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**Original Paper**

# KLF 15 Works as an Early Anti-Fibrotic Transcriptional Regulator in Ang II-Induced Renal Fibrosis via Down-Regulation of CTGF Expression

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**Key Words**
Angiotensin II • Krüppel-like factor 15 • Connective tissue growth factor • Renal fibrosis • P/CAF

**Abstract**
***Background/Aims:*** Angiotensin II (Ang II) has been regarded as an important profibrogenic cytokine in renal fibrosis. Krüppel-like factor 15 (KLF15) has been identified as an important negative transcription factor in renal fibrosis. However, little is known about the role of KLF15 in Ang II-induced renal fibrosis. ***Methods:*** In this study, we randomized mice into a control group, Ang II group or Ang II plus losartan group. KLF15 expression was examined with real-time PCR and immunofluorescence in these groups. *In vitro*, KLF15 expression was examined by Western blot in rat renal fibroblasts (NRK-49F) stimulated with Ang II, and the effect of altered KLF15 expression on the regulation of the profibrotic factor connective tissue growth factor (CTGF) was further explored with co-immunoprecipitation (CoIP) and chromatin immunoprecipitation (ChIP) analyses. ***Results:*** Compared with the control group, the murine model of Ang II-induced renal fibrosis demonstrated a significant decrease in renal KLF15 expression at 4 weeks and presented with progressive renal fibrosis at 6 weeks. Meanwhile, losartan, an angiotensin type 1 (AT1) receptor antagonist, effectively prevented the down-regulation of KLF15 expression induced by Ang II infusion. *In vitro*, NRK-49F cells stimulated with Ang II exhibited a significant decrease in KLF15 expression, accompanied by a marked increase in the expression of profibrotic factors and in the production of extracellular matrix. The up-regulation of CTGF expression induced by Ang II stimulation was inhibited by KLF15 overexpression in NRK-49F cells, and losartan treatment prevented the down-regulation of KLF15 expression and the up-regulation of CTGF expression induced by Ang II stimulation. Furthermore, CoIP and ChIP assays revealed that the transcription regulator KLF15 directly

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bound to the co-activator P/CAF and repressed its recruitment to the CTGF promoter. ***Conclusions:*** Ang II down-regulates KLF15 expression via the AT1 receptor, and KLF15 is likely to inhibit Ang II-induced CTGF expression by repressing the recruitment of the co-activator P/CAF to the CTGF promoter.



## Introduction

Chronic kidney disease (CKD) is a major health and economic burden that is increasing in incidence and prevalence. Without effective therapeutic interventions, the progression of CKD eventually leads to end stage of renal disease [1]. Renal fibrosis, characterized by glomerulosclerosis and tubulointerstitial fibrosis, is a common final pathological manifestation of CKDs with different aetiologies [2]. Although the reasons for renal fibrosis progression are multifactorial and the underlying mechanism(s) remains unclear, it has been widely recognized that the inappropriate activation of the renin-angiotensin (RAS) system and the increased local generation of angiotensin II (Ang II) play critical roles in renal fibrosis [3].

It is widely accepted that transforming growth factor-β (TGF-β) is one of the main targets of Ang II-induced renal fibrosis [4]. Basic studies have revealed that directly blocking TGF-β is beneficial in reducing renal fibrosis [5, 6]. However, clinical trials using direct TGF-β inhibition to treat renal fibrosis have not achieved the expected benefits [7]. Connective tissue growth factor (CTGF), known as the downstream mediator of TGF-β, has been regarded as a new promising anti-fibrotic target [8-11]. Ang II can directly up-regulate CTGF expression by activating Smad3 signalling via the angiotensin type 1 (AT1) receptor independent of the TGF-β pathway, resulting in the relentless accumulation and deposition of extracellular matrix (ECM) due imbalanced ECM synthesis and degradation [12].

Krüppel-like factor 15 (KLF15) is a transcriptional regulator with a wide range of functions in regulating cell differentiation, proliferation, apoptosis and fibrosis, robustly expressed in heart, liver, adipose tissues, kidneys, etc [13]. Our previous study indicated that KLF15, which is expressed in glomeruli and interstitium, is significantly down-regulated in the renal tissues of a 5/6 nephrectomised rat CKD model and that *Klf15*$^{-/-}$ mice are more susceptible to renal fibrosis than wild-type mice [14]. Furthermore, our research group has reported the molecular mechanism of KLF15 in the regulation of TGF-β-induced renal fibrosis [15]. However, the role of KLF15 in Ang II-induced renal fibrosis is still unclear.

Herein, we performed a series of studies to elucidate the essential role of KLF15, as a transcriptional repressor, in the regulation of CTGF expression in Ang II-induced renal fibrosis.

## Materials and Methods

*Animals and treatment*

Animal usage and all experimental procedures were approved by the Ethics Committees of the Second Military Medical University and followed the guidelines for the Care and Use of Laboratory Animals by the National Research Council.

Eight-week-old male C57BL6 mice weighing 20-22 g were randomly assigned to the following 3 groups (n = 8 per group): the mice in the control group were infused with normal saline; the mice in the Ang II group were infused with Ang II (MB1677, Melone Pharmaceutical Co., Ltd, Dalian, China) for 6 weeks; and the mice in the Ang II plus losartan group were infused with Ang II and given losartan (500 mg/L, MB25206, Melone Pharmaceutical Co., Ltd, Dalian, China) orally in the drinking water for 6 weeks. Additional groups of mice (n = 5 per group) were studied after 4 weeks of chronic infusion with Ang II alone or with Ang II and losartan together. An osmotic mini-pump (Alzet2006, Alzet, Cupertino, CA, USA) was subcutaneously implanted to provide a constant infusion of Ang II (2000 ng/kg/min) or normal saline. The doses of Ang II and losartan were chosen based on published data [16, 17].

*Blood pressure, blood and urine examination, renal morphology*

Blood pressure was measured via the noninvasive tail-cuff method (Softron Corp., Tokyo, Japan) prior to the mice being sacrificed. Systolic blood pressure values were derived from an average of five measurements per animal. Serum creatinine, blood urea nitrogen and urinary microalbumin/creatinine (MA/Cr) were determined using commercial kits (Cobas c 111, Roche, Basel, Switzerland). For histological analysis, the renal tissues were fixed with 4% buffered formaldehyde, embedded in paraffin, and sliced into four-micrometre-thick sections for Masson's trichrome and periodic acid-Schiff staining. The extent of glomerular sclerosis was assessed as described previously [14]. At least 30 glomeruli from each kidney were graded according to the following criteria: 0, no sclerosis; 1, less than 25% cross-sectional sclerosis; 2, 25–50% sclerosis; 3, 50–75% sclerosis; and 4, more than 75% sclerosis. The sclerosis index was determined by the mean score of each glomerulus in each kidney. Similarly, the severity of interstitial fibrosis was graded in sections from the cortex of each kidney: 0, no evidence of interstitial fibrosis; 1, interstitial fibrosis present in less than 10% of the section; 2, interstitial fibrosis present in 10-25% of the section; 3, interstitial fibrosis present in 25-50% of the section; 4, interstitial fibrosis present in 50-75% of the section; and 5, interstitial fibrosis present in >75% of the section.

