Angiotensin-converting enzyme-2 improves diabetic nephropathy by targeting Smad7 for ubiquitin degradation

ZIYE CHEN, XINPAN CHEN, YU BAI, ZONGLI DIAO and WENHU LIU

Department of Nephrology, Beijing Friendship Hospital, Capital Medical University, Beijing 100050, P.R. China

Received December 26, 2019; Accepted July 7, 2020

DOI: 10.3892/mmr.2020.11372

Abstract. Angiotensin-converting enzyme 2 (ACE2), an important component of the renin-angiotensin system, protects against renal tubulointerstitial fibrosis, but its level of involvement in the mechanism of diabetic nephropathy (DN) currently remains unclear. Herein, the effects of ACE2 in DN and the associated mechanisms were investigated using serum and renal biopsy specimens from patients with DN and control participants, and human renal proximal tubular epithelial cells (HRPTEpiCs). The present study determined that the circulating concentration of ACE2 was high, but renal ACE2 expression was markedly lower, and there was abundant expression of Arkadia, an E3 ubiquitin ligase, in patients with DN. *In vitro*, ACE2 attenuated high-glucose-induced tubular epithelial to mesenchymal cell transition (EMT), which was demonstrated by increased expression of α-SMA and loss of E-cadherin expression, as demonstrated by western blot analysis and reverse transcription-quantitative PCR. Adenovirus-mediated ACE2 overexpression was also revealed to significantly inhibit Arkadia expression and alleviated high-glucose-induced EMT, while ACE2 inhibition had the opposite effects. Furthermore, western blot analysis demonstrated that ACE2-alleviated EMT was associated with downregulated Arkadia and increased SMAD family member 7 (Smad7) protein, followed by TGF-β/Smad pathway inhibition in HRPTEpiCs. In conclusion, ACE2 is protective in DN, which may be due to the inhibition of Arkadia-mediated Smad7 degradation, whereby TGF-β/Smad-mediated EMT is ameliorated in high-glucose-stimulated HRPTEpiCs.

Correspondence to: Professor Wenhu Liu, Department of Nephrology, Beijing Friendship Hospital, Capital Medical University, 95 Yong An Road, Xi Cheng, Beijing 100050, P.R. China
E-mail: wenhuliu@mail.ccmu.edu.cn

Abbreviations: ACE2, angiotensin-converting enzyme 2; DN, diabetic nephropathy; HRPTEpiCs, Human renal proximal tubular epithelial cells; EMT, epithelial to mesenchymal cell transition; RAS, renin-angiotensin system; TGF-β, transforming growth factor-β

Key words: ACE2, diabetic nephropathy, Smad7, Arkadia, TGF-β/Smad pathway

Introduction

Diabetic nephropathy (DN) is the most common cause of end-stage renal disease worldwide (1). One of the main mechanisms of DN is renin-angiotensin system (RAS) activation, and inhibition of the RAS can slow the development of diabetic kidney injury (2). Angiotensin-converting enzyme 2 (ACE2) is a component of the RAS that has counter-regulatory functions to the classic ACE/angiotensin II (Ang II)/Ang II receptor type 1 (AT1R) axis (3). In human renal tissues, ACE2 is highly expressed in renal tubular epithelial cells, and low tubular ACE2 expression has been revealed to be associated with the progression of renal fibrosis in DN (4). However, the mechanisms by which ACE2 protects against high-glucose-induced renal fibrosis remain unclear. Moreover, circulating ACE2 has not been well studied in patients with DN.

Tubulointerstitial fibrosis is essential in the development of end-stage renal disease in DN. The renal tubular epithelial to mesenchymal cell transition (EMT) plays a key role in this process. It has been demonstrated that the transforming growth factor-β (TGF-β)/SMAD family member (Smad) signaling pathway is a potent initiator and regulator of EMT (5). Previous studies have revealed that TGF-β-induced EMT is primarily activated by Smad3 phosphorylation and inhibited by Smad7, a negative feedback regulator of the TGF-β1/Smad signaling pathway that protects against fibrosis by inducing receptor degradation, which halts the recruitment and phosphorylation of Smad3 (5,6). A previous study by Liu *et al* (7) revealed that the level of Smad7 protein was dynamically modulated by the ubiquitin-proteasome degradation pathway, and this was mainly attributed to the E3 ubiquitin ligase Arkadia (also referred to as ring finger protein 111). However, whether this process occurs in DN and the mechanisms involved have yet to be determined.

The purpose of the present study was to investigate the protective mechanism of ACE2 in DN. To this end, renal tissue from patients with DN and primary human renal proximal tubular epithelial cells (HRPTEpiC) stimulated with a high glucose concentration were studied. In addition, the serum ACE2 expression was measured in DN patients with microalbuminuria.

