

## RESEARCH ARTICLE

# Diabetes-induced upregulation of kallistatin levels exacerbates diabetic nephropathy via RAS activation

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## Abstract

Kallistatin is an inhibitor of tissue kallikrein and also inhibits the Wnt pathway. Its role in diabetic nephropathy (DN) is uncertain. Here we reported that serum kallistatin levels were significantly increased in diabetic patients with DN compared to those in diabetic patients without DN and healthy controls, and positively correlated with urinary albumin excretion. In addition, renal kallistatin levels were significantly upregulated in mouse models of type 1 (Akita, OVE26) and type 2 diabetes (*db/db*). To unveil the effects of kallistatin on DN and its underlying mechanism, we crossed transgenic mice overexpressing kallistatin with OVE26 mice (KS-tg/OVE). Kallistatin overexpression exacerbated albuminuria, renal fibrosis, inflammation, and oxidative stress in diabetes. Kallikrein activity was inhibited while the renin-angiotensin system (RAS) upregulated in the kidney of KS-tg/OVE mice compared to WT/OVE mice, suggesting a disturbed balance between the RAS and kallikrein-kinin systems. As shown by immunostaining of endothelial makers, renal vascular densities were decreased accompanied by increased HIF-1 $\alpha$  and erythropoietin levels in the kidneys of KS-tg/OVE mice. Taken together, high levels of kallistatin exacerbate DN at least partly by inducing RAS overactivation and hypoxia. The present study demonstrated a positive correlation between kallistatin levels and DN, suggesting a potential biomarker for prognosis of DN.

## KEYWORDS

diabetic nephropathy, fibrosis, hypoxia, kallikrein, kallistatin, kidney, RAS

**Abbreviations:** 3-NT, 3-Nitrotyrosine; ACE, angiotensin converting enzyme; ACR, albumin/creatinine ratio; BUN, blood urea nitrogen; CTGF, connective tissue growth factor; DN, diabetic nephropathy; DR, diabetic retinopathy; GFR, glomerular filtration rate; HIF-1 $\alpha$ , hypoxia inducible factor; HMC, human mesangial cells; ICAM-1, intercellular adhesion molecule-1; KKS, kallikrein-kinin system; KS-tg, kallistatin transgenic; NOX, NADPH oxidase; PAS, periodic acid-Schiff; RAS, renin-angiotensin system; TGF- $\beta$ 1, transforming growth factor 1; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; UACR, urinary albumin/creatinine ratio; WT, wild-type.

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## 1 | INTRODUCTION

Diabetic nephropathy (DN) is a common complication of diabetes characterized by proteinuria, decreased glomerular filtration, and renal fibrosis.<sup>1</sup> Multiple mechanisms are involved in the pathogenesis of DN, including oxidative stress, inflammation, and disturbed balance between the renin-angiotensin system (RAS) and kallikrein-kinin system (KKS).<sup>2-4</sup> RAS and KKS are two counter-balancing systems that regulate a wide array of physiological functions, such as blood pressure, local blood flow, inflammation, angiogenesis, and organ injury responses. Accumulating evidence supports protective effects of KKS and detrimental effects of overactivated RAS on DN.<sup>5-8</sup> As the kidney expresses components of both RAS and KKS, the intrarenal interaction of KKS and RAS plays a major role in the pathological processes of the kidney injury, especially under diabetic conditions. Also, the activated intrarenal RAS/oxidative stress/inflammatory cytokines axis has been demonstrated to play an important role in DN.<sup>9</sup>

Kallistatin is a serine proteinase inhibitor and was initially identified as a tissue kallikrein-binding protein.<sup>10</sup> Kallistatin is mainly produced by the liver and also broadly expressed in other tissues, such as the eye, kidney, heart, and blood vessels.<sup>11,12</sup> Kallistatin inhibits the activity of tissue kallikrein and regulates blood pressure.<sup>13</sup> Later, kallistatin was identified to function as an inhibitor of Wnt signaling, through which it confers anti-angiogenesis, anti-inflammation, anti-fibrosis, anti-oxidative stress, and anti-tumor activities.<sup>14-18</sup>

In our previous studies, we found that type 1 diabetic patients with microvascular complications showed significantly increased levels of serum kallistatin.<sup>19</sup> However, the vitreous level of kallistatin was reported to be decreased in patients with diabetic retinopathy (DR).<sup>20</sup> Overexpression of kallistatin in the retina attenuated ischemia-induced retinal neovascularization.<sup>21</sup> Another study subsequently identified elevated circulation levels of kallistatin as a marker of microvascular complications in children and adolescents with type 1 diabetes.<sup>22</sup> The increased circulating levels of kallistatin in diabetic patients have been confirmed by multiple groups.<sup>19,22,23</sup> Recently, transient overexpression of kallistatin by ultrasound-microbubble-mediated gene transfer was reported to protect against DN in *db/db* mice.<sup>24</sup> However, the exact changes of kallistatin levels and its impacts on DN have not been fully investigated in diabetic patients with DN or DN animal models.

Although a variety of spontaneously or experimentally induced hyperglycemic animals are used as models of DN, most of them display features of early stages of DN, such as microalbuminuria.<sup>25</sup> OVE26 mice on the FVB background are a transgenic model of type 1 diabetes and develop diabetes as early as the first weeks of life.<sup>26</sup> OVE26 mice recapitulate features similar to advanced human DN such as

albuminuria, mesangial matrix expansion, declined glomerular filtration rate (GFR), and interstitial fibrosis.

In the present study, we assessed serum kallistatin levels in type 2 diabetic patients with and without DN, as well as the correlation between kallistatin levels and the progression of kidney injury. In addition, we induced diabetes in transgenic mice that constantly overexpress human kallistatin in multiple tissues by cross-breeding them with OVE26, to investigate the impacts of elevated kallistatin levels on the DN.

## 2 | MATERIALS AND METHODS

### 2.1 | Patients and controls

Human subjects were recruited from Tianjin Medical University Chu Hsien-I Memorial Hospital. The diagnosis of diabetes and DN was based on the criteria reported previously.<sup>27,28</sup> Patients with other kidney or urinary tract diseases such as urinary tract infection, chronic nephritis, and primary hypertension were excluded. The study was performed in accordance with the Helsinki Guidelines and approved by the ethics committee of Tianjin Medical University Chu Hsien-I Memorial Hospital, and written informed consent was obtained from each subject.

### 2.2 | Animal models

The kallistatin transgenic (KS-tg) mice in the C57BL/6 background were generated as reported previously.<sup>21,23</sup> KS-tg mice were brought into the FVB background by continuous breeding with FVB mice. The F7 generation descendants of KS-tg mice were then crossbred with OVE26 mice (FVB background) to obtain KS-tg/OVE mice with wild-type OVE26 (WT/OVE) mice as controls. *db/db* (B6-BKS-Lep<sup>db</sup>/J) mice, Akita mice, and their respective non-diabetic control mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Serum and urinary creatinine levels were measured by HPLC.<sup>29</sup> UAE was measured by a murine microalbuminuria ELISA kit (Exocell, Philadelphia, PA). The use, treatment, and care of all animals in this study were approved by the Institutional Animal Care and Use Committee at the University of Oklahoma.

### 2.3 | ELISA and Western blot analysis

Kallistatin levels were measured using a human kallistatin ELISA kit (R&D Systems, Minneapolis, MN) and a mouse kallistatin ELISA kit (Fine Biological Technology, Wuhan, China); fibronectin was measured using a mouse fibronectin ELISA kit (Assaypro, St. Charles, MO), ICAM-1 using a

mouse ICAM-1 ELISA kit (R&D systems, Minneapolis, MN), Ang II by a ELISA kit (Enzo Life Sciences, Farmingdale, NY), and erythropoietin (EPO) using a mouse EPO ELISA kit (R&D systems, Minneapolis, MN). Western blot analysis was performed as described.<sup>30</sup> The primary antibodies used were purchased from the following companies: mouse kallistatin (Serpina3C) (R&D Systems, Minneapolis, MN); TGF- $\beta$ 1 (R&D Systems, Minneapolis, MN), T $\beta$ R-I and T $\beta$ R-II (Novus Biologicals, Littleton, CO); CTGF (Santa Cruz, Dallas, TX); TNF- $\alpha$  (Cell Signaling Technology, Danvers, MA); 3-NT, NOX4, and NOX2 (Abcam, Cambridge, MA); ACE (R&D systems, Minneapolis, MN); HIF-1 $\alpha$  (Novus Biologicals, Littleton, CO) and  $\beta$ -actin (Sigma-Aldrich, St. Louis, MO).