*Cell culture and KLF15 overexpression*

The NRK-49F cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA) and were grown in DMEM supplemented with 5% foetal bovine serum (Gibco, Grand Island, NY, USA). NRK-49F cells were grown to 70% confluence and made quiescent in DMEM supplemented with 0.5% foetal bovine serum for 48 h before stimulation with Ang II (MB1677, Melone Pharmaceutical Co., Ltd, Dalian, China). Then, cells were incubated with Ang II with or without (losartan MB25206, Melone Pharmaceutical Co., Ltd, Dalian, China) for 1 h prior to the addition of different concentrations of Ang II for designated times. Using plasmids kindly provided by Prof. Feng Zheng (Mount Sinai School of Medicine, New York, NY, USA), a GFP-KLF15 adenovirus vector (Ad-GFP-KLF15) produced by R&S Biotech (Shanghai, China) was used for KLF15 overexpression. NRK-49F cells were grown to 70% confluence in DMEM/10%FBS and made quiescent for 48 h in DMEM/0.5%FBS before infection. Then, cells were infected with the Ad-GFP-KLF15 vector or control adenovirus (Ad-GFP) vector at a multiplicity of infection (MOI) of 200 for 48 h and were exposed to Ang II ($10^{-6}$ M) for 24 h. Cells were harvested and saved for real-time PCR or Western blot analysis.

*Western blot and immunofluorescence*

The renal tissue sample from each animal was ground and suspended in lysis buffer (1 ml of lysis buffer with 5 µl of protease inhibitor mixture, 10 µl of 100 mM NaF, 10 µl of 100 mM $Na_3VO_4$ and 10 µl of 100 mM PMSF) at 4 °C for 30 min; NRK-49F cells were washed twice with ice-cold PBS and lysed in the same way as the tissue. The lysate was centrifuged at 13, 000 g and 4 °C for 15 min. The supernatant was removed, and the protein concentration was measured using a BCA protein assay kit (Pierce, Rockford, IL). Total protein was separated on an SDS-PAGE (6% or 10% acrylamide) gel and transferred to a PVDF membrane. After blocking at room temperature for 1 h in 3% BSA/PBS, the membranes were respectively incubated at 4 °C overnight with anti-GAPDH antibody (1:1000, Santa Cruz, SC-365062); anti-KLF15 antibody (1:200, Santa Cruz, SC-34827); anti-CTGF antibody (1:200, Santa Cruz, SC-25440); anti-TGF-β1 antibody (1:200, Santa Cruz, SC-146); anti-Smad2/3 antibody (1:400, CST, 5678); anti-P-Smad2/3 antibody (1:400, CST, 8828); or anti-fibronectin antibody (1:400, Santa Cruz, SC-9068). The membrane was washed three times for 10 min in PBST and incubated with a secondary antibody for 1 h at room temperature. Then, the signal was detected with enhanced chemiluminescence detection reagents. The relative density of protein bands was quantified by using Image lab 4 software (Bio-Rad, USA).

KLF15 staining was performed as previously described [14]. In brief, four-micrometre-thick sections of renal tissue, prepared from paraffin-embedded tissues, were blocked with 5% normal donkey serum in PBS for 10 min at room temperature and then incubated with a goat anti-rat KLF15 antibody (1:200, Santa Cruz, SC-34827) at 4 °C overnight. The reaction was visualized under a fluorescence microscope with a donkey anti-goat IgG–FITC antibody (1:100, Santa Cruz, SC-2024). Negative controls for immunofluorescence staining were performed by substituting the primary antibody with PBS.

**Table 1.** Primer sequences for real-time PCR. KLF15, Krüppel-like factor 15; Abbreviations: CTGF, connective tissue growth factor; TGF-β, transforming growth factor-β; FN, fibronectin; COLI, Collagen I; COLIII, Collagen III; COLIV, Collagen IV

| Mouse | Primer sequences | Rat | Primer sequences |
|---|---|---|---|
| KLF15 | Forward 5'-GCCAGAAGTTTCCCAAGAACC-3'<br>Reverse 5'- GGGACACTGGTACGGCTTCA-3' | KLF15 | Forward 5'-TCCTCCAACTTGAACCTGT -3'<br>Reverse 5'-CTTGGTGTACATCTTGCTGC-3' |
| TGF-β1 | Forward 5'- GGGAAGCAGTGCCCGAACCC-3'<br>Reverse 5'- TGGGGGTCAGCAGCCGGTTA-3' | TGF-β1 | Forward 5'-GCCAGCTCCTGTCCAAACTAA-3'<br>Reverse 5'-TTGTTGCGGTCCACCATTA-3' |
| CTGF | Forward 5'-CTGACCTGGAGGAAAACATTA-3'<br>Reverse 5'-TTAGCCCTGTATGTCTTCACAC-3' | CTGF | Forward 5'-AACCGTGTGTCATTGTCAT -3'<br>Reverse 5'- CACCTTAGTGTGCGTTCTG -3' |
| FN | Forward 5'-GGACCAGGTTGATGATACTTC-3'<br>Reverse 5'-TGTGCTACTGCCTTCTACTGA-3' | FN | Forward 5'- GTGATCTACGAGGGACAGC -3'<br>Reverse 5'- GCTGGTGGTGAAGTCAAAG -3' |
| COLI | Forward 5'-TGACTGGAAGAGCGGAGAGTA-3'<br>Reverse 5'-GACGGCTGAGTAGGGAACAC-3' | COLI | Forward 5'-ATCCTGCCGATGTCGCTAT-3'<br>Reverse 5'-CCACAAGCCTGCTGTAGGT-3' |
| COLIII | Forward 5'-TCTTATTTTGGCACAGCAGTC-3'<br>Reverse 5'-GTGGCTCCTCATCACAGATTA-3' | COLIII | Forward 5'- CTGGTCCTGTTGGTCCATCT -3'<br>Reverse 5'- ACCTTTGTCACCTCGTGGAC -3' |
| COLIV | Forward5'-GCCTCCAGGAACGACTACTCTTA-3'<br>Reverse 5'-GCGGAATCTGAATGGTCTGA-3' | COLIV | Forward 5'-GCCCTACGTTAGCAGATGTACC-3'<br>Reverse 5'-TATAAATGGACTGGCTCGGAAT-3' |
| GAPDH | Forward 5'-GGTGAAGGTCGGTGTGAAC-3'<br>Reverse 5'-GACTGTGCCGTTGAATTTG-3' | GAPDH | Forward 5'- ATGCTGGTGCTGAGTATGTC -3'<br>Reverse 5'- AGTTGTCATATTTCTCGTGG -3' |

