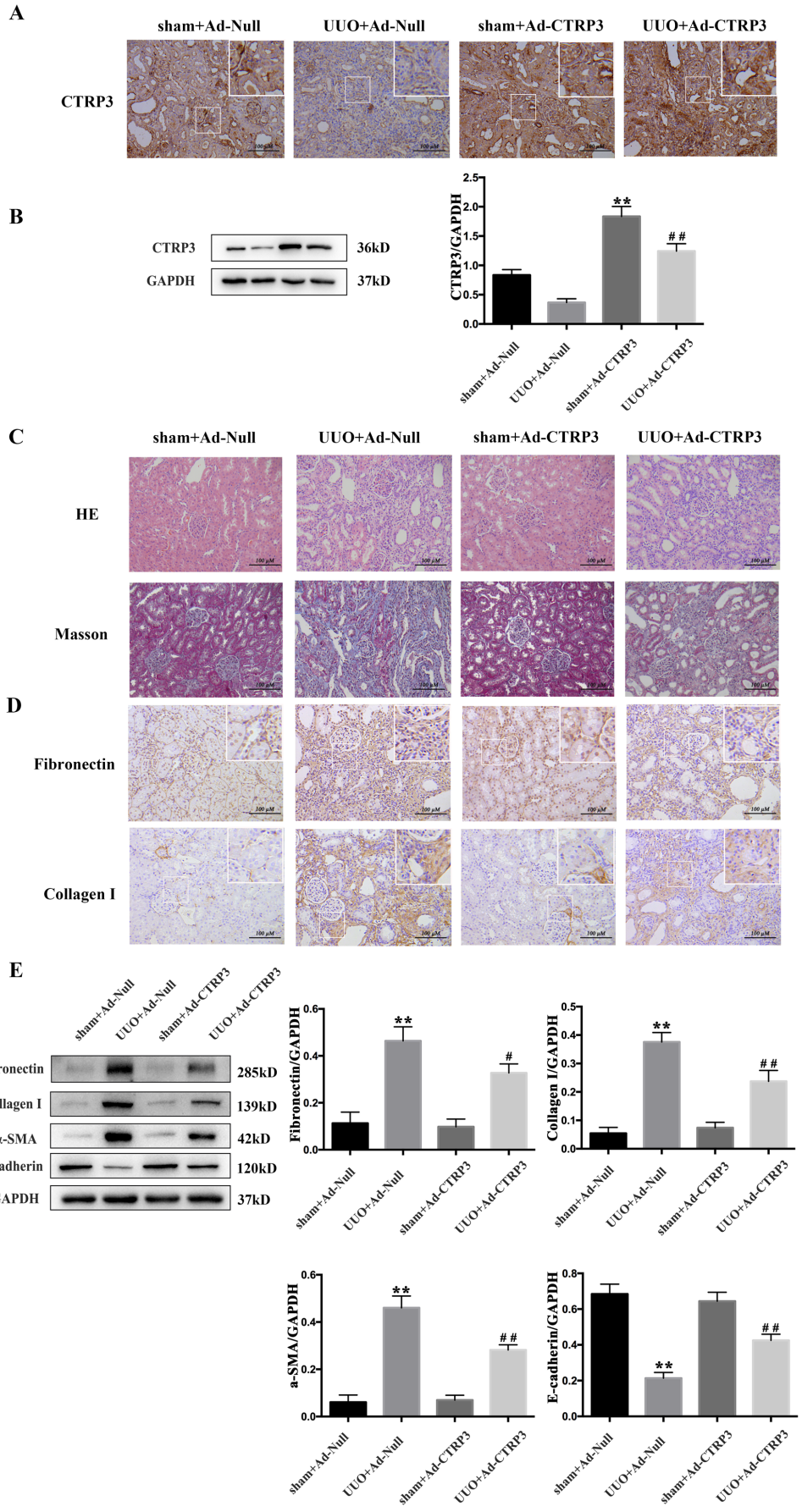