## 2.4 | Tissue kallikrein activity assay

Tissue kallikrein activities were assayed by amidase hydrolysis of the selective chromogenic substrate H-D-ValLeu-Arg-para-nitroanilide (S-2266; Chromogenix, Orangeburg, NY).<sup>31,32</sup> The chromogenic substrate was added in the presence or absence of aprotinin (Sigma-Aldrich, St. Louis, MO), an inhibitor of tissue kallikrein. The para-nitroaniline (pNa) formed in these reactions was detected at a wavelength of 405 nm. The results were expressed as OD value. Only the activity of the reaction inhibited by aprotinin was attributed to kallikrein activity.

## 2.5 | Real-time PCR analysis

The real-time PCR was performed on the Bio-Rad CFX96 Real-time system using SYBR Green PCR Master Mix kit (Applied Biosystems, Foster City, CA).<sup>33</sup> Sequences of primers used in this study are listed in Supplemental Table S1.

## 2.6 | Histology, immunohistochemistry, and Picro-Sirius red staining

Glomerulosclerosis was semi-quantified by a score system as described previously,<sup>34</sup> graded from 0 to +4: grade 0, normal; grade 1, slight glomerular damage including abnormalities such as a mild increase in mesangial sclerosis and/or hyalinosis with focal adhesions, <25% involvement of the glomerulus; grade 2, 25%-50% involvement of the glomerulus; grade 3, 50%-75%; and grade 4, sclerosis occupying >75% of the glomerulus. Tubulointerstitial damage was evaluated using a 0-5 scale as described previously<sup>34,35</sup> depending on the percentage with tubular dilatation, cast formation, interstitial infiltration, and fibrosis (grade 0, no changes; grade 1, <10%; grade 2, 10%-25%; grade 3, 25%-50%; grade 4, 50%-75%; and grade 5, 75%-100%).

IHC was performed as described previously<sup>29</sup> using primary antibodies against kallistatin (Sino Biological, China), collagen I (Abcam, Cambridge, MA), HIF-1 $\alpha$  (Novus Biologicals, Littleton, CO), and CD31 (Abcam, Cambridge, MA). Isolectin GS-IB<sub>4</sub> (Thermo Fisher Scientific) was used to label vasculature in the kidney.

Picro-Sirius red staining (Sigma-Aldrich, St. Louis, MO) was performed according to the manufacturer's instruction. Quantification of IHC and Picro-Sirius red staining images were obtained by measuring the integrated optical density using the Image Pro Plus 6.0 software. Values were means of 8 images of each sample.

## 2.7 | Cell culture

Primary human mesangial cells (HMC) (Cambrex Bio Science, Walkersville, MD) were cultured in the mesangial cell basal medium (Cambrex Bio Science, Walkersville, MD) with 0.1% GA-1000 (Cambrex Bio Science, Walkersville, MD) and 5% fetal bovine serum at 37°C in 5% CO<sub>2</sub> atmosphere.

## 2.8 | Construction of adenoviral vectors and human kallistatin gene delivery

Adenovirus expressing human kallistatin (Ad-KS) and a control adenovirus expressing green fluorescence protein (Ad-GFP) were constructed as described previously.<sup>36</sup>

## 2.9 | Statistical analysis

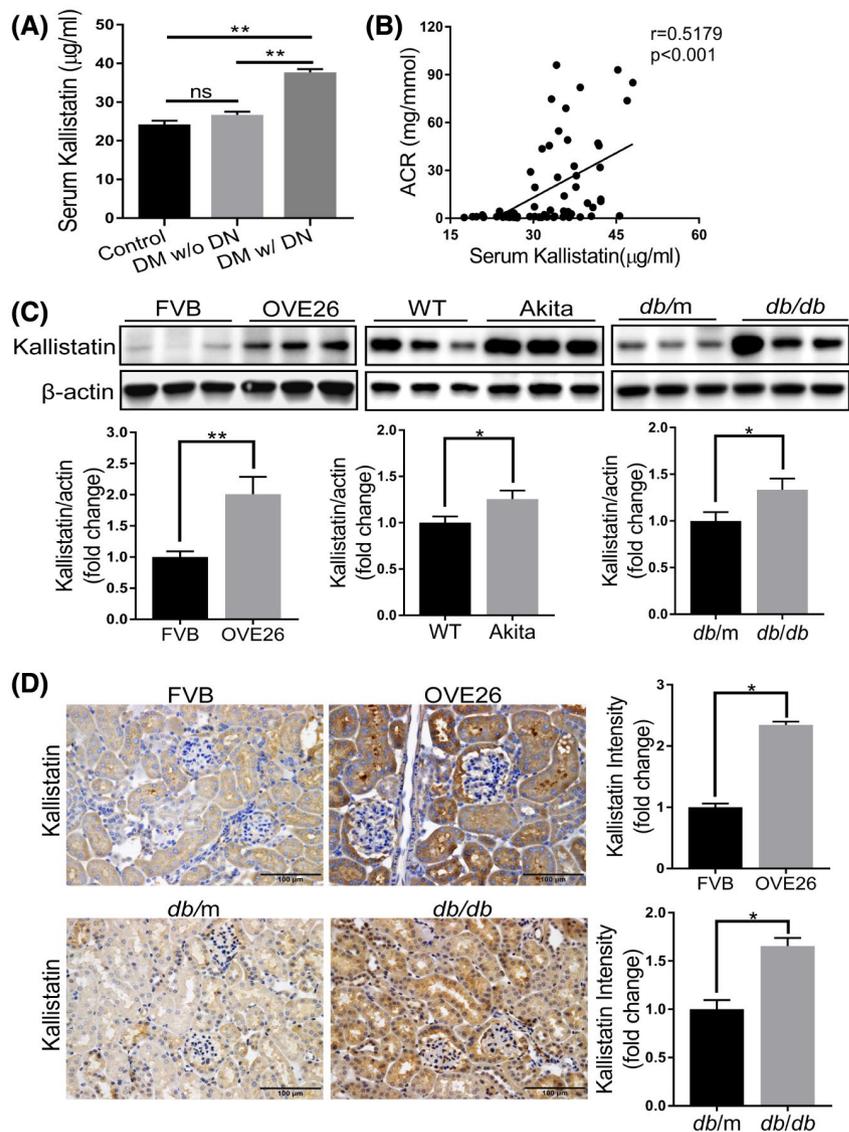
SPSS version 19.0 software was used for statistical analysis. All values were expressed as mean  $\pm$  SEM. Student's *t*-test was used for comparison of two groups. The correlation between serum kallistatin levels and clinical parameters was determined by Spearman's correlation coefficient. *P* < .05 was considered statistically significant.