*Real-time PCR*

Total RNA was extracted from renal tissues or NRK-49F cells by TRIzol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized with 2 μg of total RNA, and real-time PCR reactions using SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) were performed on an ABI Prism 7900HT sequence-detection system (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. The PCR amplification reaction mixture (20 μl) contained 2 μl of cDNA, 0.4 μl of sense and antisense primers, and 10 μl of SYBR Green I. After the initial denaturation at 95 °C for 5 min, the reaction was cycled 40 times. Each cycle consisted of denaturation at 95 °C for 5 s, primer annealing at 60 °C for 30 s, and primer extension at 72 °C for 15 s. The expression of GAPDH was used as the control to normalize the relative gene expression levels of KLF15, CTGF, TGF-β1, fibronectin, and type I, type III and type IV collagen. The primer sequences are listed in Table 1.

*Co-immunoprecipitation (CoIP) and chromatin immunoprecipitation (ChIP)*

Nuclear protein was extracted from HEK293T cells with a NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit (Thermo Scientific, Rockford, USA), and the experimental procedures were in accordance with the kit instructions. For CoIP, the presence of a bimolecular complex of P/CAF and KLF15 was measured by immunoprecipitation with anti-P/CAF antibody and immunoblotting with anti-KLF15 antibody. ChIP assays were performed as previously described. In brief, NRK-49F cells were infected for 48 h with GFP-KLF15 adenoviral vectors or with GFP adenoviral vectors as the control, followed by incubation with Ang II ($10^{-6}$ M) for 4 h before cross-linking for 10 min with 1% formaldehyde. Anti-P/CAF antibody (Santa Cruz, SC-8999) was used for ChIP. PCR was performed with specific CTGF promoter primers (sense: 5'-tcggggcggaggttggtgtc-3', antisense: 5'-tttctaggggcccgtggtatctgc-3') for CTGF promoter amplification.

*Statistical analysis*

The results are expressed as the means ± standard deviations (SDs). Significance was determined by one-way ANOVA and Student's t-test using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). Comparisons with p values <0.05 were considered statistically significant.

## Results

*Characteristics of the Ang II-induced renal fibrosis mouse model*

After 6 weeks of Ang II infusion, the mice in the Ang II group demonstrated significant weight loss, but the mice in the control and Ang II plus losartan group showed a marked gain in body weight (Fig. 1A). There was also a slight reduction in kidney weight/body weight and a progressive increase in heart weight/body weight after chronic Ang II infusion

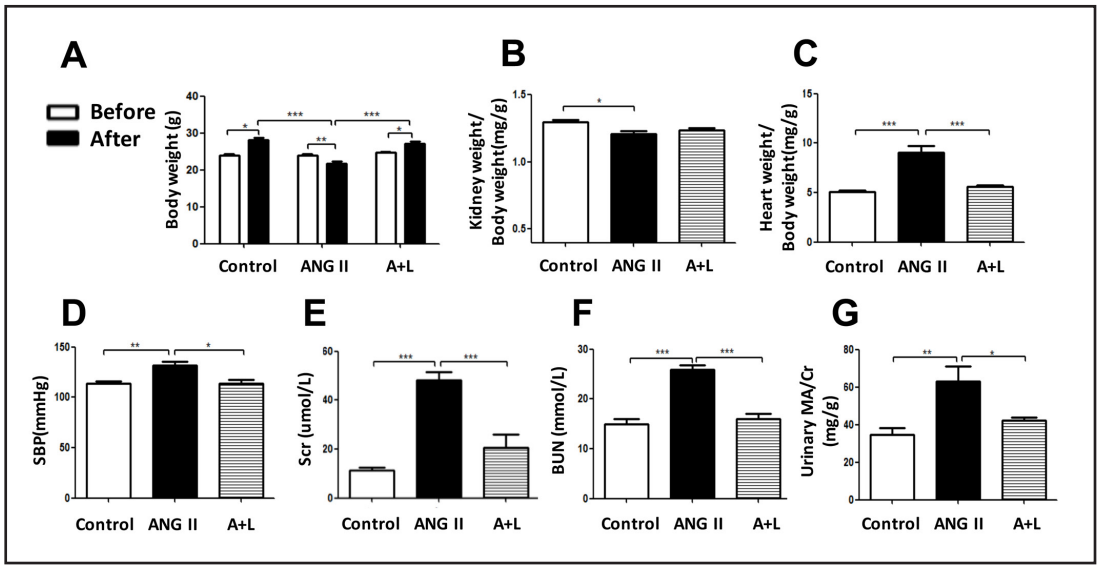


**Fig. 1.** General mouse data after 6 weeks of Ang II infusion. Body weight (A), kidney weight/body weight (B), heart weight/body weight (C), systolic blood pressure (D), serum creatinine (E), blood urea nitrogen (F) and urinary MA/Cr (G) of the control group, Ang II group and Ang II plus losartan (A + L) group. Data are expressed as the means ± standard deviations (SDs). * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

(Fig. 1B, C). Additionally, systolic blood pressure was significantly increased in the Ang II infusion group, while no change was found in the control or Ang II plus losartan group (Fig. 1D). Renal function and the urinary excretion of MA/Cr were measured in these three groups. After 6 weeks of Ang II infusion, serum creatinine, blood urea nitrogen and urinary MA/Cr were progressively increased in the Ang II group, while losartan administration effectively protected renal function and prevented the urinary excretion of microalbumin (Fig. 1E-G). Periodic acid-Schiff staining and Masson's trichrome staining showed that typical glomerulosclerosis, tubular dilation, protein cast formation, and extracellular matrix accumulation were found in the renal interstitium after chronic Ang II infusion for 6 weeks. However, losartan treatment effectively improved the glomerulosclerosis and interstitial fibrosis induced by Ang II (Fig. 2).

