Protective role of kallistatin in renal fibrosis via modulation of Wnt/β-catenin signaling

Wai Han Yiu[‾†1], Ye Li[‾†1], Sarah W. Y. Lok[1], Kam Wa Chan[1], Loretta Y. Y. Chan[1], Joseph C. K. Leung[1], Kar Neng Lai[1], James H.L. Tsu[2], Julie Chao[3], Xiao-Ru Huang[4], Hui Yao Lan[4] and Sydney C. W. Tang*[1]

[1]Department of Medicine, The University of Hong Kong, Queen Mary Hospital, Hong Kong

[2]Department of Surgery, The University of Hong Kong, Queen Mary Hospital, Hong Kong

[3]Department of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, South Carolina, USA

[4]Department of Medicine and Therapeutics, and Li Ka Shing Institute of Health Sciences, The Chinese University of Hong Kong, Hong Kong

[‾†]These authors contributed equally to this work.

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[*]Corresponding author

Prof. Sydney C.W. Tang,

Division of Nephrology, Department of Medicine,

The University of Hong Kong, Queen Mary Hospital,

102 Pokfulam Road, Hong Kong

Tel: (852) 2255 3879

Fax: (852) 2855 1143

Email: scwtang@hku.hk

Abstract

Kallistatin is a multiple functional serine protease inhibitor that protects against vascular injury, organ damage and tumor progression. Kallistatin treatment reduces inflammation and fibrosis in the progression of chronic kidney disease (CKD), but the molecular mechanisms underlying this protective process and whether kallistatin plays an endogenous role are incompletely understood. In this study, we observed that renal kallistatin levels were significantly lower in patients with CKD. It was also positively correlated with estimated glomerular filtration rate (eGFR) and negatively correlated with serum creatinine level. Unilateral ureteral obstruction (UUO) in animals also led to downregulation of kallistatin protein in the kidney, and depletion of endogenous kallistatin by antibody injection resulted in aggravated renal fibrosis, which was accompanied by enhanced Wnt/β-catenin activation. Conversely, overexpression of kallistatin attenuated renal inflammation, interstitial fibroblast activation and tubular injury in UUO mice. The protective effect of kallistatin was due to the suppression of TGF-β and β-catenin signaling pathways and subsequent inhibition of epithelial to mesenchymal transition (EMT) in cultured tubular cells. In addition, kallistatin could inhibit TGF-β-mediated fibroblast activation via modulation of Wnt4/β-catenin signaling pathway. Therefore, endogenous kallistatin protects against renal fibrosis by modulating Wnt/β-catenin-mediated EMT and fibroblast activation. Downregulation of kallistatin in the progression of renal fibrosis suggests a valuable clinical implication and therapeutic potential in CKD.

Keywords: renal fibrosis; β-catenin signaling; fibroblast activation; EMT

Introduction

Renal fibrosis is a key process driving the progression of chronic kidney disease (CKD) to end-stage renal disease (ESRD) regardless of etiology [1]. Indeed, it is a maladaptive repair characterized by persistent inflammation, increased numbers of myofibroblast and accumulation of extracellular matrix (ECM) [2]. Although it is widely accepted that CKD is a chronic inflammatory disease, we do not fully understand how inflamed kidney cells trigger the pro-fibrotic process and still cannot identify an effective therapeutic target to halt renal fibrosis. It has been reported that developmental signaling pathways including TGF-β, Wnt, Notch and Hedgehog are involved in both kidney repair and development of CKD. Transient activation of these pathways during kidney injury is important for regeneration of the damaged renal cells; however, sustained activation promotes fibrosis [3, 4].

Wnt signaling has been implicated in kidney development [5, 6]. β-catenin activation following the canonical Wnt pathway regulates transcriptional activities in different cell lineages including Wolffian duct, ureteric epithelium, mesenchyme and renal stroma [7]. In the adult kidneys, Wnt/β-catenin signaling is relatively silent, but becomes re-activated in both renal tubules and interstitium after injury. We and others have shown that re-activation of Wnt/β-catenin signaling during acute kidney injury (AKI) protects tubular epithelial cells from apoptosis [8, 9] and promotes cell cycle progression [10, 11], thereby accelerating renal recovery and proliferation. However, prolonged activation of Wnt/β-catenin pathway is detrimental and leads to the progression of CKD. Aberrant expression of Wnt/β-catenin signaling is associated with pathogenesis and progression of renal fibrosis in experimental model of obstructive nephropathy and diabetic nephropathy [12, 13]. Wnt/β-catenin signaling activates intrarenal renin-angiotensin system (RAS), which is associated with vasoconstriction, inflammation and fibrosis in CKD [14]. Transgenic mice with overexpression of stabilized β-catenin in renal tubules exhibit aggravated proteinuria,

enhanced inflammation and impaired renal function compared with wild type control in protein overload model [15]. In addition, Wnt/β-catenin directly controls the expression of key mediators in kidney fibrosis including Snail-1 for EMT, plasminogen activator inhibitor 1 (PAI-1) and matrix metallopeptidase-7 (MMP7) for ECM accumulation and fibroblast specific protein-1 (FSP-1) for fibroblast activation [16]. Therefore, modulation of Wnt/β-catenin signaling pathway has been suggested as a promising therapeutic strategy for CKD [17].