# 3 | RESULTS

## 3.1 | Elevated kallistatin levels in DN patients and diabetic animal models

We measured serum levels of kallistatin in type 2 diabetic patients with DN (UACR > 30 mg/g, *n* = 36), type 2 diabetic patients without DN (UACR < 30 mg/g, *n* = 32), and healthy controls (*n* = 30) by ELISA. There was no difference in the serum kallistatin level between diabetic patients without DN and healthy controls (Figure 1A, 26.7  $\pm$  0.82  $\mu$ g/mL

**FIGURE 1** Elevated kallistatin levels in DN patients and animal models. A, Enzyme-linked immunosorbent assay (ELISA) measurement of serum kallistatin in diabetic patients with DN (DM w/DN) and without DN (DM W/O DN) and healthy controls. B, Correlation between serum kallistatin levels and the albumin-to-creatinine ratio (ACR) in diabetic patients. C, Western blot analysis and densitometry quantification of endogenous renal kallistatin expression in 5-month-old Akita mice and OVE26 mice, 6-month-old *db/db* mice, and their non-diabetic controls. D, Representative images and quantification of immunohistochemical staining of kallistatin in kidney sections of 5-month-old OVE26 mice, 6-month-old *db/db* mice, and their non-diabetic controls. Scale bar = 100  $\mu$ m; \* $P$  < .05, \*\* $P$  < .01.  $r$ , Spearman's correlation coefficient. All values are expressed as mean  $\pm$  SEM;  $n$  = 5-7 per group



vs  $24.2 \pm 0.96 \mu\text{g/mL}$ ). However, diabetic patients with DN showed a significant increase in the kallistatin serum levels compared with those in patients without DN ( $37.8 \pm 0.78 \mu\text{g/mL}$  vs  $26.7 \pm 0.82 \mu\text{g/mL}$ ,  $P < .001$ ) and healthy controls ( $37.8 \pm 0.78 \mu\text{g/mL}$  vs  $24.2 \pm 0.96 \mu\text{g/mL}$ ,  $P < .001$ ). When considering the diabetic patients with and without DN as one group, there was a positive correlation between the serum kallistatin level with albumin/creatinine ratio (ACR) (Figure 1B,  $r = 0.5179$ ,  $P < .001$ ) and serum very low-density lipoprotein cholesterol (Supplemental Figure S1,  $r = 0.4211$ ,  $P < .001$ ) in the diabetic patients. There was no correlation between the serum kallistatin level and BMI, HbA1C, blood urea nitrogen (BUN), or the serum creatinine level. The clinical parameters of patients are shown in Table 1.

To determine the renal kallistatin expression in DN animal models, we examined endogenous renal kallistatin levels using Western blot analysis. Akita, OVE26, and *db/db* mice showed increased renal kallistatin levels compared to that in their age- and genetic background-matched non-diabetic control mice

(Figure 1C). As further shown by immunohistochemistry staining, renal kallistatin was increased in both the type 1 and type 2 diabetic mice (OVE26 and *db/db*) in multiple types of kidney cells (Figure 1D). These results suggested that both circulating and renal kallistatin levels were upregulated in DN.

Similar to the observation in diabetic humans, serum kallistatin levels in diabetic OVE26 (WT/OVE) mice (5-month-age) were significantly higher than those in age-matched non-diabetic WT/FVB mice ( $1.99 \pm 0.15 \mu\text{g/mL}$  vs  $1.55 \pm 0.23 \mu\text{g/mL}$ ,  $P < .002$ , Supplemental Figure S2A).

### 3.2 | Generation of kallistatin overexpression transgenic mice

To investigate the impacts of upregulated kallistatin on the kidney, we generated KS-tg mice.<sup>21</sup> We measured total levels of mouse and human kallistatin. As shown in Supplemental Figure S2A, circulating kallistatin levels in non-diabetic

**TABLE 1** Clinical characteristics of diabetic patients

Characteristics	Diabetic W/O DN	Diabetic W/DN
Total (n)	32	36
Males (n)	19	22
Females (n)	13	14
Age [years (median, range)]	57 (33-79)	59 (36-78)
Diabetes duration [years (median, range)]	8 (0.5-18)	12 (3-29)
BMI, kg/m <sup>2</sup> (mean ± SD)	25.04 ± 7.1	26.14 ± 3.3
HbA1C [% (mean ± SD)]	9.47 ± 1.7	8.49 ± 1.3
UACR [mg/mmol (median, range)]	0.98 (0.21-3.13)	19.74 (0.72-96.1)
Cholesterol (mmol/L)	5.16 ± 1.5	5.77 ± 1.3
HDL-C (mmol/L)	1.36 ± 0.4	1.27 ± 0.3
LDL-C (mmol/L)	3.12 ± 0.9	3.73 ± 1.6
VLDL-C (mmol/L)	0.59 ± 0.4	0.72 ± 0.4
Serum creatinine (μmol/L)	61.44 ± 1.4	77.72 ± 32.1
BUN (mmol/L)	5.35 ± 1.3	6.01 ± 1.9
Serum kallistatin (μg/mL)	26.7 ± 4.5	37.7 ± 4.7

Abbreviations: BMI, body mass index; BUN, blood urea nitrogen; DN, diabetic nephropathy; Diabetic W/DN, diabetic patients with DN; Diabetic W/O DN, diabetic patients without DN; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; n, number; UACR, urinary albumin/creatinine rate; VLDL-C, very low-density lipoprotein cholesterol.

transgenic mice (KS-tg/FVB mice) were approximately 2-fold higher than those in wild-type FVB mice (WT-FVB). Renal kallistatin levels were approximately 2.5-fold higher in the transgenic mice compared to those of WT/FVB mice under non-diabetic condition (Supplemental Figure S2B).

There was no difference in the body weight or the blood glucose level between KS-tg/FVB mice and WT/FVB mice (Supplemental Figure S3). The kallistatin overexpression has no effect on the renal function at non-diabetic condition, as shown by the left kidney weight, 24-h urine volume, and creatinine clearance (Supplemental Figure S3).

### 3.3 | Aggravated albuminuria and renal structural damage in kallistatin transgenic mice with diabetes

To investigate the effect of kallistatin overexpression on renal structure and function under diabetic conditions, we induced diabetes in KS-tg mice by crossing them with OVE26 mice on the FVB background (KS-tg/OVE). There were no significant differences in the blood glucose level, urine volume, and body and kidney weights between KS-tg/OVE mice and WT/OVE mice at the age of 20 weeks (Supplemental Figure S3). However, both UAE and urinary albumin/

creatinine ratio (UACR) were dramatically increased in KS-tg/OVE mice compared to WT/OVE mice (Figure 2A,B).

Periodic acid-Schiff staining (PAS) was performed on the kidney sections from mice at 20 weeks of age. As shown in Figure 2C–E, kallistatin overexpression obviously exacerbated the diabetes-induced glomerular and tubular damage, including increased glomerular matrix expansion, more severe glomerulosclerosis, augmented tube lumen, and increased protein casts. Under non-diabetic conditions, however, there were no detectable differences between WT/FVB and KS-tg/FVB mice in kidney function and histology (Figure 2C–E).