Materials and methods

Blood samples and renal tissue collection. Blood samples were collected from 10 patients with DN and 10 non-diabetic volun-

teers from December 2018 to March 2019. Diabetic renal tissue specimens were obtained from five patients with a pathological diagnosis of DN and control specimens were obtained from five patients with minimal change in nephropathy without renal interstitial fibrosis. None of the recruited participants had liver dysfunction, heart failure, malignancy and none were pregnant. The non-diabetic volunteers were those who visited the hospital for routine physical examination and had no history of diabetes or nephropathy with normal blood glucose and serum creatinine concentrations. The blood samples and renal tissues were obtained from Beijing Friendship Hospital, following a protocol approved by the Ethics Committee of Beijing Friendship Hospital, Capital Medical University. Each participant provided their written informed consent before being included in the study.

Serum ACE2 measurements. Venous blood was placed in disposable sterile test tubes and centrifuged at 1,000 x g, 25˚C for 10 min to isolate serum. The serum samples were then transferred to cryovials and frozen at -80˚C until assayed.

The serum ACE2 expression levels were measured using a commercial ELISA kit (cat. no. E01A0499; Shanghai BlueGene Biotech Co., Ltd.), according to the manufacturer's instructions.

Histopathological examination. Renal sections were fixed in 4% neutral-buffered paraformaldehyde for 24 h at 25˚C, embedded in paraffin, and cut into 4 μm-thick sections, which were then prepared for Periodic acid-Schiff (PAS), Periodic Schiff-Methenamine Silver (PASM) or Masson's modified trichrome histological staining. The stained renal tissue sections were scored as previously described (8). All the scoring was performed by one observer who was blinded to the group from which the tissue specimens originated. The data were recorded and used to compare the diabetic with the non-diabetic groups.

Immunohistochemistry. Paraffin-embedded sections of renal tissues were deparaffinized in xylene, rehydrated through an alcohol gradient from 99.5% to 70% alcohol, and underwent antigen retrieval in citrate buffer (cat. no. P0081; Beyotime Institute of Biotechnology, Inc.). Endogenous peroxidase activity was blocked with 3% H_2O_2, and then the sections were incubated with 5% normal goat serum at 37˚C for 30 min. Sections were then incubated overnight at 4˚C with primary antibodies targeting ACE2 (1:100; cat. no. ab87436; Abcam) or Arkadia (1:100; cat. no. OM290104; Omnimabs), followed by incubation with a biotinylated secondary antibody (1:2,000; cat. no. SP-9000; OriGene Technologies, Inc.) for 30 min at room temperature. After thorough rinsing, the antigens were detected by 3,3'-diaminobenzidine staining (OriGene Technologies, Inc.). Sections were counterstained with hematoxylin, dehydrated and cover-slipped. The stained renal tissue sections were scored as aforementioned.

Cell culture and treatments. The HRPTEpiCs were purchased from ScienCell Research Laboratories, Inc., (cat. no. 4100). Cells were cultured in epithelial cell medium (EpiCM) supplemented with 2% fetal bovine serum and epithelial cell growth supplement (EpiCGS; all from ScienCell Research Laboratories, Inc.), benzylpenicillin (100 U/ml), and streptomycin (100 μg/ml) at 37˚C in a humidified atmosphere containing 5% CO_2 as described in previous studies (9,10). Actively growing cells in their second to third passage were used for experiments. Cells were seeded at a density of $2.5x10^5$ cells/well in 6-well plates. When cell confluency reached 70-80%, the medium was replaced with serum-free epithelial cell medium. After serum starvation for 12 h, cells were incubated in medium containing a normal (5.5 mmol/l D-glucose) or high glucose concentration (30 mmol/l D-glucose; Sigma-Aldrich; Merck KGaA) for 4, 12, 24, 48 or 72 h (9). Subsequently, the cells were stimulated with a high glucose concentration for 48 h in the presence or absence of adenoviruses overexpressing ACE2. For treatment with an ACE2 inhibitor, the ACE2 inhibitor DX600 (0.5 mmol/l; BioVision, Inc.) was added to high glucose medium for 48 h. Finally, the cells were treated with small interfering (si)RNA targeting Arkadia or scramble siRNA for 48 h while remaining in high glucose medium. The cells were then collected by trypsin digestion for subsequent analysis.

Adenovirus transduction and siRNA transfection. Adenoviruses overexpressing ACE2 (pHBAd-BHG; Hanbio Biotechnology Co., Ltd), siRNA targeting *Arkadia* (5'-TTC CATGCAGAAAGAGATTTGTAAA-3') and a scrambled siRNA (5'-TTCTCCGAACGTGTCACGTAA-3') were purchased from Hanbio Biotechnology Co., Ltd. HRPTEpiCs were either infected with adenoviruses using the polybrene method at an MOI of 100 or transfected with 100 nmol/l siRNA at 37˚C and in 5% CO_2 for 6 h. All transfections were carried out using Transfection Reagent (Hanbio Biotechnology Co., Ltd.,). The medium was then replaced with fresh epithelial cell medium after 6-h incubation. Empty adenovirus and scrambled siRNA were used as negative controls. HRPTEpiCs were used for experiments 48 h after transfection.