Kallistatin was first identified as a unique serine proteinase inhibitor that binds strongly to human tissue kallikrein, via its active site, for inhibiting tissue kallikrein's bioactivity. Kallistatin is mainly produced in the liver and widely distributed in plasma, body fluids and various tissues associated with cardiovascular function such as heart, kidney and blood vessels [18]. Previous studies also found that kallistatin interacts with cell surface heparin sulfate proteoglycans, via its heparin binding site, to regulate different signaling pathways that involves in various biological functions including vasodilation, angiogenesis, oxidative stress, inflammation and fibrosis [19]. Growing evidence suggest that kallistatin has beneficial effects against hypertension, organ damage and cancer development in animal models and cultured cells [20, 21]. In the model of CKD, kallistatin gene transfer ameliorates glomerulosclerosis and renal fibrosis in diabetic mice via reduction of AGE-RAGE-induced oxidative stress [22]. Likewise, administration of kallistatin protects against AKI and attenuates renal damage in septic mice by inhibiting inflammation and apoptosis [23]. Mechanistically, kallistatin, via its heparin-binding domain, regulates multiple signaling pathways at different cellular conditions. For example, kallistatin inhibits angiogenesis and promotes apoptosis against tumor growth by blocking NF-κB signaling pathway [24, 25]. As a key regulator of vascular homeostasis, kallistatin reduces oxidative stress, inhibits apoptosis and senescence in endothelial cells via activation of eNOS signaling [26, 27]. The heparin

binding domain of kallistatin is also found to be crucial for antagonizing Wnt signaling. It blocks the canonical Wnt-mediated β-catenin signaling pathway by interacting with Wnt coreceptor low-density lipoprotein receptor-related protein 6 (LRP6) in breast cancer cells and cultured retinal cells [28, 29]. Kallistatin overexpression suppresses overactivation of Wnt pathway and attenuates retinal inflammation and vascular leakage in diabetic mice [29]. In addition, kallistatin treatment inhibits Ang II-induced aortic aneurysm formation by blocking Wnt signaling pathway in Ang II-infused ApoE deficient mice [30]. Since prolonged activation of Wnt signaling is associated with the progression of CKD and kallistatin is known to inhibit Wnt/β-catenin signaling, we hypothesized that kallistatin might act as an endogenous Wnt inhibitor and protect the kidney against renal fibrosis via antagonizing pro-fibrotic Wnt-mediated signaling pathway.

In this study, we sought to investigate the effect of kallistatin on renal inflammation, fibrosis and Wnt signaling activation by depletion and overexpression of kallistatin in the animal model of obstructive nephropathy, and evaluate the relationship between renal kallistatin level of patients with CKD and renal function.

Materials and Methods

Human renal tissue specimens

The use of tissue specimens for this study was approved by the Research Ethics Committee/ Institutional Review Board of the University of Hong Kong/ Hospital Authority Hong Kong West Cluster. Renal tissue biopsy were obtained from CKD patients including diabetic nephropathy, IgA nephropathy and membranous nephropathy (n=13). Normal portions of renal tissues removed from nephrectomy specimens for the treatment of solitary renal carcinoma in the opposite pole were used as control (n=5). All subjects provided written informed consent. The clinical data of CKD patients were listed in **Table 1**. Paraffin-embedded sections (4 μm) were prepared using a routine procedure.

Animal studies

All work with animals were approved by the Committee on the Use of Live Animals in Teaching and Research of the University of Hong Kong and was performed in accordance with the NIH Guide for the Care and Use of Laboratory Animal. All animal works were conducted in the Centre for Comparative Medicine Research (CCMR) at University of Hong Kong.

Renal kallistatin overexpression in a mouse model of UUO

Male BALB/c mice at the age of 8 weeks were obtained from CCMR, University of Hong Kong. Mice were anaesthetized with Midazolam/ Fentanyl/ Medetomidine (1:1:0.5 ; 10 ml/kg body weight) via intraperitoneal injection (I.P.) for the surgical procedures. Mice were euthanized with injection of pentobarbital (150 mg/kg; I.P.) after experiments. Kallistatin expressing plasmids (pTRE-KS) were transferred into the kidney of mice using the ultrasound-mediated, microbubble gene transfer technique as previously described [22]. Briefly, pTRE-KS and Tet-On plasmids (Clontech, Mountain View, CA) were mixed with

Sonovue (Bracco, Milan, Italy) and injected via tail vein into mice. Immediately after injection, ultrasound (1 MHz, 2 W/cm^2 for 5 min) was applied on the left kidney. A group of age-matched mice injected with empty pTRE and Tet-On plasmids, followed by ultrasound procedure was served as control. Doxycycline solution (Sigma-Aldrich, St Louis, MO) was injected intraperitoneally and added into the drinking water for transgene induction. One week after gene transfer, mice were subjected to a procedure of unilateral ureteral obstruction on the left kidney, as previously described. Sham-operated mice had their ureters exposed and manipulated without ligation. All mice were sacrificed on day 7 after UUO. Kidneys were collected for further analyses.

Depletion of endogeneous kallistatin in a rat model of UUO

Male Sprague Dawley (SD) rats weighing around 200-220 g were obtained from CCMR, University of Hong Kong. One day before surgery, rats were given daily intravenous injection of 0.5 mg anti-rat KS antibody (a kind gift from Prof. Julie Chao at Medical University of South Carolina) until sacrifice. Control rats were given same amount of normal rabbit IgG. The procedure of UUO or sham operation were performed on the left kidney and all rats were sacrificed on day 7 after UUO. Kidneys were collected for further analyses.

Histological and immunohistochemical staining

Kidneys were fixed in 10% neutral-buffered formalin and paraffin-embedded. Tissue sections (4μm) were subjected to periodic acid-Schiff (PAS) and Sirius Red staining by standard protocol. Histological changes of the renal cortex were evaluated according to the severity of tubular dilatation, atrophy and cast formation using a semiquantitative scoring system from 0-5, as previously described [22]. Immunohistochemical staining was performed using Envision Plus system (Dako, Carpinteria, CA). Paraffin-embedded kidney sections were incubated with primary antibodies against F4/80 (Serotec, Oxford, UK), Col-1 and Col-33 (Southern

Biotech, Birmingham, AL), α-SMA (Sigma), kallistatin (Abcam, Cambridge, MA), β-catenin (BD Bioscience, San Jose, CA) followed by peroxidase conjugated secondary antibodies. All sections were counterstained with hematoxylin. The positive staining of F4/80 from twenty different fields of kidney sections were quantified by Image J software (NIH).