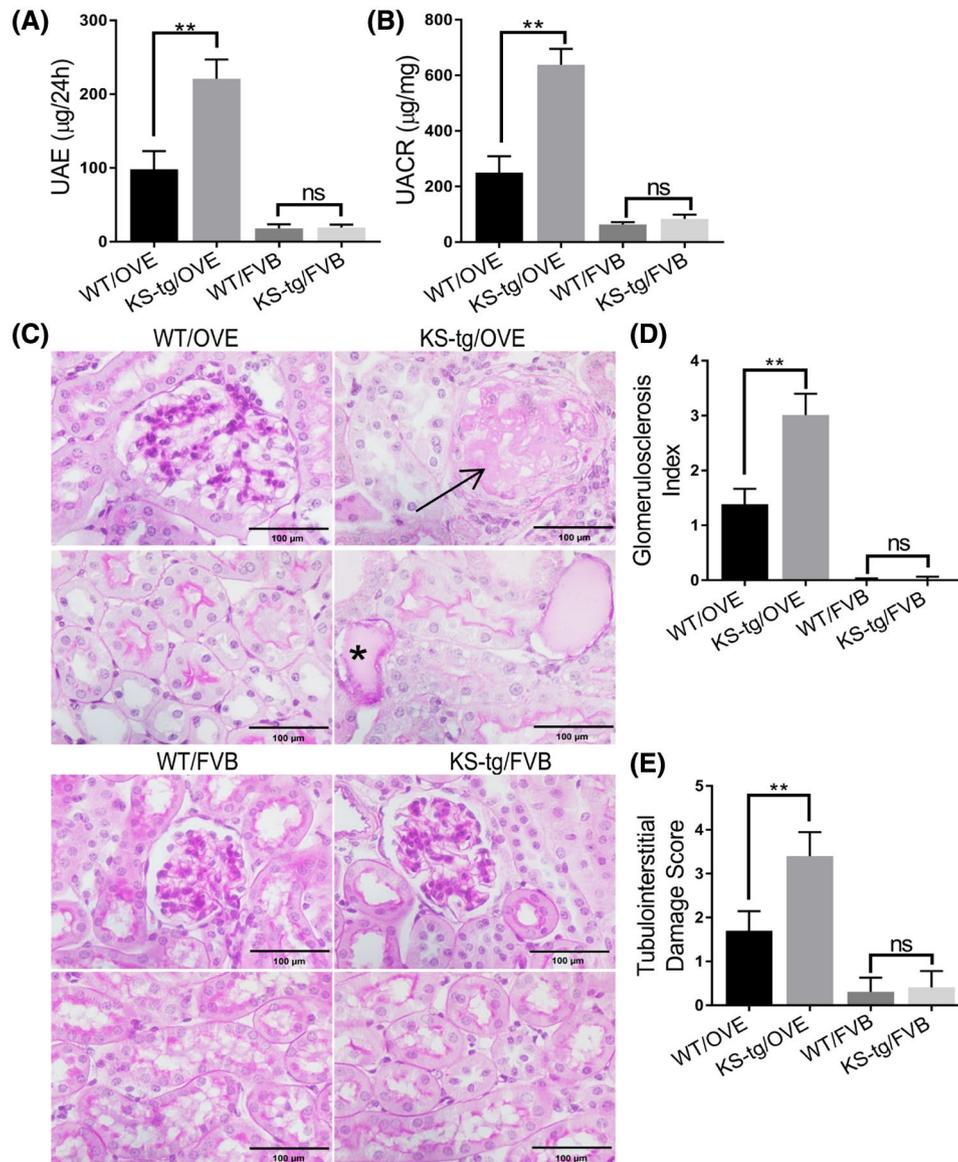
### 3.4 | Kallistatin promotes diabetes-induced renal fibrosis

To further evaluate the effect of kallistatin overexpression on renal fibrosis in diabetic kidneys, we measured the expression of fibrosis-related proteins. As shown by Western blot analysis, connective tissue growth factor (CTGF), TGF-β1, TGF-β1 receptor 1 (TβR-I), and TβR-II were significantly increased in KS-tg/OVE mice compared to WT/OVE mice (Figure 3A–E). Renal fibronectin levels were significantly increased in KS-tg/OVE mice compared to those in WT/OVE mice (Figure 3F). Picro-Sirius red staining showed that KS-tg/OVE mice developed a more severe collagen deposition in both the glomerular and tubulointerstitial areas, compared with WT/OVE mice (Figure 3G,H). The collagen IV expression was also remarkably increased in the kidneys of KS-tg/OVE mice (Figure 3I,J). These results indicated that kallistatin overexpression exacerbated renal fibrosis under diabetic conditions.

### 3.5 | Overexpression of kallistatin promotes inflammation and oxidation in diabetic kidneys

As increased inflammation and oxidative stress also contribute to the development of DN,<sup>37</sup> we measured inflammatory proteins including intercellular adhesion molecule-1 (ICAM-1) and tumor necrosis factor-α (TNF-α) in diabetic kidneys. ICAM-1 was increased significantly in the kidney of KS-tg/OVE mice, compared to that in WT/OVE mice (222.3 ± 18.6 pg/μg vs 154.8 ± 16.4 pg/μg, *P* < .05, Figure 4A). However, serum ICAM-1 showed no significant differences between these two groups (Figure 4B). Western blot analysis showed that renal levels of TNF-α were significantly increased in KS-tg/OVE mice over those in the WT/OVE mice (Figure 4C). These results suggest that overexpression of kallistatin aggravated inflammation in diabetic kidneys.

Renal oxidative stress was determined by levels of 3-nitrotyrosine (3-NT). As shown by Western blot analysis, renal 3-NT levels were significantly increased in KS-tg/OVE mice



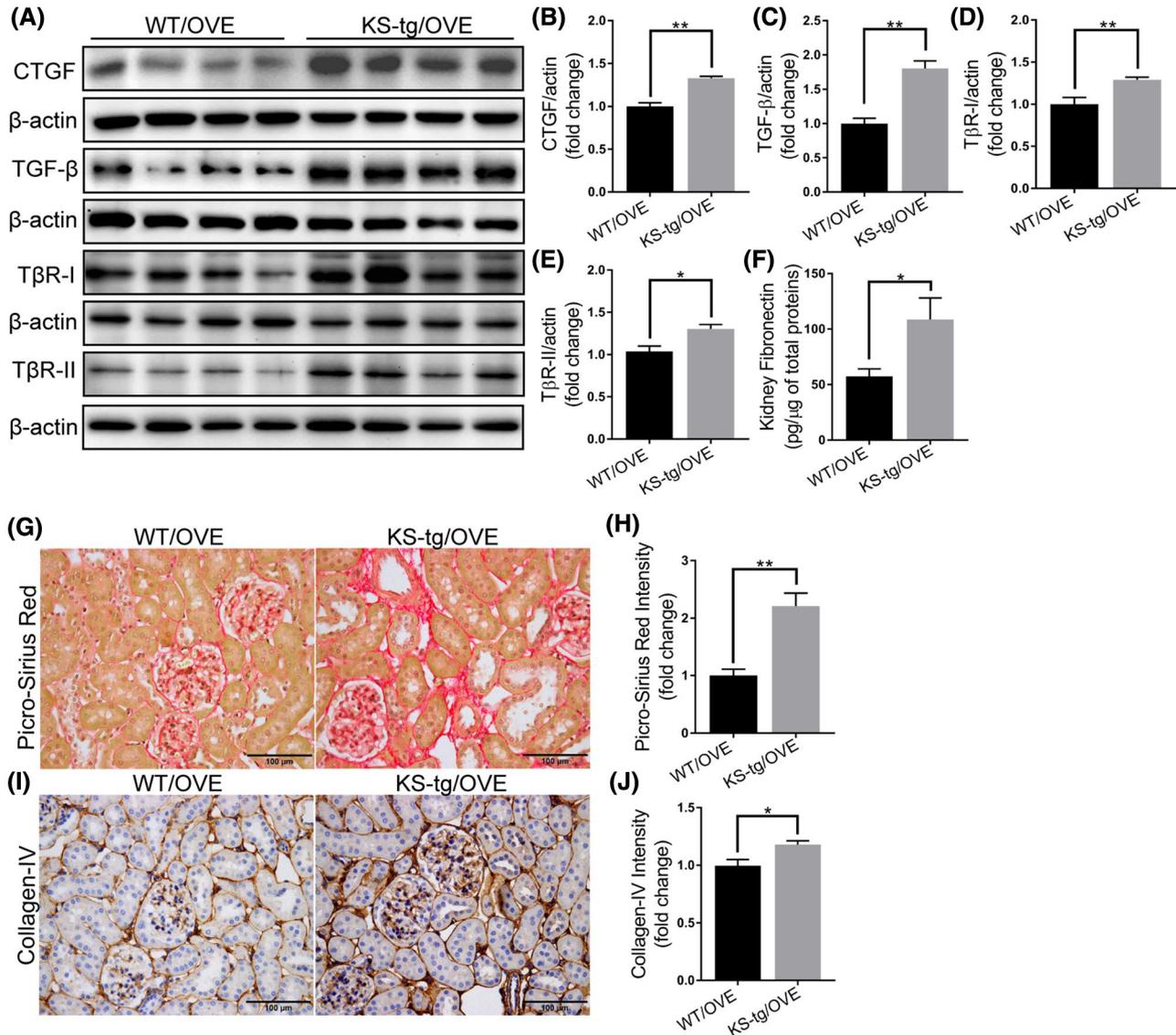
**FIGURE 2** Deleterious effect of kallistatin overexpression on DN. A, Urinary albumin excretion (UAE, µg/24 hours) in WT/OVE, KS-tg/OVE, WT/FVB, and KS-tg/FVB mice at the age of 5 months. B, The urinary albumin-to-creatinine ratio (UACR) of WT/OVE, KS-tg/OVE, WT/FVB, and KS-tg/FVB mice at the age of 5 months. C, Representative images of kidney sections with Periodic Acid-Schiff (PAS) staining from WT/OVE, KS-tg/OVE, WT/FVB, and KS-tg/FVB mice at the age of 5 months. D, Glomerulosclerosis index. E, Tubulointerstitial damage score. Scale bar = 100 µm; \*\* $P < .01$ . All values are expressed as mean  $\pm$  SEM;  $n = 6$  per group

over those in WT/OVE mice. NADPH oxidases (NOXs) are the crucial proteins regulating ROS generation in the diabetic kidney. Kallistatin overexpression resulted in a significant increase in renal NOX4 expression in diabetic animals. In contrast, there was no significant change in renal NOX2 levels in KS-tg/OVE mice, compared to those in WT/OVE mice (Figure 4C).