Western blot analysis. Western blotting was performed as previously described (9). Briefly, after blocking nonspecific binding with 5% BSA (Sigma-Aldrich; Merck KGaA), the membranes were incubated overnight at 4˚C with primary antibodies. Primary antibodies against ACE2 (1:1,000; cat. no. 4355), Smad3 (1:1,000; cat. no. 9523), phosphorylated (p)-Smad3 (1:1,000; cat. no. 9520), E-cadherin (1:1,000; cat. no. 14472), α-smooth muscle actin (α-SMA; 1:1,000; cat. no. 19245) were purchased from Cell Signaling Technology, Inc. Antibodies targeting Arkadia (1:500; cat. no. ab174624), Smad7 (1:1,000; cat. no. ab190987), TGF-β (1:1,000; cat. no. ab92486) or β-actin (1:1,000; cat. no. ab8226) were purchased from Abcam. After the first incubation, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:2,000; cat. no. ZB-2301 or ZB-2305; ZSGB-BIO; OriGene Technologies, Inc.). Target proteins were detected and analyzed using Immobilon Western Chemiluminescent HRP Substrate (EMD Millipore). Densitometry analysis was performed using ImageJ software (v1.52; National Institutes of Health).

Reverse transcription-quantitative PCR (RT-qPCR). RNA was extracted from cells with TRIzol® Reagent (Invitrogen; Thermo Fisher Scientific, Inc.), following the manufacturer's protocol. Complementary DNA (cDNA) was generated using a RevertAid cDNA Synthesis kit (Fermentas; Thermo Fisher

Table I. Clinical characteristics of study subjects.

Characteristics	Non-diabetic (n=10)	Diabetic (n=10)	P-value
Age, years	59.0±14.7	62.6±17.0	0.248
Sex, male/female	5/5	5/5	-
SBP, mmHg	135.2±15.4	138.1±14.3	0.423
DBP, mmHg	78.5±9.8	80.2±9.7	0.527
Glucose, mmol/l	4.8±0.4	9.5±2.3	0.002
Serum creatinine, μmol/l	56.6±7.5	57.1±10.7	0.454
UACR, mg/g	-	112.5±11.8	-
ACE2, ng/ml	10.2±2.6	24.7±3.9	0.007

Data are presented as the mean ± SD. SBP, systolic blood pressure; DBP, diastolic blood pressure, UACR, urine albumin-to-creatinine ratio; ACE2, angiotensin-converting enzyme 2.