Cell culture model

Human immortalized proximal tubule epithelial (HK-2) cells and rat kidney interstitial fibroblast (NRK-49F) cells (ATCC, Manassas, VA) were cultured in DMEM/F-12, supplemented with penicillin (100 IU/ml), streptomycin (100 μg/ml) and 10% fetal bovine serum (Invitrogen, Carsbad, CA) at 37ºC in 5% CO_2 atmosphere. To overexpress kallistatin in tubular cells, human kallistatin plasmid (pCMV-KS) or control plasmid (pCMV-empty) was transfected into HK-2 cells using Lipofectamine®2000 (Invitrogen). Transfected HK-2 cells were treated with TGF-β (R&D Systems, Minneapolis, MN) for various time points as indicated. Cell lysate and supernatant were collected for various analyses. Serum-starved NRK-49F cells were treated with conditioned medium from HK-2 transfected cells and were harvested for further analyses.

Real-time qPCR analysis

Total RNAs were extracted from renal cortical tissue or cultured cells and reverse transcribed into cDNA as previously described [22]. Quantitative real-time PCR was performed using SYBR Green reagent on the StepOne Plus Real-Time PCR System (Applied Biosystems, Carlsbad, CA). The primer sequences for mRNA detection were listed in Table 2.

Western blot analysis

Total proteins were prepared from cortical kidney tissue using RIPA lysis buffer (Millipore,

Bedford, MA). Equal amount of proteins were resolved in 4-12% SDS-PAGE gel (Invitrogen), transferred to a PVDF membrane (Millipore). After blocking, membrane was incubated overnight with primary antibodies against α-SMA (Sigma), Col-1 (Southern Biotech), β-catenin, p-GSK3β, GSK3β, histone, vimentin, p-smad3 and total smad3 (Cell Signaling Technology, Beverly, MA), TGF-β, kallistatin (Abcam), renin, AT1 (Santa Cruz Biotechnology, Santa Cruz, CA), Agt (Immuno-Biological Laboratories, Japan). The immunocomplex was visualized with ECL prime chemiluminescence (GE Health, Buckinghamshire, UK) using the ChemiDoc XRS system (Bio-Rad, Hercules, CA). Quantification of protein bands was performed by the ImageJ program (NIH, Bethesda, MD) and was normalized to GAPDH level.

Statistics

All the data were obtained from at least 3 independent experiments and expressed as mean ± SEM. Differences between multiple groups were evaluated with one-way analysis of variance followed by Bonferroni's comparison using GraphPad Prism, version 4 (GraphPad Software, San Diego, CA). Data were considered statistically significant at $P < 0.05$ (*$P< 0.05$; **$P<0.01$ and ***$P<0.001$)

Results

Reduced renal kallistatin expression in development and progression of CKD

We first examined the expression level of kallistatin (KS) in human renal biopsies from patients with various stages of CKD. As shown in **Figure 1A**, KS was predominantly expressed in the renal tubular epithelium and its expression was significantly reduced in patients with CKD versus control subjects. KS levels progressively decreased from CKD stage 2 to 4 (**Figure 1B**) and the magnitude of KS reduction was correlated positively with estimated glomerular filtration rate (eGFR) ($r=0.9396$, $p< 0.0001$) (**Figure 1C**) and negatively with serum creatinine ($r=-0.8625$, $p<0.0001$) (**Figure 1D**). To validate this observation, we assessed the expression level of KS in the animal model of UUO with a robust induction of tubulointerstitial fibrosis. Immunohistochemical staining of kidney tissues showed that KS expression was significantly lower in UUO rats than in sham-operated rats at day 7 after UUO (**Figure 1E**), confirming the association of renal KS loss with the development of progressive renal fibrosis.

Depletion of kallistatin aggravates renal fibrosis in UUO rat

To determine whether reduction of kallistatin expression contributes to the pathophysiology of CKD, we injected anti-KS antibody daily into UUO rats in order to deplete endogenous KS. On day 7 after UUO, KS expression was significantly reduced in kidney tissues of UUO rats receiving control IgG compared with those that underwent sham operation. Renal KS expression was further decreased in UUO rats receiving anti-KS antibody as shown by Western blot (**Figure 2A**). Immunohistochemical staining confirmed the reduction of KS expression mainly in renal tubules of the kidney and it was further decreased after the administration of anti-KS antibody (**Figure 2B**). As shown by PAS staining, ligated kidneys from UUO rats receiving control IgG displayed characteristics of renal damage including

tubular atrophy, dilated tubules, infiltration of inflammatory cells and tubulointerstitial fibrosis, and injection of anti-KS antibody further exacerbated such morphological abnormality (**Figure 2C**). Likewise, Sirius red staining showed that the positively stained area was significantly increased in UUO rats with anti-KS injection compared with UUO rats given control IgG, indicating that depletion of endogenous KS aggravated the accumulation of ECM proteins in UUO kidneys (**Figure 2D**). Tubulointerstitial fibrosis was further confirmed by immunohistochemistry. Compared to UUO rats given control IgG only, rats with depletion of KS exhibited a marked increase in interstitial collagen III (Col-3) deposition and α-smooth muscle actin expression (α-SMA) (**Figure 3A**), indicating the accumulation of activated fibroblasts in the renal interstitium. Consistently, cortical expression of vimentin, α-SMA and TGF-β was upregulated in obstructed kidneys from UUO rats compared to sham, and the induction of these fibrotic molecules was aggravated after anti-KS injection (**Figure 3B**). Although KS expression in sham-operated rats was significantly decreased after anti-KS injection to levels similar to the UUO group, there was no difference between the two sham groups regardless of anti-KS injection, in kidney morphology. In the absence of UUO, therefore, reduction of KS *per se* did not affect the expression of fibrosis-related molecules (**Figure S1**). Taken together, our data suggest that blockade of KS in the kidney aggravates the deposition of ECM proteins and fibroblast activation, resulting in severe tubular damage in the UUO model. Endogenous KS may have a protective role in tubulointerstitial fibrosis.