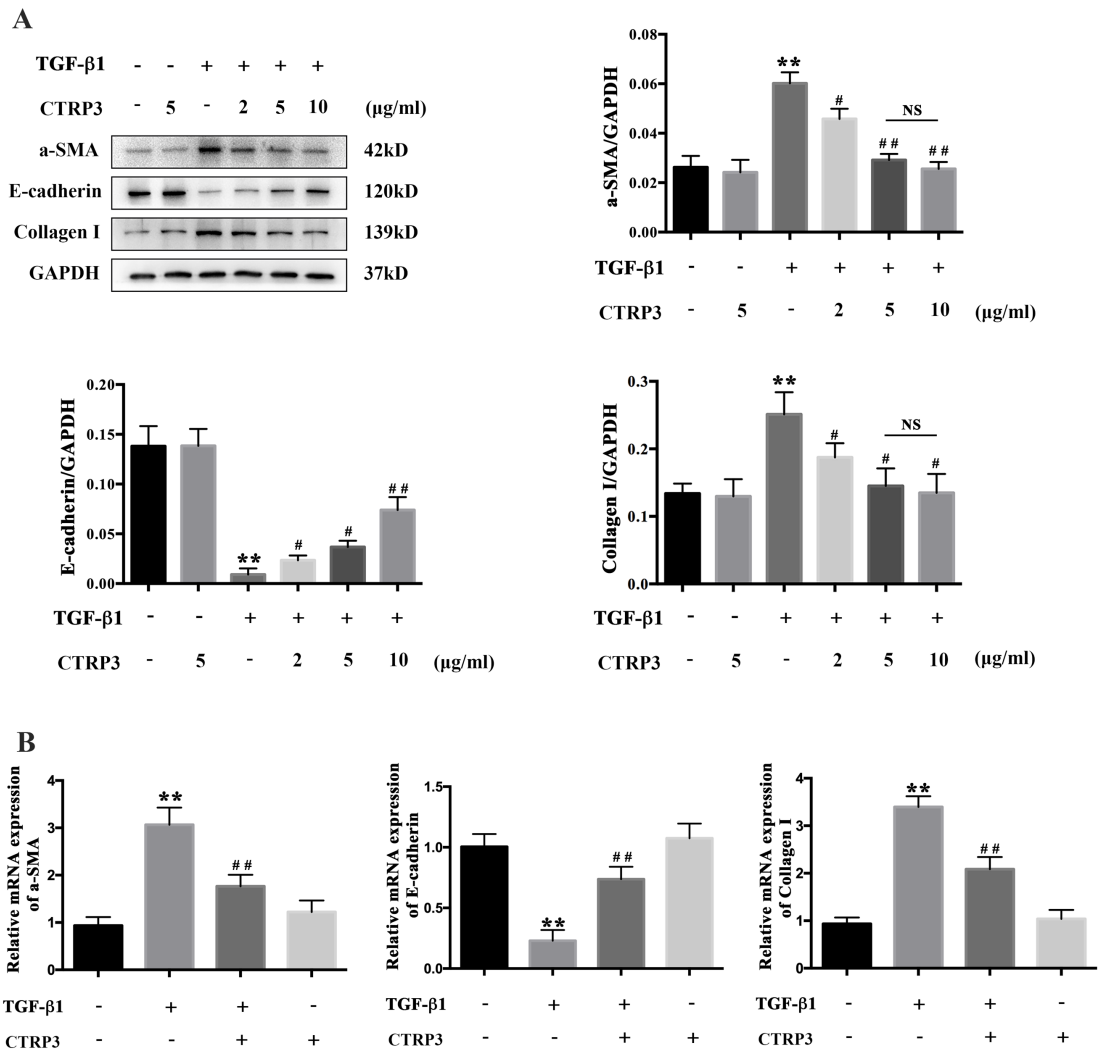