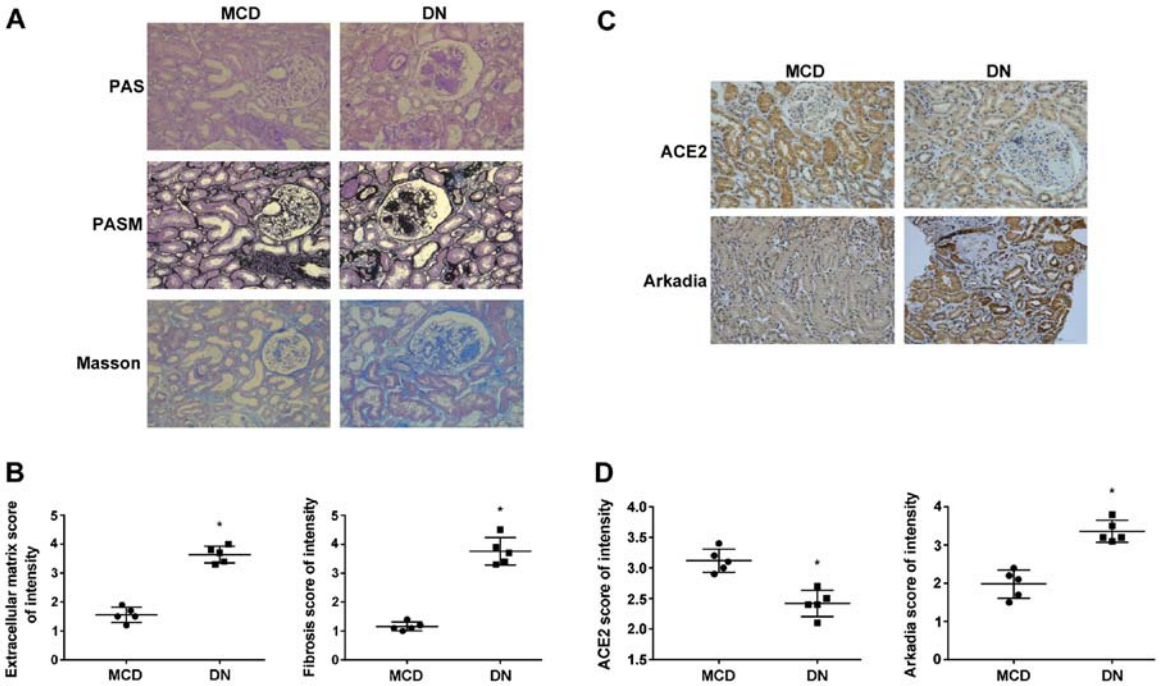

Figure 1. Deposition of extracellular matrix and collagen, and the expression of ACE2 and Arkadia in diabetic and non-diabetic human kidneys. (A) Periodic acid-Schiff staining, Periodic Schiff-Methenamine Silver staining and Masson's trichrome staining of renal sections, Scale bar, 100 μm. (B) Scored results of the PASM and Masson staining (1 = weakest; 4 = strongest). Each dot represents an individual specimen. (C) Immunohistochemical staining for ACE2 and Arkadia, Scale bar, 100 μm. (D) Scored results of the immunohistochemical staining (1 = weakest; 4 = strongest). Each dot represents an individual specimen. *P<0.05 compared with the minimal change nephropathy samples. ACE2, angiotensin-converting enzyme 2; MCD, minimal change nephropathy; DN, diabetic nephropathy; PAS, Periodic acid-Schiff; PASM, Periodic Schiff-Methenamine Silver.

Scientific, Inc.). RT-qPCR was performed using a SYBR1 Taq™ kit (Takara Bio, Inc.,) based on the ABI PRISM 7000 system. The thermocycling conditions consisted of an initial denaturation at 95˚C for 5 min, followed by 40 cycles at 95˚C for 20 sec, 55˚C for 20 sec, and 72˚C for 20 sec. *β-actin* was used as the reference gene. The results were analyzed using the $2^{-\Delta\Delta Cq}$ method (9). The primer sequences were as follows: *ACE2* forward, 5'-CATTGGAGCAAGTGTTGGATCTT-3' and reverse 5'-GAGCTAATGCATGCCATTCTCA-3'; *Arkadia* forward, 5'-CCACATAGGATGCACCCAAAC-3' and reverse, 5'-AATTCCCAGTTCCCAGGCA-3'; *Smad7* forward, 5'-CCTTAGCCGACTCTGCGAACTA-3' and reverse, 5'-CCAGATAATTCGTTCCCCCTGT-3'; *E-cadherin* forward, 5'-GAGAACGCATTGCCACATACAC-3' and reverse, 5'-GCACCTTCCATGACAGACCC-3'; *α-SMA* forward, 5'-ACTGGGACGACATGGAAAAG-3' and reverse, 5'-CATCTCCAGAGTCCAGCACA-3'; and *β-actin* forward, 5'-TGGGTCAGAAGGACTCCTATG-3' and reverse, 5'-CAGGCAGCTCATAGCTCTTCT-3'.

Statistical analysis. Data were expressed as the means ± standard deviations (SDs). Comparisons between groups were performed using one-way analysis of variance, followed by the Student-Newman-Keuls test. Statistical analyses were performed using SPSS v17.0 software (SPSS, Inc.). P<0.05 was considered to indicate a statistically significant difference.