Blockade of kallistatin is associated with activation of Wnt/β-catenin signaling in UUO kidneys

Kallistatin inhibits tumor progression by blocking Wnt/β-catenin signaling pathway [31]. Since activation of Wnt/β-catenin pathway is linked to the pathogenesis of kidney fibrosis [16], we next evaluated the effect of KS depletion on Wnt/β-catenin signaling in the UUO model by detecting GSK3β and β-catenin levels in kidney tissues. In the absence of Wnt signaling, β-catenin is sequestered and phosphorylated by a destructive complex of GSK3β, axin, casein kinase 1 and adenomatous Polyposis Coli, where phosphorylated β-catenin is subjected to degradation by the ubiquitin-proteasome system. Upon Wnt activation, GSK3β is phosphorylated at Ser9 and results in inhibition of its activity on phosphorylation of β-catenin. Thus, β-catenin is dephosphorylated, and accumulate in cytoplasm and subsequently migrate to the nucleus where it initiates transcription of target genes. As shown in Western blotting, the expression levels of phosphorylated GSK3β at Ser9 and dephosphorylated β-catenin (active form) were significantly induced in the UUO group compared to the sham-operated group, indicating the activation of Wnt/β-catenin signaling after UUO, and the elevated levels were further augmented after injection of anti-KS antibody (**Figure 4A**). Consistently, immunohistochemical staining also demonstrated that β-catenin accumulated in the tubular epithelium of UUO kidneys, and its expression was further enhanced after injection with anti-KS antibody (**Figure 4B**). In addition, gene expression of Wn4, β-catenin and Axin2, the direct downstream target of Wnt/β-catenin signaling, was significantly higher in UUO kidneys compared with sham-operated control, and KS depletion resulted in further induction of these molecules (**Figure 4C**). Our results indicate that kallistatin deficiency increased the activation of Wnt/β-catenin signaling in UUO kidneys.

Kallistatin attenuates Wnt/β-catenin and TGF-β signaling *in vitro*

We next examined the effect of kallistatin overexpression on Wnt/β-catenin and TGF-β signaling, two important fibrogenic signaling pathways involved in CKD. In cultured tubular epithelial cells, KS expression was markedly increased after transfection of KS expression

plasmid in HK-2 cells (**Figure 5A**). Likewise, KS secretion in culture medium was also upregulated from cells transfected with KS plasmid (**Figure 5B**). As shown in **Figure 5C**, KS overexpression significantly reduced TGF-β-induced cytoplasmic β-catenin expression and inhibit its nuclear translocation . KS overexpression also suppressed the downstream targets (PAI-1, fibronectin (Fn) and Snail) and the negative regulator of Dickkopf-related protein 1 (DKK1) of Wnt/β-catenin signaling as evidenced by quantitative real-time PCR (qPCR) analysis (**Figure 5D**). It has been shown that crosstalk between Wnt/β-catenin and TGF-β signaling modulates renal fibrosis in injured tubules [32]. We therefore investigated whether KS overexpression in HK-2 cells could block TGF-β-mediated fibrotic responses. As shown in **Figure 6A,** transfection of KS plasmid repressed TGF-β- induced endogenous TGF-β expression and phosphorylation of smad3 (p-smad3). Consistently, KS also significantly inhibited mRNA expression of TGF-β and collagen I (Col-1) upon TGF-β treatment (**Figure 6B**). Together, these results show that KS could attenuate TGF-β-mediated Wnt/β-catenin activation and its downstream smad3 signaling pathway and fibrotic responses in tubular epithelial cells.

Kallistatin gene transfer ameliorates inflammation and renal injury in UUO model

Given that reduced renal KS expression led to worsening of tubulointerstitial fibrosis in the obstructed kidneys, we explored whether KS overexpression would ameliorate renal damage in UUO model. Gene transfer of KS plasmid was performed in the obstructed kidney of UUO mice by ultrasound, microbubble-mediated method as previously described [22]. Induction of KS overexpression (**Figure 7A**) significantly alleviated tubule damage (**Figure 7B**). UUO kidneys overexpressed with KS plasmid also showed a decreasing trend of interstitial fibrosis

(**Figure 7C**). In addition, KS overexpression attenuated infiltration of F4/80 positive macrophages in UUO kidneys (**Figure 8A and 8B**). In addition, the increased mRNA expression levels of CCL-2 and TNF-α in the UUO kidney were significantly lower in mice with KS overexpression (**Figure 8C**), indicating a reduction of proinflammatory responses after KS gene transfer. Given that Wnt/β-catenin and TGF-β signaling pathways are important drivers of renal fibrosis, we determined the effect of KS overexpression on these pathways. Compared to sham-operated mice, UUO kidneys exhibited higher TGF-β and β-catenin expression, which became significantly suppressed after KS gene transfer (**Figure 9A**). Moreover, qPCR confirmed the activation of Wnt/β-catenin pathway as shown by increased mRNA levels of Wnt4 and Axin2 in UUO kidneys and these molecules were attenuated by KS overexpression (**Figure 9B**). In addition, components of the intrarenal renin-angiotensin system (RAS) including angiotensinogen (Agt), renin and angiotensin II type 1 receptor (AT1) were all significantly increased in UUO kidneys, whereas upregulation of renin and AT1 were attenuated after KS gene transfer. However, KS overexpression did not affect Agt expression in UUO kidneys (**Figure 9C and 9D**). Together, these results suggest that KS overexpression protects against kidney injury through amelioration of inflammation, modulation of Wnt/β-catenin and TGF-β signaling pathways and suppression of intrarenal RAS.