*Fibrotic cytokine expression and ECM production are increased in the Ang II-induced renal fibrosis mouse model*

Real-time PCR demonstrated that the mRNA expression levels of TGF-β1, CTGF, fibronectin, and type I, III and IV collagen were significantly up-regulated in the Ang II infusion group (Fig. 3A-F). Consistent with mRNA expression, the protein levels of CTGF, TGF-β1 and phosphorylated Smad2/3 were also increased in the Ang II group (Fig. 3G). Moreover, in accordance with the renal morphology observed above, gene and protein expression levels of fibrotic cytokines and the production of extracellular matrix were returned to baseline after administering losartan (Fig. 3A-G). These results indicated that fibrotic cytokine expression and ECM production were increased after Ang II infusions, while Ang II inhibition suppressed these effects.

*In vivo, KLF15 expression is down-regulated in Ang II-induced renal fibrosis via the AT1 receptor*

Next, to elucidate the role of KLF15 in Ang II-induced renal fibrosis, we measured KLF15 expression after Ang II infusion. After 4 weeks of Ang II infusion, the KLF15 mRNA expression level was drastically decreased. In contrast, losartan effectively increased KLF15 expression in the Ang II plus losartan group (Fig. 4A). Similar results were also demonstrated by immunofluorescence methods that showed KLF15-positive cells present in the renal

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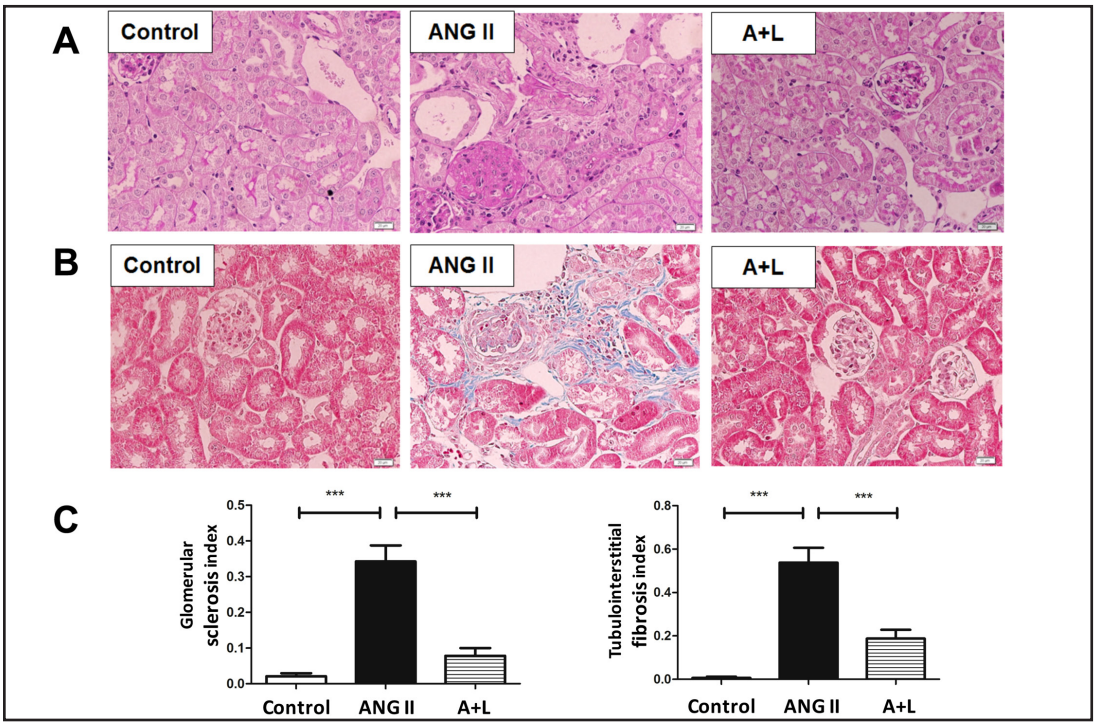


**Fig. 2.** Renal pathology of mice after 6 weeks of Ang II infusion. Representative photomicrographs of sections stained with periodic acid-Schiff to assess glomerulosclerosis, arterio-arteriolar sclerosis, and tubulointerstitial damage (pink stain) of the control group, Ang II group and Ang II plus losartan (A + L) group (×400, A). Representative photomicrographs of sections stained with Masson's trichrome to assess the extracellular matrix (blue stain) of the control group, Ang II group and Ang II plus losartan (A + L) group (×400, B). Semiquantitative measurements of glomerulosclerosis and interstitial fibrosis revealed that Ang II infusion increased the glomerulosclerosis index and interstitial fibrosis score, while losartan treatment attenuated these parameters (C). Data are expressed as the means ± standard deviations (SDs). *** p<0.001.

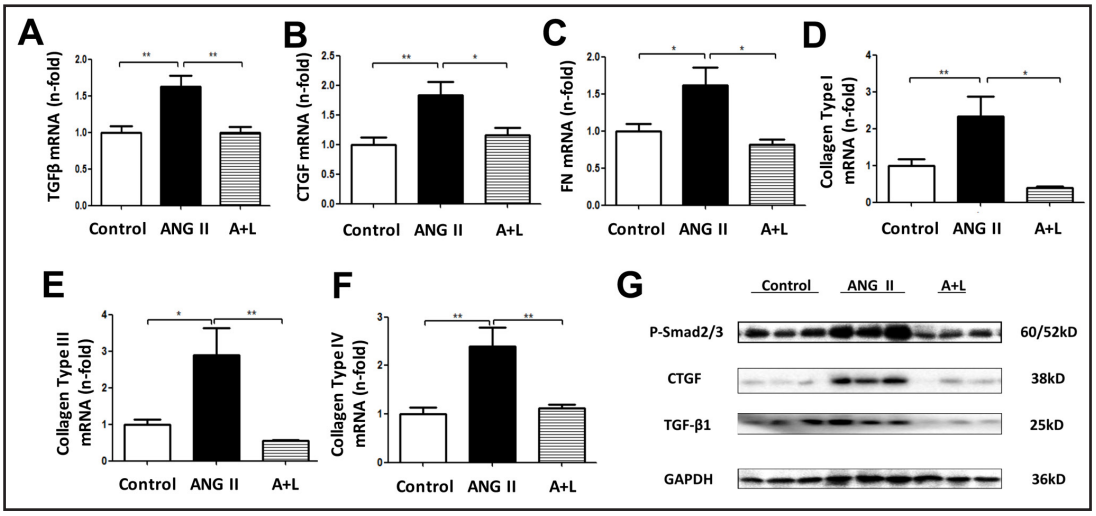


**Fig. 3.** Fibrotic cytokine expression and ECM production were increased in the Ang II-induced renal fibrosis mouse model. Real-time PCR revealed that TGF-β1 (A), CTGF (B), fibronectin (C), Type I (D), Type III (E) and Type IV collagen (F) mRNA expression levels were increased by Ang II and were reduced by Ang II plus losartan. Western blots showed that the phosphorylation level of Smad2/3 and expression of CTGF and TGF-β1 were elevated in mice infused with Ang II but were restored by treatment with losartan (G). Data are expressed as the means ± standard deviations (SDs). * p<0.05, ** p<0.01.