A
sham+Ad-Null
UUO+Ad-Null
sham+Ad-CTRP3
UUO+Ad-CTRP3
CTRP3
B
CTRP3
GAPDH
36kD
37kD
CTRP3/GAPDH
C
HE
Masson
D
Fibronectin
Collagen I
E
Fibronectin
Collagen I
α-SMA
E-cadherin
GAPDH
285kD
139kD
42kD
120kD
37kD
Fibronectin/GAPDH
Collagen I/GAPDH
a-SMA/GAPDH
E-cadherin/GAPDH

Figure 3. Adenoviral CTRP3 delivery improves renal interstitial fibrosis in the UUO rats.

(**A-B**) Location and expression of CTRP3 in each group determined by immunostaining and western blotting. Scale bar = 100 μm (×200). (**C**) Renal histological changes in each group revealed by HE staining. Scale bar = 100μm (200×). Renal fibrosis in each group was determined by Masson's trichrome staining. Blue indicates collagen fibers; red represents muscle fibers. Scale bar = 100 μm (×200). (**D**) Location and expression of renal fibronectin and collagen-I of each group were presented by immunostaining. Scale bar = 100 μm (×200). (**E**) Western blotting confirmed that, in UUO group, CTRP3 delivery reduced the expressions of fibronectin, collagen-I and α-SMA, whereas increased the expression of E-cadherin, compared to the UUO + Ad-Null group. Results were normalized to GAPDH expression. *P<0.05 compared with the sham + Ad-Null groups, **P<0.01 compared with the sham + Ad-Null groups; $^{\#}P$<0.05 compared with the UUO + Ad-Null groups, $^{\#\#}P$<0.01 compared with the UUO + Ad-Null groups; n = 3 for each group.



612

613 **Figure 4. CTRP3 attenuates TGF-β1-induced fibrosis in HRPTEpiCs.**

614 (**A**) HRPTEpiCs were treated with 5 ng/ml TGF-β1 with or without various
615 concentrations of recombinant globular CTRP3 (2, 5, and 10 μg/ml) for 48 hours.
616 Western blotting demonstrated that CTRP3 attenuated the expression of collagen-I and
617 α-SMA, and decreased expression of E-cadherin in a dose-dependent manner. (**B**)
618 HRPTEpiCs were treated with 5 ng/ml TGF-β1 with or without recombinant globular
619 CTRP3 (5 μg/ml) for 48 hours. qPCR revealed that CTRP3 attenuated the mRNA
620 expression of collagen-I and α-SMA, and decreased mRNA expression of E-cadherin.
621 Results were normalized to GAPDH expression. **P*<0.05 compared with the control
622 group; ***P*<0.01 compared with the control group; $^{\#}P$<0.05 compared with the TGF-

β1-stimulated group; $^{\#\#}P<0.01$ compared with the TGF-β1-stimulated group; n = 3 for each group.