### 3.6 | Anti-fibrosis effect of kallistatin in cultured mesangial cells

A previous study has shown that a transient kallistatin gene delivery protected against renal inflammation and fibrosis

in *db/db* mice.<sup>24</sup> However, we demonstrated here that the transgenic expression of kallistatin in the kidney deteriorated DN phenotypes. To address these conflicting observations in renal fibrosis, we examined the direct regulation of kallistatin on fibronectin secretion in primary HMC. HMC were treated with high glucose (30 mM) in the presence of serial concentrations of human kallistatin for 3 days. Kallistatin significantly inhibited the fibronectin secretion into the culture media in a concentration-dependent manner (Figure 5A). HMC were also co-treated with 5 ng/mL of TGF- $\beta$ 1 and different concentrations of kallistatin. TGF- $\beta$ 1-induced fibronectin production was significantly attenuated by kallistatin (Figure 5B). These results demonstrated an anti-fibrotic



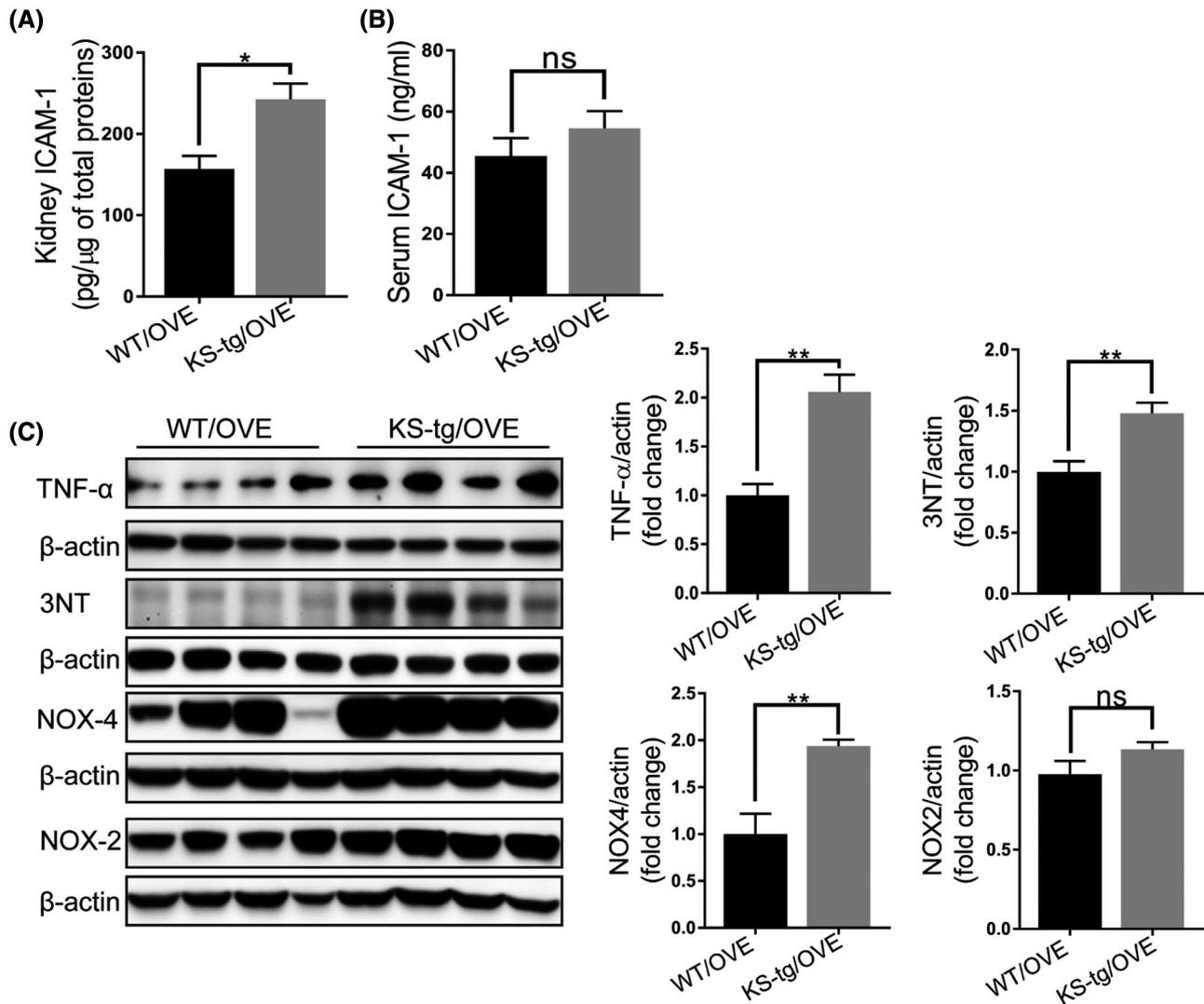
**FIGURE 3** Kallistatin overexpression promotes diabetes-induced renal fibrosis. A, Western blot analysis and (B-E) densitometry quantification of connective tissue growth factor (CTGF), transforming growth factor 1 (TGF- $\beta$ 1), TGF- $\beta$ 1 receptor 1 (T $\beta$ R-I), and T $\beta$ R-II in kidney homogenates of WT/OVE and KS-tg/OVE mice at 5 months of age. F, Enzyme-linked immunosorbent assay (ELISA) measurement of fibronectin in kidney homogenates of WT/OVE and KS-tg/OVE mice at 5 months of age. G, Representative images and (H) densitometry quantification of Picro-Sirius red staining of kidney sections at 5 months of age. I, Representative images and (J) densitometry quantification of immunohistochemistry (IHC) staining of collagen-IV in kidney sections at 5 months of age. Scale bar = 100  $\mu$ m; \* $P < .05$ , \*\* $P < .01$ . All values are expressed as mean  $\pm$  SEM; n = 5-6 per group

effect of kallistatin in cultured renal cells, and the exacerbated DN phenotypes in diabetic KS-tg mice may be mediated through a different mechanism.