Kallistatin suppresses renal interstitial fibroblast activation and ECM production

Interstitial fibroblasts play a key role in the development of renal fibrosis. In response to injury, resident fibroblasts differentiate into myofibroblasts that express α-SMA and produce ECM proteins. We therefore investigated the effect of KS on fibroblast activation in both UUO animals and TGF-β-treated fibroblasts. Immunohistochemistry and qPCR results showed that KS overexpression after gene transfer suppressed the enhanced expression of α-SMA and Col-1 in renal interstitium of UUO kidneys compared to sham-operated kidneys

(**Figure 10A and 10B**). The inhibitory effect of KS on fibroblasts was further demonstrated by a reduction of increased mRNA expression of fibroblast specific protein-1 (FSP-1) in UUO kidneys (**Figure 10C**). To confirm the inhibitory effect of KS on kidney fibroblasts, we performed *in vitro* study in which NRK49F cells were pretreated with conditioned medium from HK-2 cells that overexpressed KS prior to TGF-β stimulation. Secreted KS from transfected HK-2 cells could attenuate the induced expression of α-SMA and Col-1 in TGF-β-stimulated NRK49F cells in kidney fibroblasts compared with pretreatment of conditioned medium without KS (**Figure 10D)**. Together, these results suggest that KS suppresses TGF-β-activated interstitial fibroblasts, thereby reducing the accumulation of ECM proteins in the UUO model.

Discussion

Many studies have shown a protective role of endogenous kallistatin against oxidative stress, inflammation, angiogenesis and apoptosis in various organ injuries, including kidney [20, 33]. Kallistatin treatment prevents sepsis-induced kidney injury through suppression of inflammatory responses [23]. The anti-oxidative effect of kallistatin is associated with increased endothelial nitric oxide synthase (eNOS) expression and nitric oxide levels in experimental model of salt-induced renal injury [34]. Our previous study has also demonstrated that overexpression of kallistatin by gene transfer ameliorates diabetic nephropathy in *db/db* mice by inhibiting NF-κB-mediated inflammation, TGF-β-activated fibrosis and reducing AGE-induced oxidative stress [22]. However, it is unclear whether kallistatin plays an endogenous role in CKD and how kallistatin participates in the pathological mechanism of renal interstitial fibrosis.

The present study provide, for the first time, evidence on a correlation between kallistatin expression and the progression of CKD. Our findings on clinical specimen showed that kallistatin levels in the kidneys of patients with CKD from distinct causes were significantly lower than those in healthy controls. Of particular interest, the reduction of kallistatin levels in CKD patients increase with increasing stages of CKD and a decline in renal function, suggesting that loss of kallistatin is associated with the progression of renal injury. To test this hypothesis, we injected anti-KS antibody into UUO rats and showed that depletion of the endogenous kallistatin exacerbated the severity of renal fibrosis in the obstructed kidneys through activation of TGF-β and Wnt/β-catenin signaling pathways, which play important roles in regulating kidney fibrosis [35, 36]. This result confirms an endogenous role of kallistatin in the development and progression of CKD. Moreover, our findings demonstrated that secreted kallistatin from tubular epithelium could inhibit the activation of fibroblasts, providing a novel insight into the protective mechanism of kallistatin against tissue fibrosis.

Kallistatin was first discovered as tissue kallikrein binding protein for its ability to inhibit tissue kallikrein activity through its structural active site [37, 38]. On the other hand, kallistatin, via its heparin binding domain, interacts with heparin sulfate proteoglycans to modulate multiple signaling pathways including NF-κB activation, p38 MAPK and Akt phosphorylation [26, 39, 40]. Accumulating evidence show that sustained Wnt/β-catenin reactivation is involved in the pathogenesis of renal fibrosis. Crosstalk between Wnt/β-catenin and TGF-β/Smad signaling has been reported in regulating EMT and fibrotic responses in tubular epithelial cells [41, 42] and interstitial fibroblasts [43]. Results from this study not only support our previous findings of anti-fibrotic effect of kallistatin via suppression of renal inflammation and TGF-β signaling pathway in diabetic nephropathy [22], but further reveal that kallistatin inhibits TGF-β-induced fibrotic changes via β-catenin-dependent pathway, thereby suppressing the expression of profibrotic β-catenin target genes, such as PAI-1, Fn, Snail and key components of RAS including renin and AT1. Activation of intrarenal RAS contributes to CKD progression and anti-RAS drug such as angiotensin converting enzyme (ACE) inhibitor (ACEI) and AT1 receptor blockers (ARB) remain the most commonly used therapeutic approach for CKD. Renin secreted by juxtaglomerular cells in the kidney cleaves Agt into angiotensin I (Ang I), which is then converted by ACE into angiotensin II (Ang II). Upon binding to AT1 receptor, Ang II mediates various deleterious effects including vasoconstriction, inflammatory responses and renal fibrosis. Besides, renin can directly mediate renal fibrosis through binding to its membrane receptor PRR and triggering TGF-β and matrix gene expression [14]. Kallistatin is a potent vasodilator independent of the tissue kallikrein-kinin system. Kallistatin, via its active site, activates Akt-eNOS signaling and induces NO formation in endothelial cells [33]. Here, our data showed that kallistatin suppresses renin and AT1 expression in the kidney tissues. Given that RAS genes are targets of Wnt/β-catenin signaling that contain putative TCF/LEF-binding sites in

their promoter regions [44], reduction of Wnt/β-catenin signaling by kallistatin overexpression lead to the downregulation of these RAS components and attenuating renal fibrosis in experimental model of UUO.

Kallistatin suppresses Wnt pathway activation in the retina of mice with oxygen-induced retinopathy and diabetes by antagonizing Wnt co-receptor LRP6 [29]. Similarly, kallistatin inhibits tumor growth and induces apoptosis in cancer cells by blocking Wnt-3-induced pathway [31] and activating PPAR signaling [45]. From our depletion study, we found that endogenous kallistatin could regulate expression of active β-catenin in kidney tubules by modulating the phosphorylation of GSK3β and Wnt4 expression. Wnt4 activates canonical β-catenin signaling pathway and is essential for mesenchymal to epithelial transition (MET) during kidney development [46, 47]. In adult kidneys, both Wnt4 expression and the β-catenin signaling are silenced, but re-activated after renal injury. In human CKD, Wnt4 is induced in both tubular and interstitial compartments of renal biopsies from patients [48]. In UUO model, Wnt4 is highly induced in interstitial myofibroblasts of injured kidney and canonical Wnt/β-catenin signaling plays an important role in myofibroblast differentiation, indicating a functional role of fibroblast Wnt4/β-catenin activation in renal fibrosis [49, 50]. Via inhibiting Wnt4/β-catenin signaling, our findings suggest a protective mechanism of kallistatin in fibroblast activation in UUO model. Overexpression of kallistatin significantly reduces the expression of fibroblast markers such as α-SMA, FSP-1 and Col-1 in UUO mice. Consistently, secreted kallistatin in conditioned medium from tubular epithelial cells inhibits *in vitro* TGF-β-induced fibroblast activation. Since kallistatin is mainly localized in the tubular epithelium of the kidney; therefore, it is possible that kallistatin prevents interstitial fibroblast activation via paracrine mechanism. It has been reported that kallistatin inhibits TGF-β-induced Col-1 synthesis in cultured myofibroblasts through its anti-oxidative effect, thereby preventing myocardial infarction-induced hypertrophy and fibrosis [51]. Our findings

further suggest that kallistatin prevents renal fibroblast activation via antagonizing Wnt/β-catenin signaling pathway.