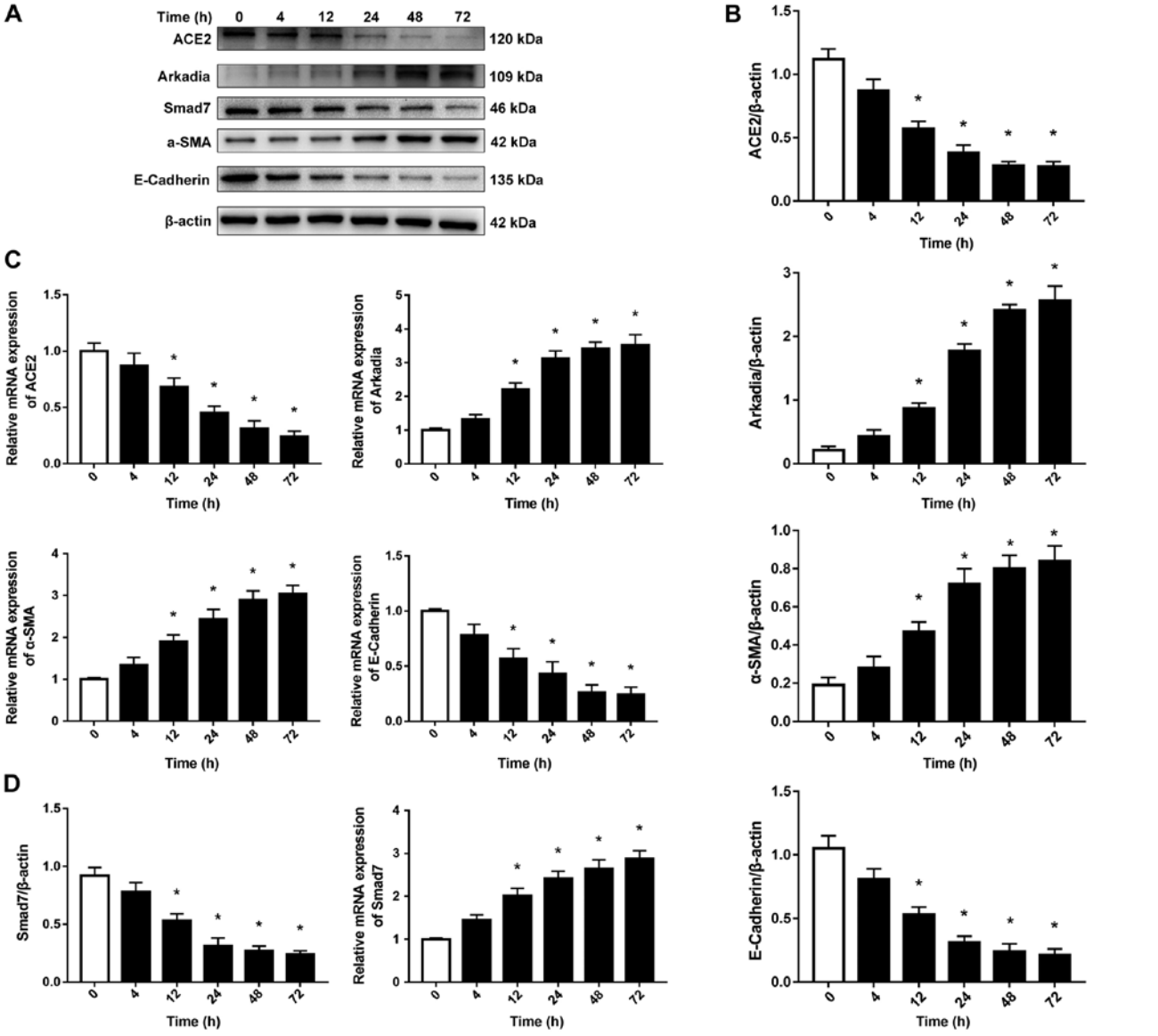

Figure 2. ACE2, Arkadia, Smad7, α-SMA and E-cadherin expression in HRPTEpiCs stimulated with high glucose. HRPTEpiCs were incubated in a high glucose medium (30 mmol/l D-glucose) for the indicated periods of time. (A) Western blotting and (B) its semi-quantification demonstrated that high glucose increased the expression of Arkadia and α-SMA, and decreased the expression of ACE2 and E-cadherin in a time-dependent manner. (C) RT-qPCR revealed that the mRNA expression levels of Arkadia and α-SMA increased, while that of ACE2 and E-cadherin decreased with time. Results were normalized to β-actin expression. (D) Semi -quantification of western blotting results revealed a gradual reduction in Smad7 protein expression level and RT-qPCR revealed that Smad7 mRNA expression level was increased in HRPTEpiCs stimulated with high glucose. *P<0.05 compared with time 0 h. ACE2, angiotensin-converting enzyme 2; α-SMA, α-smooth muscle actin; HRPTEpiCs, human renal proximal tubular epithelial cells; RT-qPCR, reverse transcription-quantitative PCR; Smad7, SMAD Family member 7.

Results

Clinical characteristics of and serum ACE2 expression levels in diabetic and non-diabetic participants. In total, 20 participants were recruited to the present study (10 diabetic and 10 non-diabetic). The characteristics of the study participants are presented in Table I. The age of the diabetic patients (59.0±14.7 years) and non-diabetic volunteers (62.6±17.0 years) were similar (P=0.248). There were no significant differences in serum creatinine or systolic and diastolic blood pressure. However, the serum ACE2 level was significantly higher in patients with diabetes compared with non-diabetic volunteers (10.2±2.6 vs. 24.7±3.9 ng/ml, P=0.007).

Expression of ACE2 is lower and Arkadia expression is higher in diabetic human kidneys. Since tubulointerstitial fibrosis is one of the major pathological alterations in DN (11), renal fibrosis was evaluated by Masson's trichrome staining in diabetic and non-diabetic human kidneys. Furthermore, immunohistochemistry was used to examine the expression of ACE2 and Arkadia in diabetic and non-diabetic human kidneys. Histologically, PAS and PASM staining demonstrated profound extracellular matrix deposition and Masson's trichrome staining revealed more severe renal fibrosis in diabetic than in non-diabetic human kidneys (Fig. 1A and B). Immunohistochemistry demonstrated significantly lower ACE2 expression in diabetic samples compared with the control kidneys, whereas the expression of Arkadia was higher (Fig. 1C and D).