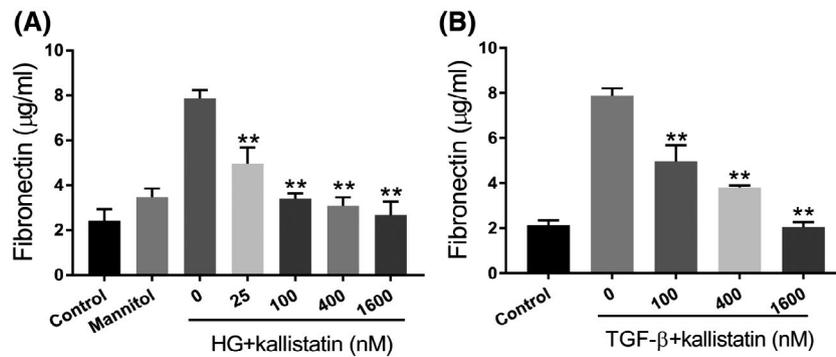
### 3.7 | Kallistatin overexpression inhibits kallikrein activity and exacerbates the activation of RAS in diabetic kidneys

Kallistatin is known to inhibit tissue kallikrein activity.<sup>13</sup> We measured tissue kallikrein activities in the kidney homogenate and urine. As expected, kallistatin

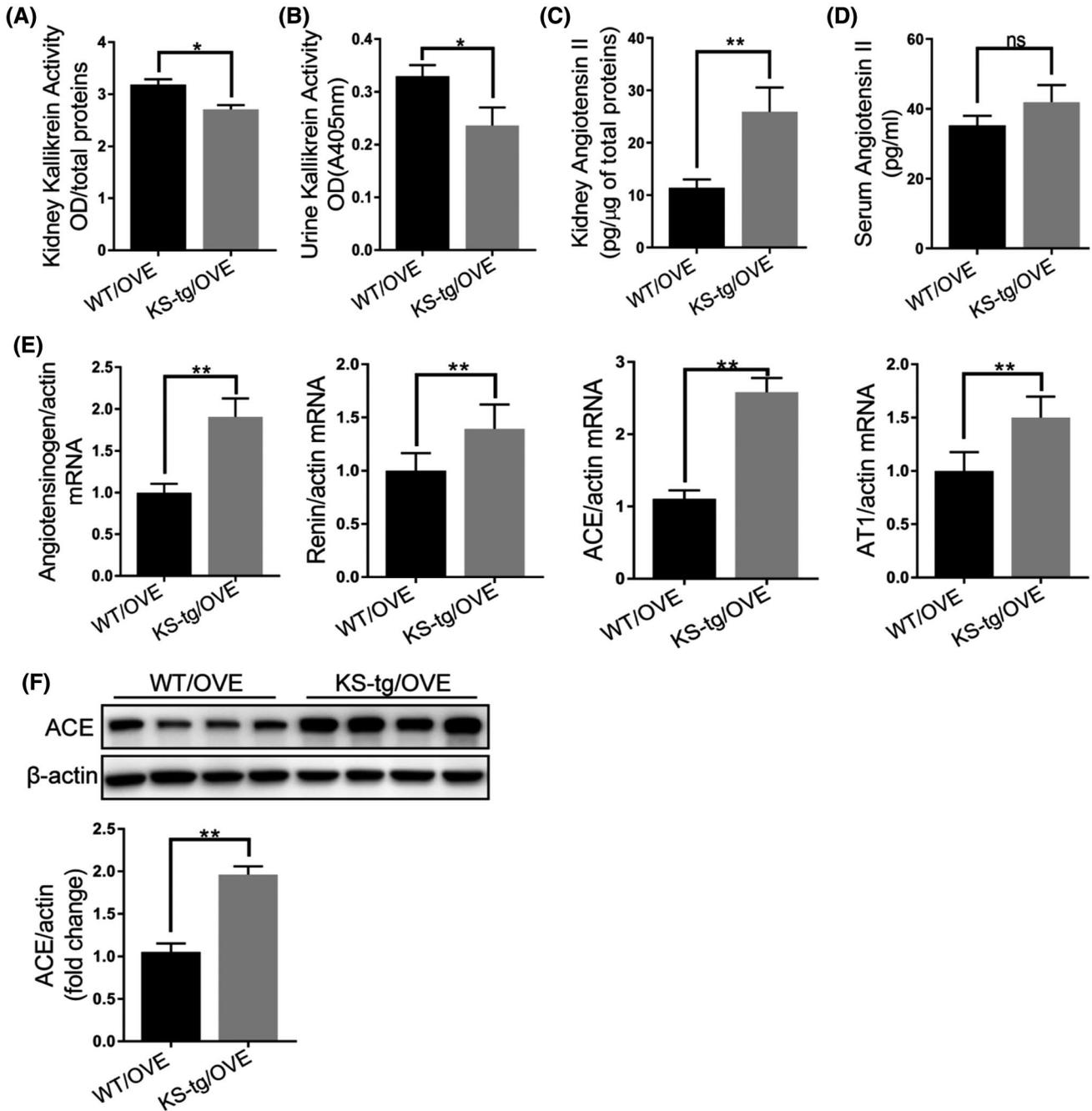
overexpression significantly decreased the renal tissue kallikrein activity in the diabetic kidney (Figure 6A). KS-tg/OVE mice also showed a diminished kallikrein activity in urine (Figure 6B). As the KKS counterbalances the RAS, and activation of the RAS contributes to the development of DN, we also measured RAS levels and activities. As shown in Figure 6E, kallistatin overexpression in diabetic kidneys resulted in significantly upregulated mRNA levels of RAS components, including angiotensinogen, renin, ACE, and angiotensin II receptor 1 (AT-1) mRNA. Renal angiotensin II (Ang II) protein levels were significantly elevated in KS-tg/OVE mice over those in WT/



**FIGURE 4** Kallistatin overexpression promotes inflammation and oxidative stress in diabetic kidneys. A, Enzyme-linked immunosorbent assay (ELISA) measurement of renal and (B) serum intercellular adhesion molecule-1 (ICAM-1). C, Western blot analysis and densitometry quantification of tumor necrosis factor-α (TNF-α), 3-nitrotyrosine (3-NT), NADPH oxidase 4 (NOX4), and NOX2 in kidney homogenates of 5-month-old WT/OVE and KS-tg/OVE mice, \* $P < .05$ , \*\* $P < .01$ . All values are expressed as mean  $\pm$  SEM;  $n = 6-8$  per group



**FIGURE 5** Anti-fibrotic effects of kallistatin in cultured human mesangial cells (HMC). A, HMC were treated with high glucose (HG, 30 mM) in the presence of serial concentrations of kallistatin for 3 days. To exclude impacts of osmolarity difference, the control cells were treated with 25 mM mannitol + 5 mM glucose under the same conditions. B, HMC were treated with 5 ng/mL TGF-β1 without or with serial concentrations of kallistatin for 3 days. Fibronectin secreted into the culture medium was measured by ELISA (mean  $\pm$  SEM,  $n = 3$ ). (\*\* $P < .01$  in all concentrations of kallistatin)



**FIGURE 6** Kallistatin overexpression inhibits tissue kallikrein activity and exacerbates the activation of RAS in diabetic kidney. A, Kidney tissue kallikrein activity in 5-month-old WT/OVE and KS-tg/OVE mice. The results were normalized by total protein concentrations. B, Urinary tissue kallikrein activity in 5-month-old WT/OVE and KS-tg/OVE mice. C, Enzyme-linked immunosorbent assay (ELISA) measurement of angiotensin-II in kidney homogenates and serum (D). E, Real-time PCR measurement of angiotensinogen, renin, angiotensin converting enzyme (ACE), and angiotensin-II receptor 1 (AT-1) mRNA levels in the kidneys from 5-month-old WT/OVE and KS-tg/OVE mice. F, Western blot analysis and densitometry quantification of ACE in kidney homogenates from 5-month-old WT/OVE and KS-tg/OVE mice. \* $P < .05$ , \*\* $P < .01$ . All values are expressed as mean  $\pm$  SEM;  $n = 8-10$  per group

OVE mice (Figure 6C,  $27.1 \pm 5.3$  pg/ $\mu$ g vs  $10.5 \pm 1.7$  pg/ $\mu$ g,  $P < .01$ ). No significant change in serum Ang II was found (Figure 6D,  $35.29 \pm 6.6$  pg/mL vs  $39.68 \pm 8.9$  pg/mL,  $P = .34$ ). Renal ACE protein levels were significantly upregulated in KS-tg/OVE kidneys, compared to those in