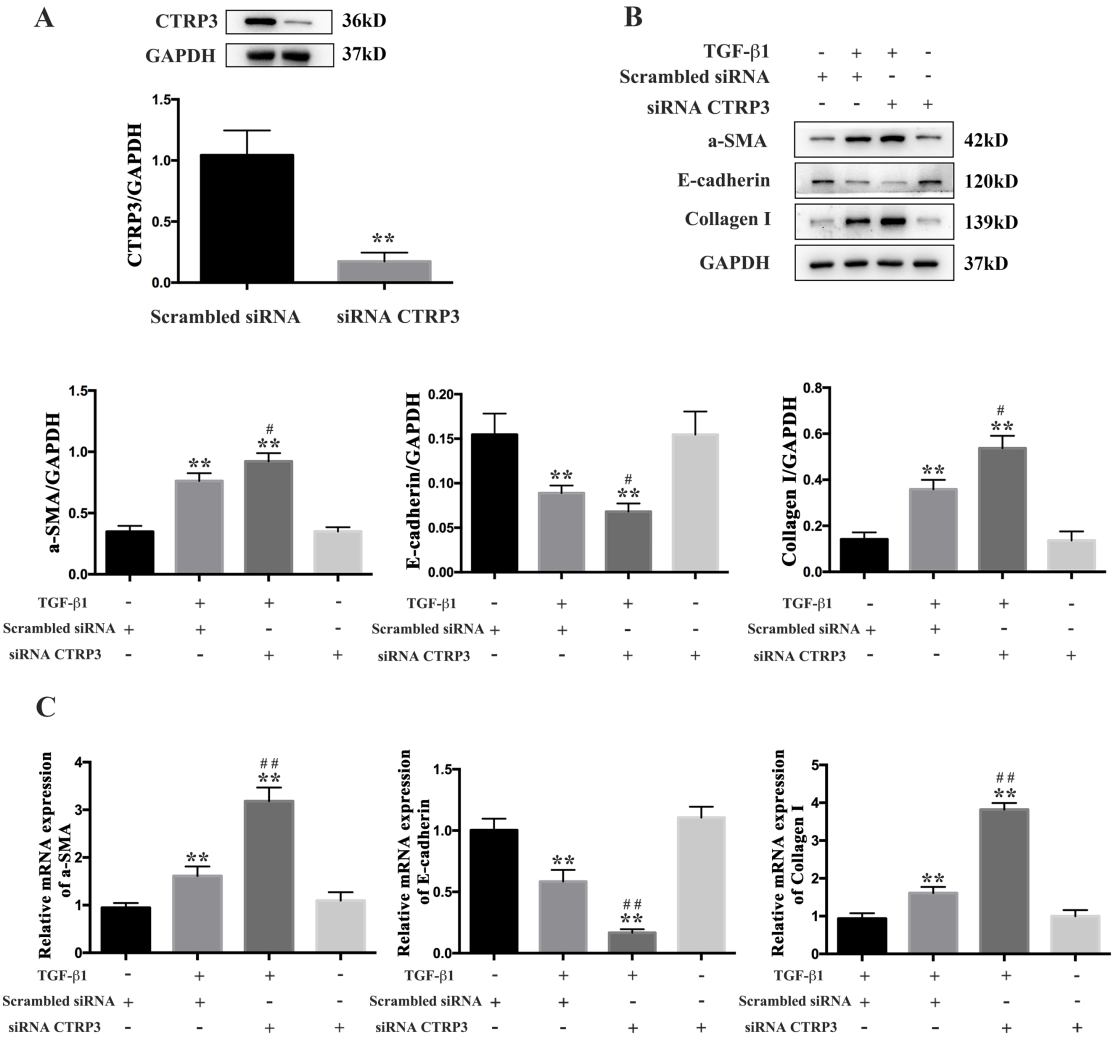


Figure 5. CTRP3 silencing facilitates TGF-β1-induced fibrosis in HRPTEpiCs.

(**A**) HRPTEpiCs were transfected with siCTRP3 or scrambled siRNA for 48 hours. Western blotting demonstrated that the expression of CTRP3 was significantly reduced by the specific siRNA. (**B**) Western blotting demonstrated that CTRP3 silencing facilitated the effect of TGF-β1 on the protein expression of collagen-I, α-SMA, and E-cadherin. (**C**) qPCR revealed that CTRP3 silencing facilitated the effect of TGF-β1-induced mRNA expression of collagen-I, α-SMA, and E-cadherin. Results were

634 normalized to GAPDH expression. *P<0.05 compared with the scrambled siRNA
635 group; **P<0.01 compared with the scrambled siRNA group; $^{\#}P$<0.05 compared with
636 the scrambled siRNA+TGF-β1 group, $^{\#\#}P$<0.01 compared with the scrambled
637 siRNA+TGF-β1 group; n = 3 for each group.