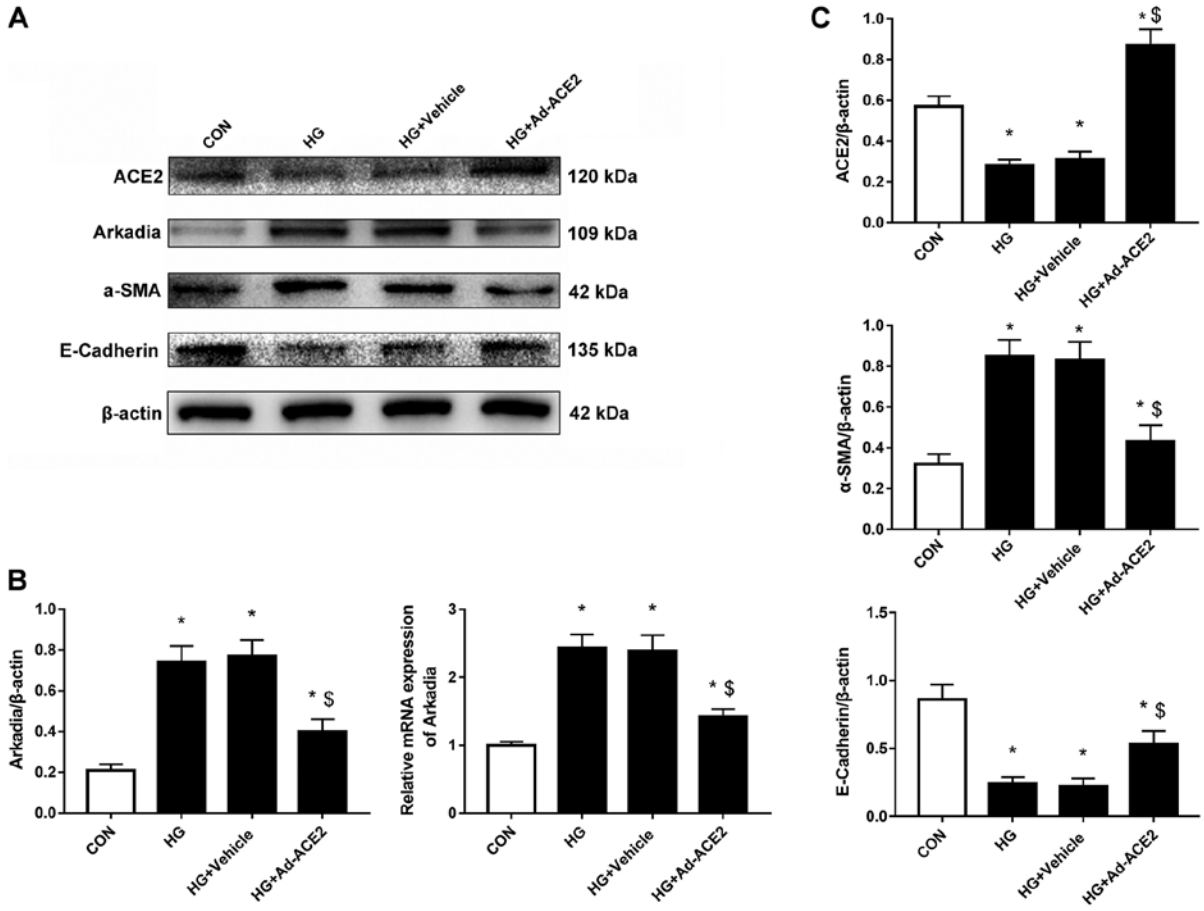

Figure 3. ACE2, Arkadia, α-SMA and E-cadherin expression when ACE2 is overexpressed. HRPTEpiCs were infected with Ad-ACE2 or empty adenovirus for 48 h. (A) Western blotting demonstrated that the expression of ACE2 was significantly induced by the specific adenovirus and reduced in high glucose conditions. ACE2 overexpression alleviated the effects of high glucose on the expression of Arkadia, α-SMA and E-cadherin. (B) Semi-quantification of the Arkadia protein and mRNA expression levels when ACE2 was overexpressed as detected by western blotting and reverse transcription-quantitative PCR, respectively. (C) Semi-quantification of ACE2, α-SMA and E-cadherin protein expression levels when ACE2 was overexpressed. Results were normalized to β-actin expression. *P<0.05 compared with control cells; $P<0.05 compared with high glucose-treated cells. ACE2, angiotensin-converting enzyme 2; α-SMA, α-smooth muscle actin; CON, control; HG, high glucose; HRPTEpiCs, human renal proximal tubular epithelial cells; Ad-ACE2, adenoviruses overexpressing ACE2.