Kallistatin may inhibit renal fibroblast activation through its direct effect on tubular epithelial cells. The origin of myofibroblasts in renal fibrosis are controversial [52] and renal fibroblasts can be derived from resident fibroblasts, from pericytes, from macrophage-to myofibroblast transition (MMT), from endothelial cells via an endothelial-mesenchymal transition (EndoMT) or from epithelial cells via EMT [53]. Our *in vitro* data show that kallistatin attenuates β-catenin nuclear translocation and expression of EMT-related genes such as Snail and PAI-1 in tubular epithelial cells, which play critical roles in the process of EMT [42, 54]. Although EMT contributes only a small population of fibroblasts in fibrotic kidney, conditional inhibition of EMT in proximal tubular cells is sufficient to maintain tubular integrity and attenuate interstitial fibrosis in experimental UUO mice [55]. Taken together, kallistatin exerts a direct protective effect on tubular epithelial cells through inhibition of TGF-β and β-catenin signaling pathways as well as EMT induction.

Reduced kallistatin levels are observed in both human diseases and animal models associated with oxidative stress and chronic inflammation [56]. In this study, we, for the first time, demonstrate the downregulation of kallistatin levels in kidney biopsies from CKD patient, and it correlates with different degrees of renal function. The lowest kallistatin level is associated with later stages of CKD, suggesting a pathogenic role in the progression of CKD. Our findings on reduced renal KS levels in patients with CKD was opposite to increased serum KS levels from patients with DN in our CKD group (**Figure S2**) and previous study in which serum kallistatin levels are increased in diabetic patients with DN compared to those diabetic patients without DN and healthy controls [57]. Since liver is the major site of kallistatin synthesis and secretion, the increase in serum level may not reflect the kidney problem. Similar phenomenon was observed in diabetic retinopathy (DR), in which serum

kallistatin levels were significantly increased in patients with DR compared to diabetic patients without rentinopathy and healthy controls, but reduced kallistatin levels were detected in the vitreous fluids of patients with DR [58, 59]. Furthermore, it is likely that proteinuria in CKD patients may lead to impaired reabsorption of circulating KS such that the renal KS level is limited to protect the kidney from injury.

However, it has been reported that renal kallstatin expression was increased in various animal models of DN[57]. The discrepancy in renal kallistatin expression found between animal model of UUO and DN may due to the difference in the severity of renal fibrosis. UUO is a progressive model of renal fibrosis with tubular dilatation and atrophy, and strong induction of fibronectin, α-SMA and ECM proteins, whereas tubulointerstatial fibrosis is less severe in most DN models compared to UUO. Since many studies have reported that kallistatin exerts protective effect against organ injury [20], it may be possible that it is upregulated at the early stage of DN and then downregulated at the later stage as renal damage persists. Indeed, our clinical findings on reduced renal KS levels are in line with our animal study, in which depletion of kallistatin leads to aggravated renal fibrosis in UUO rats. Previous study also reported that kallistatin deficiency results in enhanced renal dysfunction and tissue fibrosis in a salt-induced hypertensive rat [60]. In endothelial cells, kallistatin expression is downregulated by oxidative stress via JNK/FOXO1 pathway, and the reduced kallistatin expression is inversely correlated with elevated ROS level in animal models of hypertension and cardiovascular disease [61]. Since oxidative stress plays a crucial role in kidney injury and fibrosis, it is speculated that oxidative stress may contribute to the downregulation of kallistatin expression in the tubular epithelial cells or kidney. Indeed, previous study has showed that injection of anti-KS antibody into rats leads to an increase in renal superoxide formation, NADH oxidase activity and decrease in nitrate/nitrite production [60]. This oxidative stress may cause the suppression of kallistatin expression in kidney tissues.

However, the exact molecular mechanism remains to be elucidated. Taken together, our findings indicate an endogenous protective role of kallistatin in kidney injury.

Protective role of kallistatin has been reported in other diseases such as vascular injury, sepsis, liver disease, diabetic retinopathy and in cancer development [20]. Kallistatin gene transfer protects mice from tubular apoptosis in ischemia-reperfusion injury [62] and ameliorates renal fibrosis in diabetic mice [22]. In this study, kallistatin overexpression also attenuates renal inflammation and fibrosis in UUO model. However, a recent study showed a deleterious effect of kallistatin overexpression on diabetic nephropathy due to the activation of renal renin-angiotensin system (RAS) [57]. It has been reported that kallistatin exerts double-edged roles in many biological functions [33]. It inhibits angiogenesis in VEGF-induced endothelial cells, whereas promotes vascular repair in endothelial progenitor cells. Kallistatin blocks TNF-α-induced apoptosis via its heparin-binding site, but stimulates apoptosis in breast cancer cells via its active site. The contradictory results in renal injury between our study and others may be due to difference in the experimental condition. First, our ultrasound-mediated microbubble gene transfer delivers kallistatin transgene specifically into the kidney, while kallistatin is broadly expressed in multiple tissues in transgenic mice and after adenovirus injection in other study. Second, the magnitude of kallistatin overexpression from transgenic mice may counteract the beneficial effect of kallistatin in the kidney.