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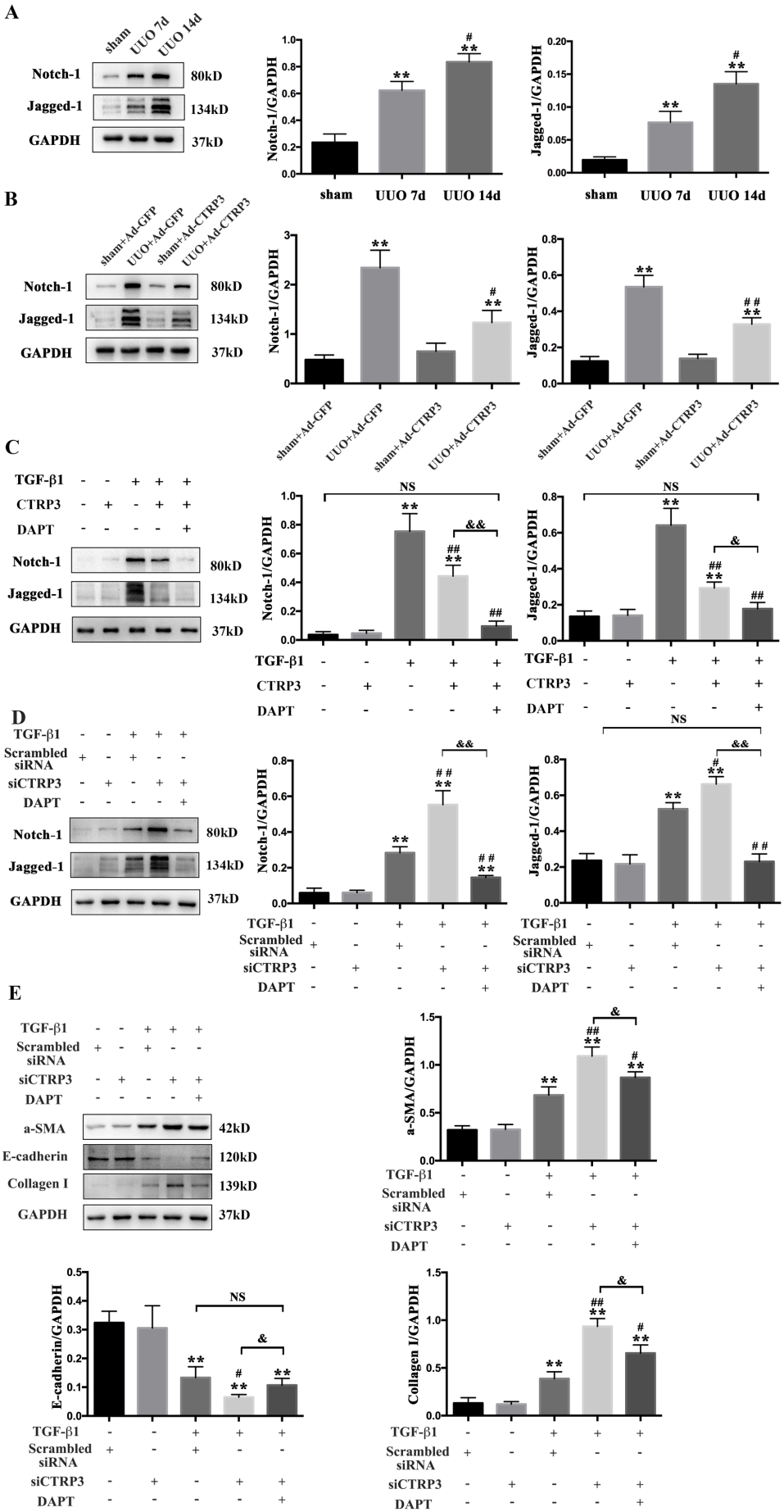
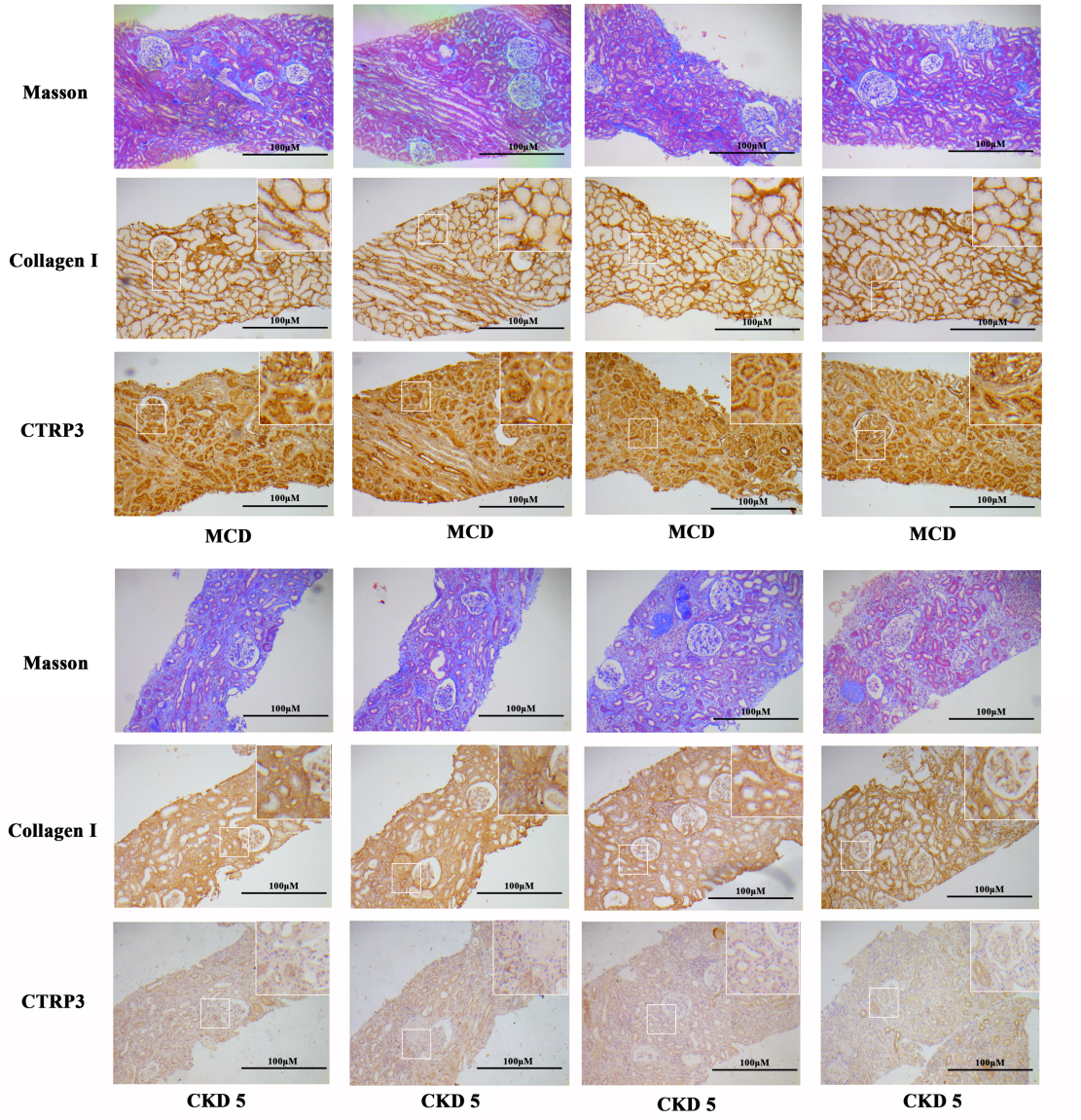