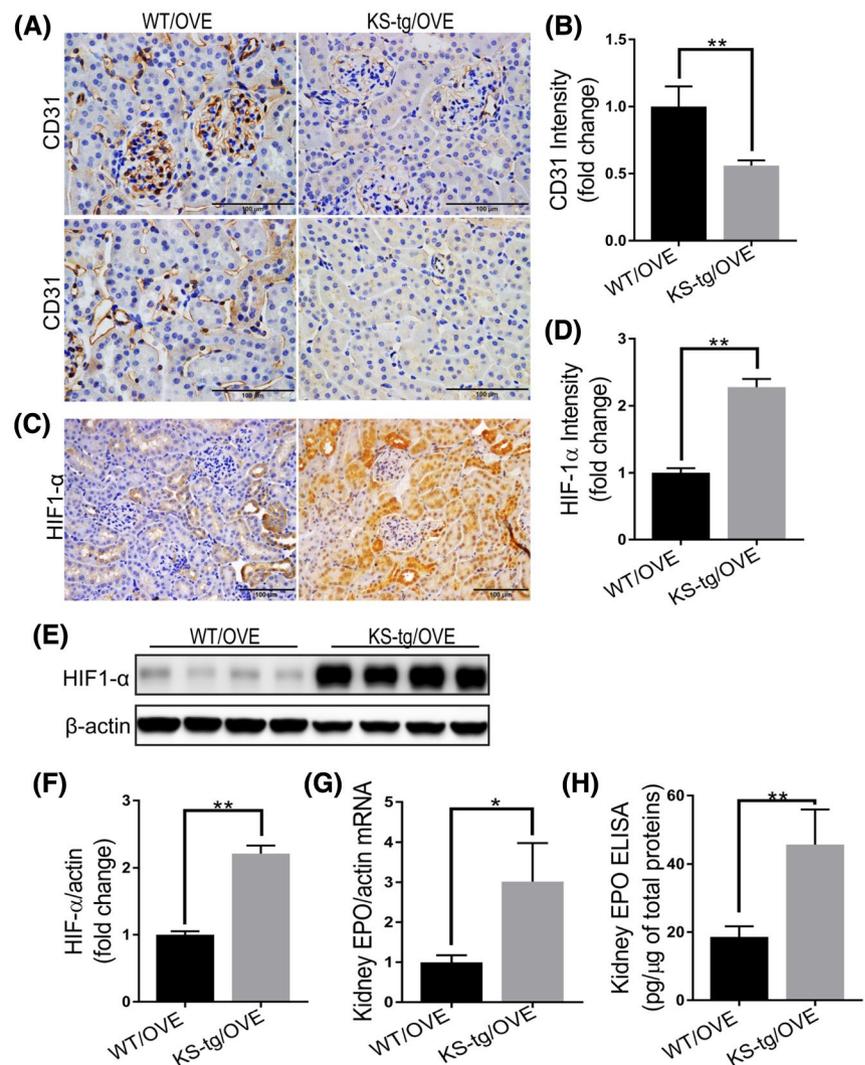
WT/OVE kidneys (Figure 6F). These results demonstrated a significant inhibition of the KKS and overactivation of the RAS in the kidney of KS-tg/OVE mice, which might contribute to the deteriorating effect of kallistatin overexpression on diabetes-induced renal damage in KS-tg mice.

### 3.8 | Kallistatin overexpression contributes to decreased capillary density and increased hypoxia in diabetic kidney

Kallistatin is a potent anti-angiogenic factor.<sup>14,21</sup> To determine whether kallistatin overexpression affects the microvascular system in the diabetic kidney, microvascular densities in the kidney were quantified by immunohistochemical staining of CD31 and staining with Isolectin IB4. As shown in Figure 7A,B, KS-tg/OVE mice showed decreased vascular densities in the kidneys, compared with those in the WT/OVE kidneys. The CD31-positive endothelial cells were significantly decreased in both intra-glomerular and tubulointerstitial capillary networks in KS-tg/OVE mice, compared to WT/OVE mice. Kallistatin overexpression also decreased microvascular densities in the diabetic renal cortex. Consistently, based on quantification of endothelial cells in kidney sections stained by Isolectin IB4, KS-tg/OVE mice also demonstrated decreased vascular densities in the kidney compared with age-matched WT/OVE mice (Supplemental Figure S4).

Declined microvascular densities could result in intra-renal hypoxia, which plays a role in the pathogenesis of DN. To evaluate the effect of kallistatin overexpression on renal hypoxia, we measured levels of HIF-1 $\alpha$  in diabetic kidneys. As shown by Western blot analysis (Figure 7E,F), HIF-1 $\alpha$  protein levels were significantly higher in KS-tg/OVE mouse kidneys than those in WT/OVE ones, suggesting that HIF-1 $\alpha$  was stabilized in the kidney of KS-tg/OVE mice. The immunohistochemistry analysis also showed dramatically upregulated levels of HIF-1 $\alpha$  and increased nuclear translocation of HIF-1 $\alpha$  in the kidney of KS-tg/OVE mice (Figure 7C,D). As erythropoietin (EPO) is a target gene of HIF-1 $\alpha$  and angiogenic factor, we measured EPO expression in the kidneys. As shown in Figure 7G,H, KS-tg/OVE kidneys had significant increases in EPO mRNA and protein levels ( $50.6 \pm 10.6$  pg/ $\mu$ g vs  $18.7 \pm 3.2$  pg/ $\mu$ g,  $P < .01$ ). These results suggest decreased microvascular densities and increased hypoxia in the kidney may contribute to the severe renal damage in diabetic kallistatin transgenic mice.

**FIGURE 7** Kallistatin overexpression aggravates renal hypoxia. A, Representative images of immunohistochemical staining and (B) densitometry quantification of CD31 (brown) in kidney sections of 5-month-old WT/OVE and KS-tg/OVE mice. C, Representative images of immunohistochemical staining and (D) quantification of hypoxia-inducible factor (HIF-1 $\alpha$ ) in kidney sections. E, Western blot analysis and (F) densitometry quantification of HIF-1 $\alpha$  in kidney homogenates. G, Real-time PCR measurement of the erythropoietin (EPO) mRNA in the kidneys. H, Enzyme-linked immunosorbent assay (ELISA) measurement of EPO in kidney homogenates. Scale bar = 100  $\mu$ m; \* $P < .05$ , \*\* $P < .01$ . All values are expressed as mean  $\pm$  SEM; n = 5-6 per group



### 3.9 | Kallistatin gene delivery exacerbates albuminuria

To further confirm the deleterious effect of kallistatin overexpression on diabetic kidneys, we injected OVE26 mice intraperitoneally with Ad-KS or the same titer of a control adenovirus Ad-GFP, with PBS only as blank control. As shown in Supplemental Figure S5, serum kallistatin levels and UAE were significantly higher in OVE26 mice injected with Ad-KS, 4 weeks after the injection, compared with OVE26 mice injected with PBS and Ad-GFP, confirming the deleterious effects of kallistatin overexpression on DN in adult mice.

## 4 | DISCUSSION

Kallistatin has been previously reported to confer anti-inflammation, anti-fibrosis, and anti-angiogenesis effects through its inhibition of canonical Wnt signaling.<sup>15,16,21</sup> Therefore, it was expected to be renoprotective against DN. This assumption was supported by a previous study which showed that a transient kallistatin gene delivery confers a renoprotective effect in *db/db* mice.<sup>24</sup> However, in the present study, we found serum kallistatin levels were elevated in patients with DN and positively correlated with ACR, implicating a deteriorating effect of kallistatin on DN. In a transgenic mouse model, we also showed that kallistatin expression promoted diabetes-induced renal inflammation, oxidative stress, and fibrosis, through inhibiting renal tissue kallikrein activity and disturbing the balance between the KKS and RAS, which could further aggravate renal hypoxia and exacerbate renal injury. These results for the first time suggest that elevated kallistatin levels in diabetic patients could contribute to the development or progression of DN and represent a potential therapeutic target for DN.