In conclusion, our study uncovers an important clinical implication of renal kallistatin expression in CKD. Reduced kallistatin level in renal biopsy is correlated with renal function at different stages of CKD, suggesting a pathogenic role in the progression of CKD. We also identify an endogenous role of kallistatin in preventing renal fibrosis by modulating TGF-β and Wnt/β-catenin pathways, inhibiting EMT and fibroblast activation; and provide evidence that kallistatin therapy may be a promising therapeutic strategy for CKD progression.

Clinical Perspectives

- Renal kallistatin was significantly lowered in patients with CKD and its expression level was negatively correlated with renal function, implying that kallistatin may become a biomarker for the progression of CKD.

- In the UUO model, depletion of kallistatin enhanced Wnt/β-catenin activation and aggravated renal fibrosis, indicating its endogenous protective role in progression of CKD.

- Kallistatin treatment ameliorated TGF-β-induced EMT and fibroblast activation in UUO mice by antagonizing Wnt/β-catenin activation, suggesting a potential novel therapeutic strategy for CKD.

Conflicts of Interest

The authors declared no competing interests

Data Availability Statement

The data underlying this article are available in the article and in its online supplementary material.

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Author contributions

WHY and YL designed experiment, analyzed data and wrote the manuscript. YL and SWYL performed experiments. KWC acquired and analyzed clinical data. LYYC and JCKL analyzed and interpreted results. JHLT provided clinical tissue specimen. JC provided anti-KS antibody. KNL, XRH and HY L reviewed and edited the manuscript. SCWT conceived and supervised the study.

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Table 1. Clinical data of CKD patients.

	All patients (n=13) mean ± SD	CKD stage 2 (n=4) mean ± SD	CKD stage 3 (n=7) mean ± SD	CKD stage 4 (n=2) mean ± SD
Gender (number)				
Male	7	2	4	1
Female	6	2	3	1
Age (year)	49.5 (±11.7)	55.0 (±15.3)	48.6 (±7.4)	41.5 (±19.1)
eGFR (ml/min per 1.73m^2)	46.6 (±18.76)	69.5 (±8.3)	40.2 (±9.2)	23.0 (±4.2)
sCr (μmol/L)	140.8 (±62.19)	87.0 (±14.2)	142.1 (±40.2)	243.5 (±58.7)
Underlying disease				
DKD	8	1	6	1
IgAN	4	3	0	1
MN	1	0	1	0

Abbreviation: SD, standard deviation; eGFR, estimated glomerular filtration rate; sCr, serum creatinine; DKD, diabetic kidney disease; IgAN, IgA nephropathy; MN, membranous nephropathy

Table 2. Primer sequences for quantitative real-time PCR.

Gene	Forward primer 5'→3'	Reverse primer 5'→3'
β-catenin (rat)	GGACAAGCCACAGGACTACAAGA	CAGTCCGAGATCAGCAGTCTCAT
Axin2 (rat)	TGGTGCATACCTCTTCCGGACTTT	TTTCCTCCATCACCGCCTGAATCT
Wnt4 (rat)	GTTCCACACTGGACTCCCTG	ACACCTGCTGAAGAGATGGC
TNF-α (mouse)	CCGATGGGTTGTACCTTGTC	GGCAGAGAGGAGGTTGACTTT
CCL-2 (mouse)	CTCTTCCTCCACCACCAT	CTCTCCAGCCTACTCATTG
Axin2 (mouse)	ACTGACCGACGATTCCATGT	TGCATCTCTCTCTGGAGCTG
Wnt4 (mouse)	CTGGAGAAGTGTGGCTGTGA	GGACTGTGAGAAGGCTACGC
α-SMA (mouse)	GTGCTATGTCGCTCTGGACTTTGA	ATGAAAGATGGCTGGAAGAGGGTC
Col-1 (mouse)	TGTGTGCGATGACGTGCAAT	GGGTCCCTCGACTCCTACA
FSP-1 (mouse)	CAGGCAAAGAGGGTGACAAG	TGCAGGACAGGAAGACACAG
GAPDH (rat/mouse)	TCCATCATGAAGTGTGACGT	GAGCAATGATCTTGATCTTCAT
PAI-1 (human)	GGGCCATGGAACAAGGATGA	CTCCTTTCCCAAGCAAGTTG
Fn (human)	CCCAACTGGCATTGACTTTT	CTCGAGGTCTCCCACTGAAG
DKK1 (human)	GGGTCTTTGTCGCGATGGTA	GGGGGCAGGTTCTTGATAGC
Snail (human)	ACCCACACTGGCGAGAAGCC	TTGACATCTGAGTGGGTCTG
TGF-β (human)	CACGTGGAGCTGTACCAGAA	GAACCCGTTGATGTCCACTT
Col-1 (human)	GTGCTAAAGGTGCCAATGGT	ACCAGGTTCACCGCTGTTAC
GAPDH (human)	GCCAAAAGGGTCATCATCTC	GGCCATCCACAGTCTTCTGG

Figure Legends

Figure 1. Reduction of kallistatin levels in renal tissues from patients with CKD and UUO animals. (**A**) Representative immunohistochemical staining of kallistatin (KS) from control subjects and patients with CKD at different stages (2-4). Bar =100 μm. (**B**) Quantitative analysis of immunohistochemical staining of kallistatin from control (n=5), CKD stage 2 (n=4), CKD stage 3 (n=7) and CKD stage 4 (n=2). (**C**) Positive correlation between kallistatin staining and eGFR from CKD patients. (**D**) Negative correlation between kallistatin staining and serum creatinine level from CKD patients. (**E**) Representative immunohistochemical staining of kallistatin and the corresponding quantitative analysis from sham-operated (n=5) and UUO (n=5) rats. Bar =100 μm. *P< 0.05; **P<0.01 and ***P<0.001 between groups as indicated.