A
sham
UUO 7d
UUO 14d
Notch-1
Jagged-1
GAPDH
80kD
134kD
37kD
Notch-1/GAPDH
Jagged-1/GAPDH
B
sham+Ad-GFP
UUO+Ad-GFP
sham+Ad-CTRP3
UUO+Ad-CTRP3
C
TGF-β1
CTRP3
DAPT
NS
D
Scrambled siRNA
siCTRP3
E
a-SMA
E-cadherin
Collagen I
42kD
120kD
139kD
a-SMA/GAPDH
E-cadherin/GAPDH
Collagen I/GAPDH

Figure 6. CTRP3 inhibits TGF-β1-induced renal fibrosis via the Notch signaling pathway.

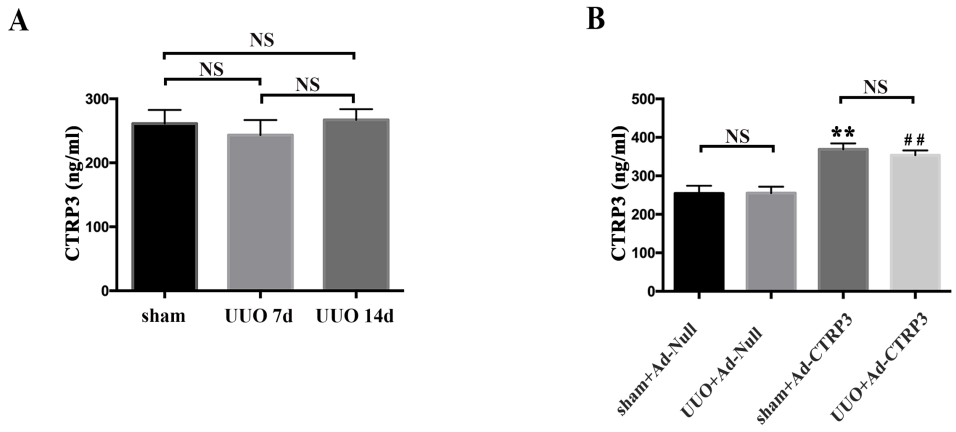
(**A**) Activation of the Notch signaling pathway in UUO rats as confirmed by western blotting. Results were normalized to GAPDH expression. **P*<0.05 compared with the sham group; ***P*<0.01 compared with the sham group; $^{\#}$*P*<0.05 compared with the UUO 7d group; $^{\#\#}$*P*<0.01 compared with the UUO 7d group; n = 3 in each group. (**B**) CTRP3 delivery inhibited the Notch signaling pathway in UUO rats as confirmed by western blotting. Results were normalized to GAPDH expression. **P*<0.05 compared with the sham+Ad-Null group; ***P*<0.01 compared with the sham+Ad-Null group; $^{\#}$P<0.05 compared with the UUO+Ad-Null group; $^{\#\#}$*P*<0.01 compared with the UUO+Ad-Null group; n = 3 in each group. (**C**) HRPTEpiCs were treated with TGF-β1, CTRP3, and/or DAPT. Western blotting demonstrated that CTRP3 attenuated TGF-β1-induced increases of Notch-1 and Jagged-1 expression. DAPT and CTRP3 co-treatment of TGF-β1-induced HRPTepiCs completely blocked activation of the Notch signaling pathway. Results were normalized to GAPDH expression. **P*<0.05 compared with the control group; ***P*<0.01 compared with the control group; $^{\#}$*P*<0.05 compared with the TGF-β1-stimulated group; $^{\#\#}$*P*<0.01 compared with the TGF-β1-stimulated group; $^{\&}$*P*<0.05 compared with the TGF-β1+CTRP3 group; $^{\&\&}$*P*<0.01 compared with the TGF-β1+CTRP3 group; n = 3 for each group. (**D**) HRPTEpiCs were transfected with siCTRP3 and/or treated with DAPT and/or TGF-β1 for 48 hours. Western blotting demonstrated that CTRP3 silencing facilitated the effect of TGF-β1 on the protein expression of Notch-1 and Jagged-1. The increased expression of Notch-1 and Jagged-1 in CTRP3-silenced cells was restored by DAPT. Results were normalized to GAPDH expression. **P*<0.05 compared with siCTRP3 or scrambled siRNA groups; ***P*<0.01 compared with siCTRP3 or scrambled siRNA groups; $^{\#}$*P*<0.05 compared with the scrambled siRNA+TGF-β1 groups; $^{\#\#}$*P*<0.01 compared with the scrambled siRNA+TGF-β1 groups; $^{\&\&}$*P*<0.01 compared with the siCTRP3+TGF-β1 groups; n = 3 for each group. (**E**) Western blotting demonstrated that CTRP3 silencing facilitated the effect of TGF-β1 on the protein expression of collagen-I, α-SMA, and E-cadherin.