Our previous study has reported that kallistatin functions as an inhibitor of the canonical Wnt pathway through binding and destabilizing Wnt co-receptor LRP6.<sup>38</sup> Supporting this finding, transgenic expression of kallistatin indeed alleviated retinal inflammation and vascular leakage in a diabetic model.<sup>21</sup> Overexpression of kallistatin also attenuated ischemia-induced retinal neovascularization.<sup>21</sup> Kallistatin also showed an anti-fibrotic activity in the retina of a diabetic model.<sup>39</sup> These effects can be all ascribed to the inhibition of Wnt signaling overactivation in diabetes, as we have shown that Wnt signaling overactivation in diabetes plays key roles in inflammation, neovascularization, and fibrosis in diabetic complications.<sup>40,41</sup> In our cultured primary renal mesangial cells, kallistatin indeed inhibited expression of a fibrotic factor. Consistently, another group reported that transient expression of kallistatin in the kidney through gene delivery displayed beneficial effects against DN.<sup>24</sup> Based on these findings, it was expected that overexpression of kallistatin in

transgenic mice should attenuate pathologies of DN. To our surprise, kallistatin transgenic mice with diabetes manifested more severe renal inflammation and fibrosis, compared to WT mice with the same diabetes duration. This disparity suggests that primary function of kallistatin in the kidney may be different from that in the retina. One possible explanation is that the KKS and RAS balance is critical for maintenance of normal kidney structure and function.

The KKS plays a pivotal role in the kidney function. Kallikrein and kinins have been reported to have pleiotropic effects on inflammation, fibrosis, and apoptosis in different experimental animal models and to have a protective effect against DN.<sup>42-44</sup> The renal kallikrein production is markedly reduced in patients with severe renal insufficiency.<sup>3</sup> Overexpression of kallikrein by a somatic gene transfer reduces salt-induced glomerular sclerosis and improves renal function in Dahl salt-sensitive rats.<sup>45</sup> The protective effects of kallikrein in renal injury were associated with increased NO bioavailability and suppressed oxidative stress.<sup>46</sup> Tissue kallikrein increases nitric oxide levels and Akt activation, and reduces TGF- $\beta$ 1 expression and reactive oxygen species formation.<sup>47</sup> As a kallikrein-binding protein, kallistatin inhibits kallikrein activity and reduces kinin generation, which could explain its deleterious effect on DN.

In contrast to the renoprotective effects of the KKS, the activation of the RAS has long been considered as a pathological mechanism of DN. All components of the KKS and the RAS are expressed abundantly in the kidney, and these two systems are in a regulated counter-balance under the physiological conditions. Under pathophysiological conditions such as DN, however, the balance between the KKS and the RAS is disturbed, leading to pathological features.<sup>44</sup> Hyperglycemia is known to activate RAS, and the local RAS in the kidney is known to contribute to the renal injury in diabetes.<sup>48</sup> Blockade of the RAS has been shown to ameliorate DN and stimulate tissue kallikrein activity.<sup>44</sup> The intra-renal Ang II is also a critical contributor to DN by stimulating renal inflammation, oxidative stress, and fibrosis.<sup>6,48,49</sup> In this study, kallistatin resulted in significant increases in renal levels of RAS components in KS-tg/OVE mice. Thus, the inhibition of the KKS and overactivation of the RAS in diabetic kallistatin transgenic mice could contribute to the deteriorating effects on DN. However, we need to point out that Ang II protein levels were measured by an ELISA kit, with the limitation of not separating its metabolites prior to analysis. However, the mRNA expression of several RAS components was upregulated, supporting our hypothesis that kallistatin upregulates the renal RAS system. It should be mentioned that Danser and others recently indicated that most of angiotensinogen, ACE, and Ang II in the kidney are derived from reuptake of these proteins after filtration.<sup>50,51</sup> Damage to the filtration barrier as seen in diabetes increases filtration of these proteins which could lead to augmentation in tubular

reuptake of these proteins, thus increasing their concentration within the kidney. Therefore, other mechanisms may also contribute to the elevated RAS levels in the diabetic kidney.

We previously demonstrated the protective effect of kallistatin in transgenic mice against DR by anti-angiogenesis and anti-inflammation through inhibition of the Wnt pathway.<sup>21</sup> However, in the present study, diabetic kallistatin transgenic mice developed exacerbated DN, contradicting to previous observations. When we treated human mesangial cells with different concentrations of recombinant kallistatin in the presence of high glucose or TGF- $\beta_1$ , we found that kallistatin significantly inhibited fibronectin expression induced by high glucose and TGF- $\beta_1$ , suggesting that kallistatin per se indeed has anti-fibrosis activity at the cellular level, consistent with its inhibitory effect on Wnt signaling and its protective effect against DN after transient gene delivery.<sup>24</sup> Thus, the deteriorating effect of kallistatin on DN is likely ascribed to renal homeostasis disturbance, outweighing its direct Wnt inhibitory effect on renal cells.

Kallistatin was identified as a potent endogenous anti-angiogenic factor.<sup>14</sup> We and other groups have demonstrated the therapeutic effects of kallistatin on tumor and ocular neovascular diseases by suppressing angiogenesis in animal models.<sup>14,21,23,52</sup> Consistent with the anti-angiogenic activity of kallistatin, microvascular densities were dramatically decreased in KS-tg/OVE mouse kidneys, compared with those in WT/OVE kidneys (Figure 7). The decreased vascular density could result in renal ischemic damage.<sup>53</sup> Ang II was also shown to induce renal hypoxia via both hemodynamic and non-hemodynamic mechanisms. Distortion and loss of peritubular capillaries are also associated with extensive tubulointerstitial injury.<sup>54</sup> The ischemia in the kidney overexpressing kallistatin is likely to contribute to the stabilization of HIF-1 $\alpha$  protein and the activation of the HIF-1 $\alpha$  signaling pathway, leading to overexpression of EPO. Chronic hypoxia plays a vital role in the pathogenesis of DN, including increasing urine albumin and promoting renal fibrosis and inflammation.<sup>53,55-57</sup> These results suggest that KS-tg/OVE mice developed chronic hypoxia in the kidney due to decreased vascular densities, which might exacerbate the injury of DN.

There are several advantages of our diabetic kallistatin transgenic models. Firstly, we used OVE26 mice at 5 months of age to better simulate pathological changes in advanced human DN. Secondly, the increased serum kallistatin levels in the transgenic mice were comparable to the kallistatin levels in DN patients, which made this model more relevant to the clinical conditions of DN patients. A disadvantage of the transgenic mice model is that the kallistatin transgene is constantly expressed after birth driven by the chicken  $\beta$ -actin promoter. To exclude the potential effect of kallistatin on the development, we delivered the kallistatin gene by intraperitoneal injection of adenovirus expressing kallistatin into adult

WT/OVE mice. Consistent with the findings in kallistatin transgenic mice, the kallistatin gene delivery also showed a deleterious effect on diabetic kidney, further supporting our conclusion.

In this study, we also demonstrated a correlation between serum kallistatin levels and the urine albumin excretion in diabetic patients, which suggests that kallistatin might become a potential biomarker for DN. Although kallistatin has anti-fibrosis, anti-oxidant, and anti-inflammation activities at the cellular level, the impaired balance between the RAS and KKS in the kidney, and the chronic hypoxia induced by kallistatin may offset its beneficial effects in the kidney. This reminds us that kallistatin may display double-edge functions in different conditions, and we should be cautious when considering kallistatin as a therapeutic agent for the long-term intervention of DN. Although the increased kallistatin expression in diabetes and its microvascular complications have been shown, the initial factors triggering its overexpression and the upstream signaling pathway remain to be further investigated.

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## DISCLOSURE

The authors declare no conflict of interest.

## AUTHOR CONTRIBUTIONS

Yanhui Yang designed the research studies, conducted experiments, acquired and analyzed the data, wrote the manuscript, and collaborated with all other authors. Xuemin He and Rui Cheng co-designed and conducted the experiments, acquired the data, and revised the manuscript. Qian Chen and Chunyan Shan collected the clinical samples and discussed the study. Liming Chen and Jian-xing Ma co-designed the studies, discussed analyses and data interpretation, revised and approved the manuscript, and collaborated with all other authors.

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## SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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