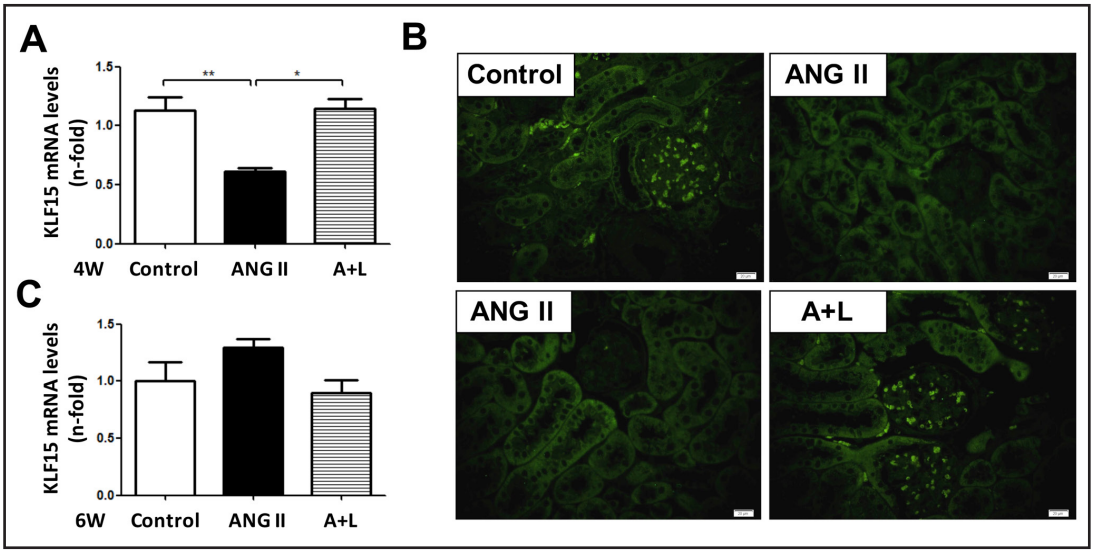


**Fig. 4.** Ang II decreased KLF15 expression via the AT1 receptor in vivo. KLF15 gene expression decreased significantly after 4 weeks of Ang II infusion (A) but returned to baseline after 6 weeks of Ang II infusion (C), as assessed by real-time PCR. Losartan completely prevented the decrease in KLF15 mRNA expression after 4 weeks of Ang II infusion (A). Representative microphotographs showed high basal expression levels of KLF15 (green), which were decreased by Ang II infusion and recovered by losartan intervention for 4 weeks (B). Data are expressed as the means ± standard deviations (SDs). * p<0.05, ** p<0.01.

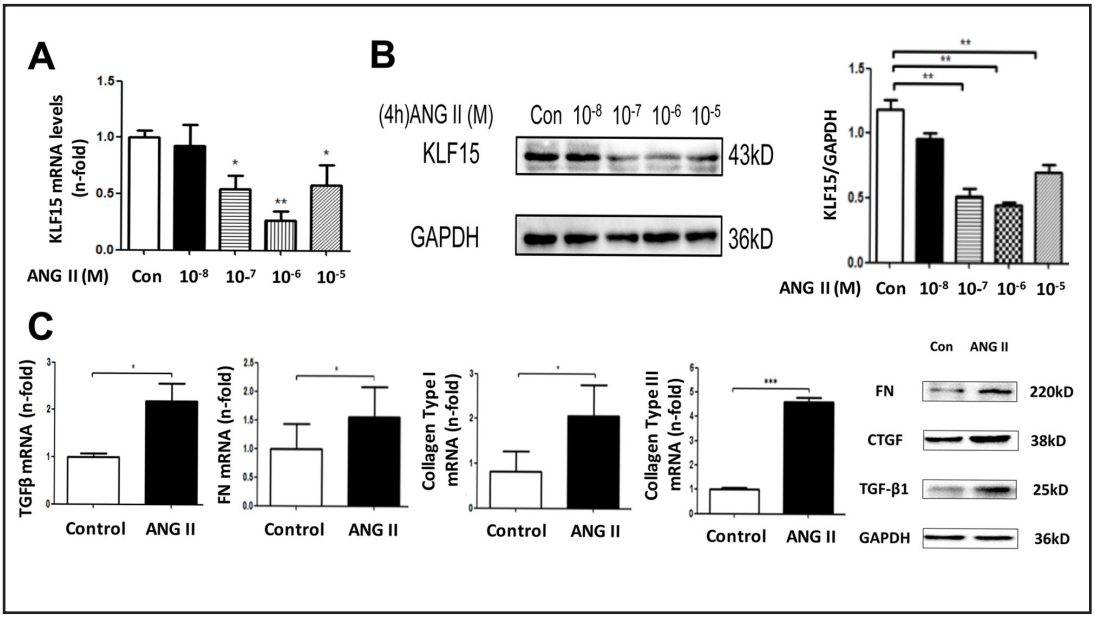


**Fig. 5.** Effect of different concentrations of Ang II on KLF15 expression. Ang II treatment for 4 h at final concentrations ranging from $10^{-7}$ to $10^{-5}$ M decreased KLF15 mRNA expression in NRK-49F cells (A). Representative Western blots (left) and densitometric analysis (right) showed that the protein expression levels of KLF15 in NRK-49F cells were significantly reduced by Ang II at final concentrations ranging from $10^{-7}$ to $10^{-5}$ M (B). Gene expression levels of TGF-β1, fibronectin, collagen I and collagen III were markedly increased after stimulation with Ang II for 24 h and were followed by significant increases in the protein expression levels of CTGF, TGF-β1 and fibronectin in NRK-49F cells after Ang II stimulation for 48 h (C). Data are expressed as the means ± standard deviations (SDs). * p<0.05, ** p<0.01, *** p<0.001.