After inhibiting activation of the Notch pathway by DAPT, the expression of α-SMA and collagen-I was downregulated, while expression of E-cadherin was upregulated compared with TGF-β1 and siCTRP3-treated cells. *P<0.05 compared with siCTRP3 or scrambled siRNA groups; **P<0.01 compared with siCTRP3 or scrambled siRNA groups; $^{\#}P$<0.05 compared with the scrambled siRNA+TGF-β1 group; $^{\#\#}P$<0.01 compared with the scrambled siRNA+TGF-β1 group; $^{\&}P$<0.05 compared with the siCTRP3+TGF-β1 group; $^{\&\&}P$<0.01 compared with siCTRP3+TGF-β1 group; n = 3 for each group.



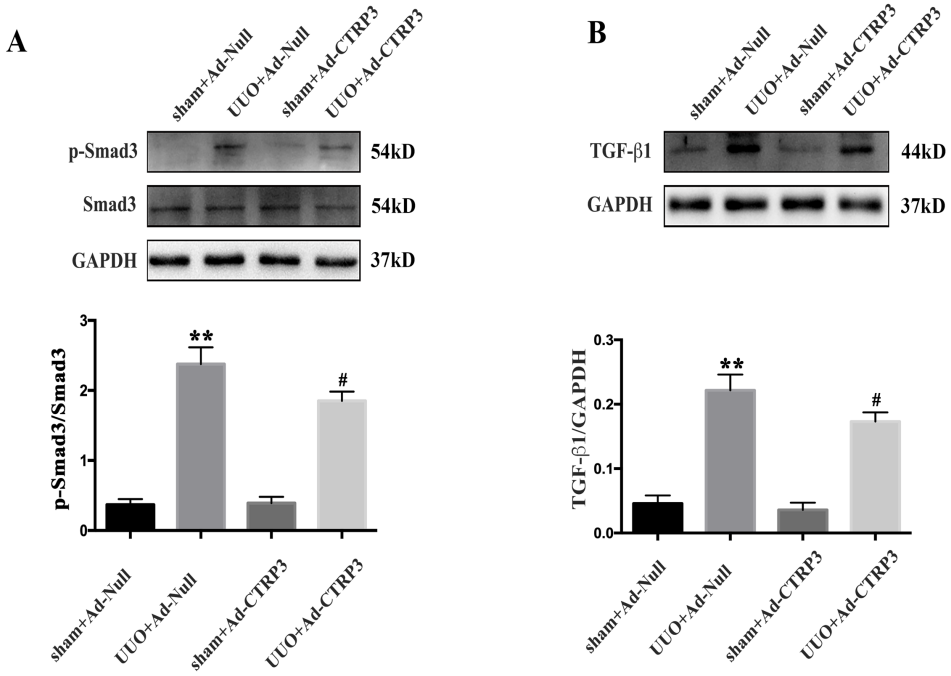
Supplementary Figure S1. Masson's trichrome staining and immunostaining of CTRP3 and collagen-I

Masson's trichrome staining and immunostaining of CTRP3 and collagen-I (CKD 5, chronic kidney disease stage 5; MCD, minimal change nephropathy). Scale bar = 100 μm (×100).



Supplementary Figure S2. Serum levels of CTRP3 in UUO rats and adenoviral CTRP3-delivered rats

(**A**) There was no significant difference in serum CTRP3 levels between sham, UUO 7d, and UUO 14d groups. (**B**) Adenoviral CTRP3 delivery significantly elevated serum CTRP3 levels. $^{**}P<0.01$ compared with sham + Ad-Null group, $^{\#\#}P<0.01$ compared with UUO + Ad-Null group; n = 6 for each group.



693

694 **Supplementary Figure S3. Adenoviral CTRP3 delivery inhibit TGF-β1 expression**
695 **and the phosphorylation of Smad3 in UUO rats.**

696 (**A-B**) CTRP3 delivery reduced the expression of TGF-β1 and p-Smad3 in the UUO
697 group compared with the UUO+Ad-Null group as confirmed by western blotting.
698 Results were normalized to Smad3 or GAPDH expression. **P*<0.05 compared with the
699 sham+Ad-Null group; ***P*<0.01 compared with the sham+Ad-Null group; $^{\#}P$<0.05
700 compared with the UUO Ad-Null group, $^{\#\#}P$<0.01 compared with the UUO + Ad-Null
701 group; n = 3 for each group.