High glucose reduces ACE2 and induces Arkadia expression and increases α-SMA and decreases E-cadherin expression in HRPTEpiCs. The present study subsequently investigated the effects of high glucose on the expression of ACE2, Arkadia and EMT in HRPTEpiCs. As revealed in Fig. 2, both the protein (Fig. 2A and B) and mRNA (Fig. 2C) expression levels of ACE2 decreased with time, while the mRNA and protein expression levels of Arkadia were increased with time in HRPTEpiCs incubated with a high glucose media. Furthermore, the expression of α-SMA, a myofibroblast marker, increased, whereas that of E-cadherin, an epithelial marker that plays a key role in maintaining the integrity of epithelial cells, decreased.

Smad7 mRNA increases while Smad7 protein expression level decreases in HRPTEpiCs stimulated with high glucose. Western blot analysis was used to determine the expression levels of Smad7 protein in HRPTEpiCs at different time-points during the stimulation of high glucose (Fig. 2A and D). High glucose gradually but significantly reduced the expression of Smad7 protein. To determine whether the decrease in Smad7 protein expression resulted from downregulation of *Smad7* mRNA expression, mRNA expression levels were assessed by RT-qPCR. In contrast to the significant decrease in Smad7 protein, the expression levels of *Smad7* mRNA increased with time in HRPTEpiCs stimulated with high glucose. These results indicated that Smad7 expression was downregulated at the protein level in the presence of a high glucose concentration.

ACE2 overexpression inhibits Arkadia expression and alleviates high-glucose-induced EMT in HRPTEpiCs. Next, ACE2 was overexpressed or inhibited in HRPTEpiCs stimulated with high glucose. Ad-ACE2 infection significantly increased the expression of ACE2 mRNA (Fig. S1A). The RT-qPCR and western blot analysis revealed that ACE2 overexpression significantly reduced both the mRNA and protein expression of Arkadia (Fig. 3A and B), whereas ACE2 inhibition increased Arkadia mRNA and protein levels (Fig. 4A and B) in HRPTEpiCs stimulated with high glucose. Accordingly, high-glucose-induced EMT, indicated by upregulation of α-SMA and downregulation of E-cadherin expression, was alleviated when ACE2 was overexpressed (Fig. 3A and C) and aggravated when ACE2 was inhibited (Fig. 4A and D).

ACE2 alleviates high-glucose-induced EMT in HRPTEpiCs by downregulating Arkadia-dependent ubiquitin degradation of Smad7 and inhibiting the TGF-β/Smad pathway. HRPTEpiCs