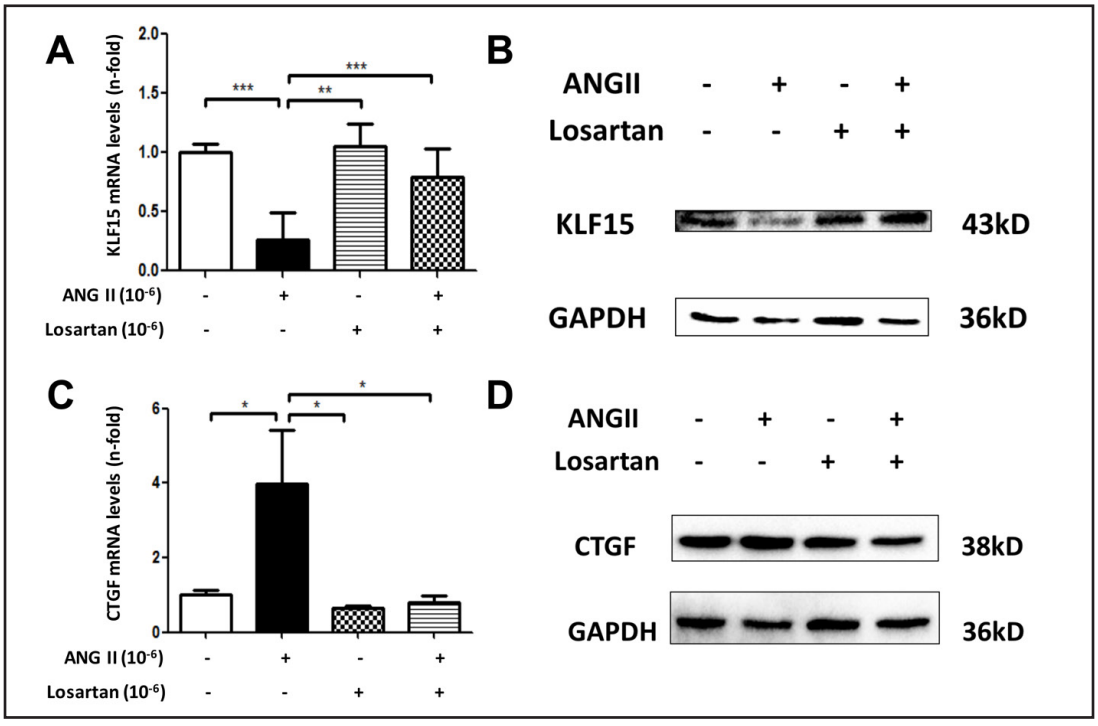


**Fig. 6.** Effect of losartan on Ang II-induced KLF15 and CTGF expression levels. The Ang II-induced down-regulation of KLF15 mRNA expression in NRK-49F cells was prevented by losartan (A). Representative Western blots also showed that pretreatment with losartan abolished the Ang II-induced down-regulation of KLF15 protein expression (B). Consistent with these results, pretreatment with losartan attenuated the Ang II-induced up-regulation of CTGF mRNA and protein expression levels in NRK-49F cells (C&D). Data are expressed as the means ± standard deviation (SD). * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

tissues of the control group and Ang II plus losartan group but not in the Ang II group (Fig. 4B). These results demonstrated that the decreased expression of KLF15 in response to Ang II was regulated by the AT1 receptor. Surprisingly, KLF15 expression was not significantly different among the three groups after 6 weeks of Ang II infusion (Fig. 4C). The reduction in KLF15 expression after 4 weeks, preceding the evident renal fibrosis at 6 weeks, implied that KLF15 might have an early anti-fibrotic effect on renal fibrosis.

*Ang II decreases KLF15 expression via the AT1 receptor in NRK-49F cells*

To further explore the effect of Ang II on KLF15 expression *in vitro*, real-time PCR and Western blot were performed in NRK-49F cells stimulated with Ang II at a final concentration of $10^{-8}$ M, $10^{-7}$ M, $10^{-6}$ M and $10^{-5}$ M for 4 h. The results confirmed that Ang II significantly decreased KLF15 expression in a dose-dependent manner (Fig. 5A, B). After treating NRK-49F cells with Ang II for 24 h, the gene expression of TGF-β1, fibronectin, collagen I and collagen III were markedly increased, and a significant increase was observed in the protein expression levels of CTGF, TGF-β1 and fibronectin after treating cells with Ang II for 48 h (Fig. 5C). All above results demonstrated that Ang II had a direct effect on KLF15 expression and that KLF15 expression was closely correlated with the expression of Ang II-induced fibrotic factors. To detect whether the AT1 receptor was involved in the decreased KLF15 expression after Ang II treatment, NRK-49F cells were exposed to Ang II ($10^{-6}$ M) for 4 h with or without pretreatment with losartan ($10^{-6}$ M). The down-regulation in KLF15 expression induced by Ang II treatment was attenuated by pretreatment with losartan (Fig. 6A, B). It is likely that the down-regulation of KLF15 expression in response to Ang II was mediated by the activation of the AT1 receptor. Furthermore, the Ang II-induced up-regulation of CTGF expression was also prevented by losartan (Fig. 6C, D).