Figure 2. Blockade of endogenous kallistatin worsens renal injury in UUO rats. (**A**) Western blotting analyses shows renal expression of kallistatin (KS) from rats with sham-operation (n=5), UUO (n=5) and UUO with anti-kallistatin (anti-KS) antibody injection (n=5) and the corresponding quantitative analysis. (**B**) Representative immunohistochemical staining of kallistatin (KS) in different groups and quantitative analysis. (**C**) Representative PAS staining and quantitative analysis (**D**) Representative Sirius Red staining and quantitative analysis. Bar =100 μm *P< 0.05; **P<0.01 and ***P<0.001 between groups as indicated.

Figure 3. Blockade of endogenous kallistatin aggravates deposition of ECM and fibroblast activation in UUO rats. (**A**) Representative immunohistochemical staining of Col-3 and α-SMA and corresponding quantitative analysis in sham (n=5), UUO (n=5) and UUO with anti-KS (n=5) group. Bar =100 μm (**B**) Western blotting analyses shows expression of vimentin, α-SMA and TGF-β protein in different groups and the corresponding quantitative analysis. *P< 0.05; **P<0.01 and ***P<0.001 between groups as indicated.

Figure 4. Blockade of kallistatin enhances the activation of Wnt/β-catenin signaling in UUO kidneys. (**A**) Western blotting analyses shows phosphorylation level of GSK3β (p-GSK3β), total GSK3β and β-catenin (active form) levels in renal tissues from rats with sham-operation (n=5), UUO (n=5) and UUO with anti-kallistatin (anti-KS) antibody injection (n=5) and the corresponding quantitative analysis. (**B**) Representative immunohistochemical staining of β-catenin in renal tissue sections. Bar =100 μm. (**C**) Quantitative real-time PCR

shows relative expression of β-catenin, Axin2 and Wnt4 in different groups (n=5 in each group). *P< 0.05; **P<0.01 and ***P<0.001 between groups as indicated.

Figure 5. Overexpression of kallistatin attenuates Wnt/β-catenin and TGF-β signaling in HK-2 cells. HK-2 cells were transfected with control (ctl) (n=5-6) or kallistatin (KS) plasmid (n=5-6) and then stimulated with TGF-β (10 ng/ml). (**A**)Western blot analyses and quantitative analysis and (**B)** ELISA measurement for kallistatin level in the cell lysates and culture supernatant after transfection with control and KS plasmid. (**C)** Western blotting analyses shows expression of β-catenin in cytoplasmic (normalized with GAPDH) and nuclear fraction (normalized with histone) of cell lysates in different groups and the corresponding quantitative analysis. (**D**) Quantitative real-time PCR shows the relative expression of PAI-1, Fn, Snail and DKK1 in different groups. *P< 0.05; **P<0.01 and ***P<0.001 between groups as indicated.

Figure 6. Overexpression of kallistatin suppresses TGF-β-mediated smad3 signaling pathway and profibrotic responses in HK-2 cells. (A) Western blotting analyses shows the expression of TGF-β, phosphorylated smad3 and total smad3 in whole cell lysates of TGF-β (10 ng/ml)-stimulated HK-2 cells transfected with control (ctl) (n=5-6) or kallistatin (KS) plasmid (n=5-6) and the corresponding quantitative analysis. (**B**) Quantitative real-time PCR shows the relative expression of TGF-β and Col-1 in different groups. *P< 0.05; **P<0.01 and ***P<0.001 between groups as indicated.

Figure 7. Kallistatin gene transfer improves renal injury in UUO mice. Control (ctl) or kallistatin (KS) plasmid were transferred into the kidney via tail vein injection, followed by ultrasound, microbubble-mediated gene transfer technique, and then subjected to sham operation (n=6) or UUO (n=7). (**A**) Representative immunohistochemical staining of KS in renal tissues of different groups and quantitative analysis. (**B**) Representative PAS staining and quantitative analysis. (**C**) Representative Sirius red staining and quantitative analysis in different groups. Bar =100 μm *P< 0.05; **P<0.01 and ***P<0.001; n.s.: not significant between groups as indicated.

Figure 8. Kallistatin gene transfer reduces renal inflammation in UUO mice. Control (ctl) or kallistatin (KS) plasmid were transferred into the kidney via tail vein injection, followed by ultrasound, microbubble-mediated gene transfer technique, and then subjected to sham operation (n=6) or UUO (n=7). (**A**) Representative immunohistochemical staining of $F4/80^+$ cells in renal tissues of different groups and (B) corresponding quantitative analysis.

Bar =100 μm (**C**) Quantitative real-time PCR shows the relative expression of TNF-α and CCL-2 in different groups. *P< 0.05; **P<0.01 and ***P<0.001 between groups as indicated.

Figure 9. Kallistatin gene transfer reduces Wnt/ β-catenin signaling and RAS in UUO mice. (**A**) Western blotting analyses shows the expression of TGF-β and β-catenin from renal cortical tissues of sham or UUO mice with control (ctl) or kallistatin (KS) plasmid and the corresponding quantitative analysis. (**B**) Quantitative real-time PCR shows the relative expression of Axin2 and Wnt4 in different groups. (**C**) Western blotting analyses with the expression of Agt, renin and AT1 of RAS and (**D**) the corresponding quantitative analysis. *P< 0.05; **P<0.01 and ***P<0.001 between groups as indicated.

Figure 10. Kallistatin suppresses fibroblast activation both *in vivo* and *in vitro*. (**A**) Representative immunohistochemical staining of α-SMA and Col-1 in renal tissues from sham-operated or UUO mice with gene transfer of control (ctl) or kallistatin (KS) plasmid (n=6-7). Bar =100 μm. (**B and C**) Quantitative real-time PCR shows relative expression of α-SMA, Col-1 and FSP-1 in different groups. (**D**) Cultured fibroblasts (NRK49F) were pretreated with conditioned medium (CM) from tubular cells (HK-2 cells) transfected with ctl and KS plasmid and then stimulated with TGF-β (10 ng/ml). Western blotting analyses show the expression of α-SMA and Col-1from whole cell lysates from stimulated fibroblasts and the corresponding quantitative analysis (n=5). *P< 0.05; **P<0.01 and ***P<0.001 between groups as indicated.