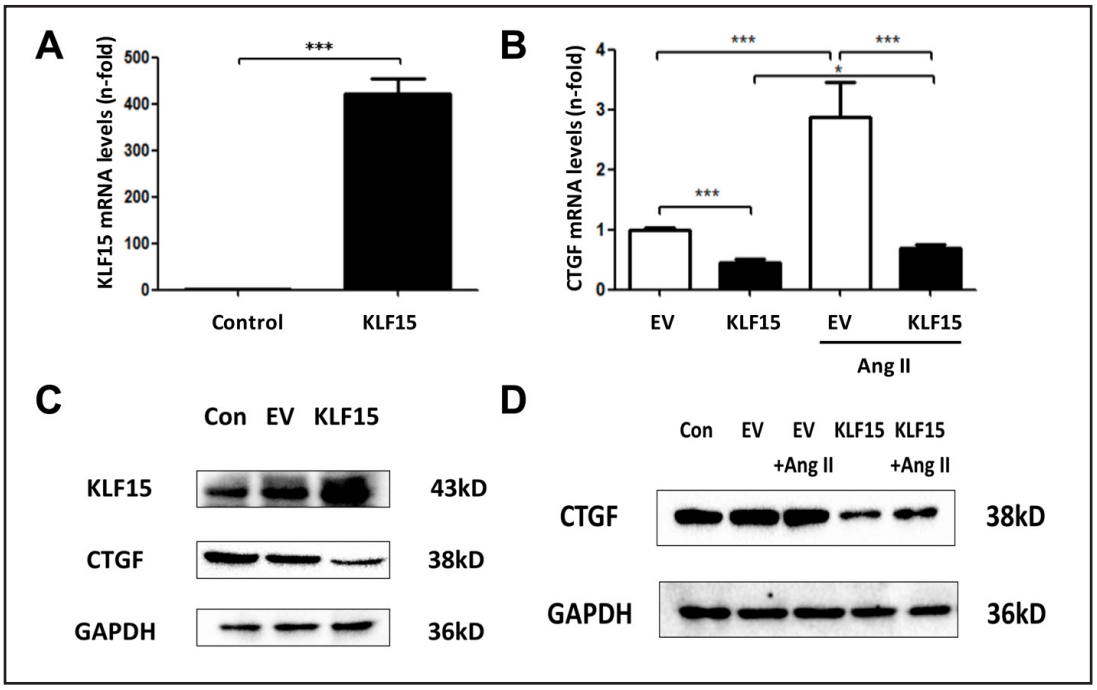


**Fig. 7.** KLF15 inhibited basal and Ang II-induced CTGF expression in NRK-49F cells. KLF15 mRNA expression was increased 400-fold after KLF15 adenoviral infection (A). Overexpression of KLF15 inhibited basal and Ang II-induced CTGF mRNA expression in NRK-49F cells (B). Western blots also showed that the overexpression of KLF15 inhibited the basal levels and Ang II-induced up-regulation of CTGF protein expression compared with those of the empty vector control (C&D). Data are expressed as the means ± standard deviations (SDs). * $p<0.05$, *** $p<0.001$.

*Overexpression of KLF15 decreases basal and Ang II-induced CTGF expression*

To verify whether the up-regulation of CTGF expression induced by Ang II is mediated by KLF15 expression, NRK-49F cells were infected with either Ad-GFP or Ad-GFP-KLF15. A progressive increase in KLF15 expression was found in NRK-49F cells infected with Ad-GFP-KLF15 and was accompanied by a decrease in the expression of CTGF (Fig. 7). Ang II treatment caused a significant decrease in KLF15 expression, followed by a marked increase in CTGF, whereas the overexpression of KLF15 attenuated the up-regulation of CTGF induced by Ang II (Fig. 7B, D). These results implied that KLF15 could inhibit the basal and Ang II-induced up-regulation of CTGF expression.

*KLF15 down-regulates CTGF transcription via co-activator P/CAF*

To clarify the relationship between the transcriptional regulator KLF15 and Ang II-induced CTGF expression, we immunoprecipitated P/CAF in HEK-293T total cell lysate and nuclear protein with an anti-P/CAF antibody. Then, the HEK-293T total cell lysate and nuclear protein were immunoblotted with an anti-KLF15 antibody. As shown in Fig. 8A, the transcription regulator KLF15 directly bound to the co-activator P/CAF. ChIP analysis also indicated that the overexpression of KLF15 competed with P/CAF and inhibited the recruitment of P/CAF to the CTGF promoter in the NRK-49F cell line (Fig. 8B). All these results confirmed that KLF15 interacted with CTGF to play an important role in Ang II-induced renal fibrosis.

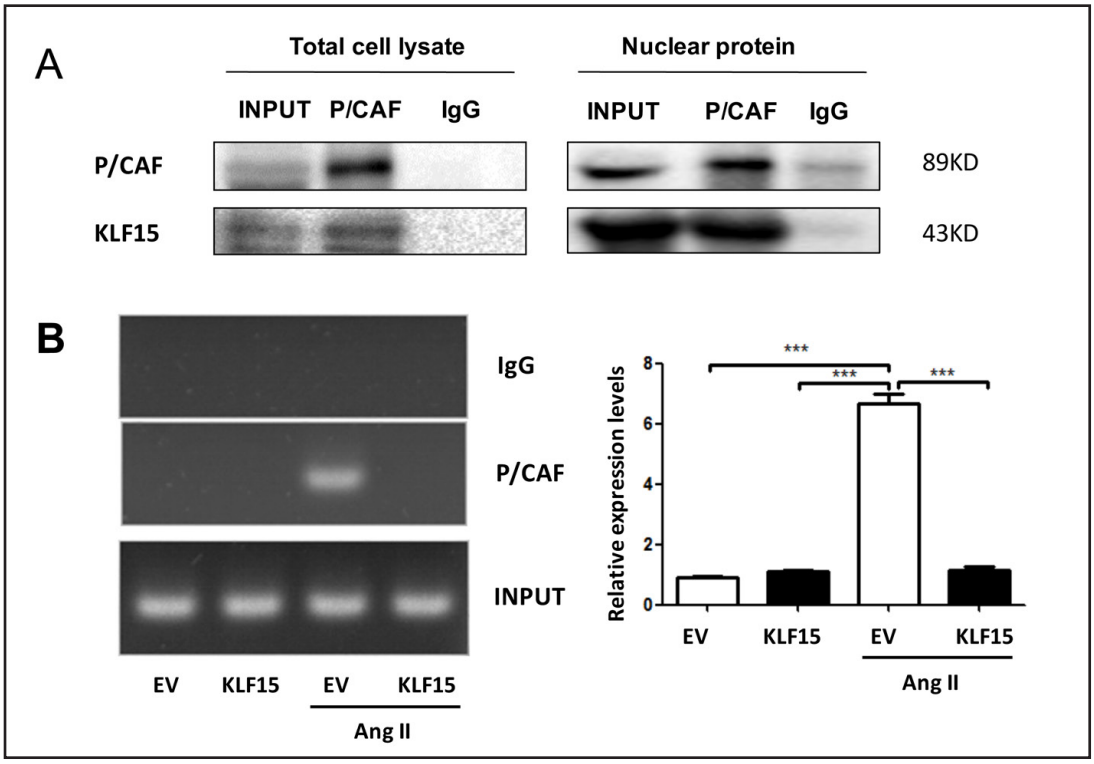


**Fig. 8.** KLF15 inhibited P/CAF recruitment to the CTGF promoter. HEK-293T cells were transfected with Ad-GFP-KLF15 and co-immunoprecipitation was performed. KLF15 interacted with P/CAF in the total cell protein and extracted nuclear protein (A). NRK-49F cells were infected with Ad-GFP or Ad-GFP-KLF15 and stimulated with Ang II. Chromatin immunoprecipitation assays were performed for P/CAF recruitment to the endogenous rat CTGF promoter using an anti-P/CAF antibody and the indicated PCR primers. Overexpression of KLF15 competed with P/CAF and inhibited the recruitment of P/CAF to the CTGF promoter (B). Data are expressed as the means ± standard deviations (SDs). *** $p<0.001$.

## Discussion

The RAS system plays an important role in renal fibrosis [3, 18, 19]. Many studies have demonstrated that TGF-β and CTGF are key factors in Ang II-induced fibrosis [20, 21]. However, directly blocking TGF-β in the treatment of renal fibrosis has not achieved effective outcomes [22]. CTGF, as a promising target for the treatment of renal fibrosis, seems to be an attractive target for anti-fibrotic therapy in kidney disease [23]. KLF15, an important transcription regulator, is greatly involved in regulating fibrosis [14, 15]. Wang et al [24]. demonstrated that *Klf15*$^{-/-}$ mice were sensitized to severe cardiac fibrosis, presenting with an exaggerated expression of CTGF, and KLF15 functioned in cardiac fibroblasts as an important inhibitor of cardiac fibrosis by inhibiting TGF-β-induced CTGF. The work of Leenders et al [25]. also showed that KLF15 overexpression in mouse heart prevented cardiac fibrosis in response to Ang II-induced hypertension. In our previous studies, KLF15 expression was significantly decreased in the renal tissues of a CKD model of 5/6 nephrectomised rats, and KLF15 mRNA and protein expression levels were down-regulated by TGF-β in renal medullary carcinoma (RMC) and NRK-49F cells. Furthermore, *Klf15*$^{-/-}$ mice were susceptible to renal fibrosis in a uninephrectomy model [14]. All these studies reiterate that KLF15 plays an important role in the regulation of CTGF expression in Ang II-induced renal fibrosis.

In the current study, we created an Ang II infusion-induced renal fibrosis murine model and found that KLF15 expression was markedly decreased after 4 weeks of chronic Ang II infusion. In addition, *in vitro* study also demonstrated that KLF15 expression was down-

regulated by Ang II stimulation in NRK-49F cells and that the expression of CTGF was mediated by KLF15. Although the level of KLF15 expression returned to baseline after 6 weeks of chronic Ang II infusion, evident extracellular matrix accumulation was found in renal tissues. Studies have demonstrated that the profibrotic transcription factor ETS-1 has a similar effect; in Ang II infusion model, ETS-1 expression was significantly increased at early timepoints but reduced to normal levels at later timepoints [26, 27]. According to the results above, we speculate that KLF15 acts as an early profibrotic transcription repressor. A negative feedback mechanism is initiated to modulate its expression.

It is well-known that the AT1 receptor plays a key role in Ang II-induced renal fibrosis, and AT1 receptor blockades have become a first-line treatment option for many kidney diseases [28, 29]. *In vivo*, after 4 weeks of Ang II infusion, we found that losartan significantly prevented KLF15 expression from decreasing in renal fibrosis. Consistent with this finding, losartan attenuated the decrease in the expression of KLF15 in NRK-49F cells exposed to Ang II. Losartan is a hyperselective AT1 receptor antagonist and is widely used as an effective treatment for Ang II-induced renal fibrosis [30]. In our study, we demonstrated the CTGF expression was mediated by KLF 15 in Ang II -induced renal fibrosis. Yang et al. [31] found that the expression of CTGF induced by Ang II was blocked by an AT1R antagonist (losartan), which might suggest that the AT1 receptor played a pivotal role in Ang II-induced renal fibrosis. Since the down-regulation of KLF15 expression in Ang II-induced renal fibrosis was attenuated by losartan treatment, we speculated that the attenuation effect was achieved by blocking the AT1 receptor. Additionally, whether KLF15 expression can be directly mediated by the AT1 receptor is still unclear. Ang II can activate many profibrotic factors, such as TGF-β, ET-1, and MCP-1, though the AT1 receptor [3], and previous studies have found that TGF-β can mediate KLF15 expression [32]. These results suggest that the down-regulation of KLF15 expression in Ang II-induced renal fibrosis is partially mediated through the TGF-β pathway, and the specific mechanism requires further study. In summary, the regulation of KLF15 expression may provide another mechanism for AT1 receptor blockades as a first-line treatment option for kidney diseases.

CTGF is an important profibrotic factor that has been well studied in TGF-β-induced renal fibrosis. However, CTGF may play an important role in renal fibrosis independent of TGF-β [23]. For example, CTGF can bind to low-density lipoprotein receptor-related protein 1 (LRP1) and epidermal growth factor receptor (EGFR) to activate profibrotic ERK signalling in the kidney [33, 34]. Moreover, many studies have demonstrated that directly decreasing CTGF expression effectively modulates kidney disease [23]. As mentioned above, Ang II-induced CTGF expression plays an important role in renal fibrosis. Based on these findings, we speculate that the Ang II-induced CTGF expression is mediated by KLF15 expression. *In vitro* studies, we determined the effects of altered KLF15 expression on the expression of CTGF in NRK-49F cells, which showed that the overexpression of KLF15 could inhibit basal and Ang II-induced CTGF expression. These results may confirm that Ang II-induced CTGF expression is regulated by KLF15. Smad activation is an important mechanism in Ang II-induced renal fibrosis. Specifically, Ang II causes the rapid activation of Smad2/3 phosphorylation and the nuclear translocation of P-Smad2/3 and Smad4 [3, 22]. In the nucleus, Smads can bind to the consensus Smad binding element (SBE) in the promoter sequence of some genes, such as CTGF, to modulate the transcription of downstream genes [35]. How does KLF15 regulate Ang II-induced CTGF overexpression? Wang et al. [24] have already found that KLF15 inhibits basal and Smad3/4-induced 3Tplux promoter activity and that the overexpression of the co-activator P/CAF rescues this inhibition in HEK-293T cells. Moreover, previous work by Itoh also showed that the co-activator P/CAF directly interacted with Smad3 and potentiated the activation of TGFβ /Smad signalling [32]. Taken together, we hypothesized that KLF15 could compete with P/CAF and inhibit the recruitment of P/CAF to the SBE in the CTGF promoter. To confirm our hypothesis, we performed a CoIP assay to confirm that KLF15 could directly interact with P/CAF in intact cells. Then, the ChIP method was further used to prove that Ang II stimulation prevented KLF15 from binding to P/CAF and induced the recruitment of P/CAF to the CTGF promoter. These results may provide key insights into the regulation of Ang II-induced CTGF expression by KLF15.

## Conclusion

In summary, our work identifies that KLF15 regulates CTGF expression by competing with P/CAF and inhibiting the recruitment of P/CAF to the CTGF promoter in Ang II-induced renal fibrosis; these findings provide key insights into renal fibrosis pathogenesis and pave the way for novel therapeutic interventions.

## Disclosure Statement

The authors declare that they have no competing interests.

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