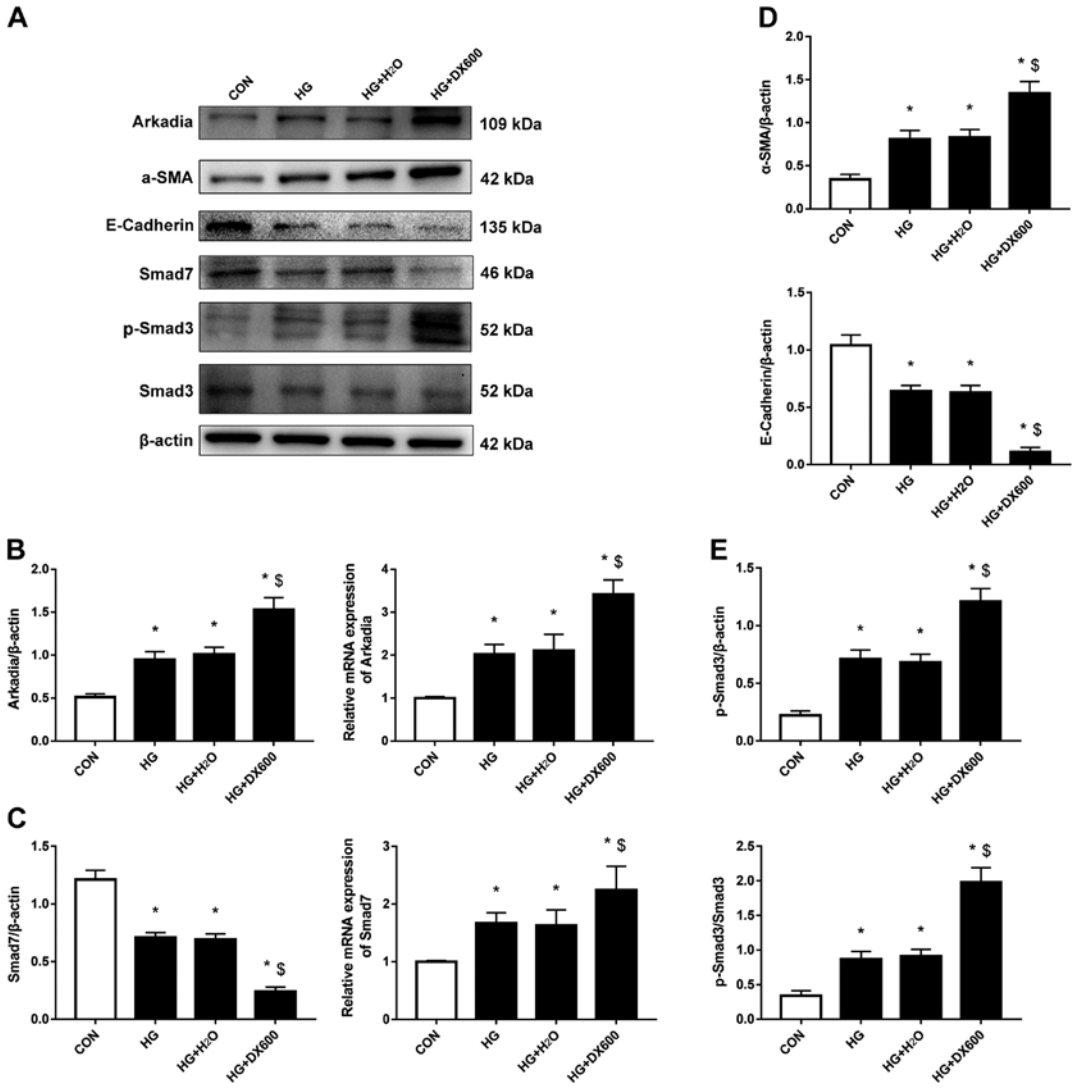

Figure 4. ACE2, Arkadia, Smad7, p-Smad3, α-SMA and E-cadherin expression when ACE2 was inhibited. HRPTEpiCs were treated with the ACE2 inhibitor DX600 for 48 h. (A) Western blotting demonstrated that ACE2 inhibition aggravated the effects of high glucose on the expression of Arkadia, α-SMA and E-cadherin. Smad7 protein expression levels were decreased in HRPTEpiCs stimulated with high glucose and further reduced by DX600 treatment, while p-Smad3 exhibited the opposite changes. (B) Semi-quantification of Arkadia protein and mRNA expression levels after ACE2 inhibition as detected by western blotting and RT-qPCR, respectively. (C) Semi-quantification of Smad7 protein and mRNA expression levels after ACE2 inhibition as detected by western blotting and RT-qPCR, respectively. (D) Semi-quantification of the expression levels of α-SMA and E-cadherin protein when ACE2 was inhibited. (E) Semi-quantification of p-Smad3 protein when ACE2 was inhibited. Results were normalized to β-actin expression. *P<0.05 compared with control cells; $^{\$}$P<0.05 compared with high glucose-treated cells. ACE2, angiotensin-converting enzyme 2; α-SMA, α-smooth muscle actin; CON, control; HG, high glucose; HRPTEpiCs, human renal proximal tubular epithelial cells; p-, phosphorylat

were stimulated with high glucose in the presence of an ACE2 inhibitor and/or *Arkadia* siRNA to explore the mechanism by which ACE2 deactivation promotes EMT when exposed to a high glucose concentration. Transfection with siArkadia significantly decreased Arkadia expression (Fig. S1A). ACE2 inhibition was found to reduce Smad7 protein expression, but induced *Smad7* mRNA expression, and substantially activated the TGF-β/Smad signaling pathway, as detected by p-Smad3, in high-glucose-stimulated HRPTEpiCs (Fig. 4A, C and E). To further confirm the effects of the ACE2/Arkadia/Smad7 axis on EMT, high-glucose-stimulated HRPTEpiCs were cotreated with an ACE2 inhibitor and *Arkadia* siRNA. All the effects of ACE2 deactivation observed were reversed following depletion of Arkadia (Fig. 5). Moreover, the expression of TGF-β, another crucial component of the TGF-β/Smad signaling pathway, was detected and exhibited a similar trend to that of p-Smad3 expression (Fig. S1B and C). Collectively, these findings indicated that ACE2 protected against high-glucose-induced renal fibrosis by inhibiting EMT, which may be at least in part due to inhibition of Arkadia-dependent ubiquitin degradation of Smad7 and the TGF-β/Smad signaling pathway.

Discussion

The present study has demonstrated the relationship between ACE2 and the ubiquitination pathway in DN. Inhibition of ACE2 appeared to suppress EMT *in vitro* and *in vivo* via an Arkadia-dependent mechanism. This process would lead to an increase in TGF-β/Smad signaling, the key regulatory pathway in EMT, by targeting Smad7 for ubiquitin-mediated degradation. Moreover, the present study also revealed that serum ACE2 was higher in patients with DN with microproteinuria.

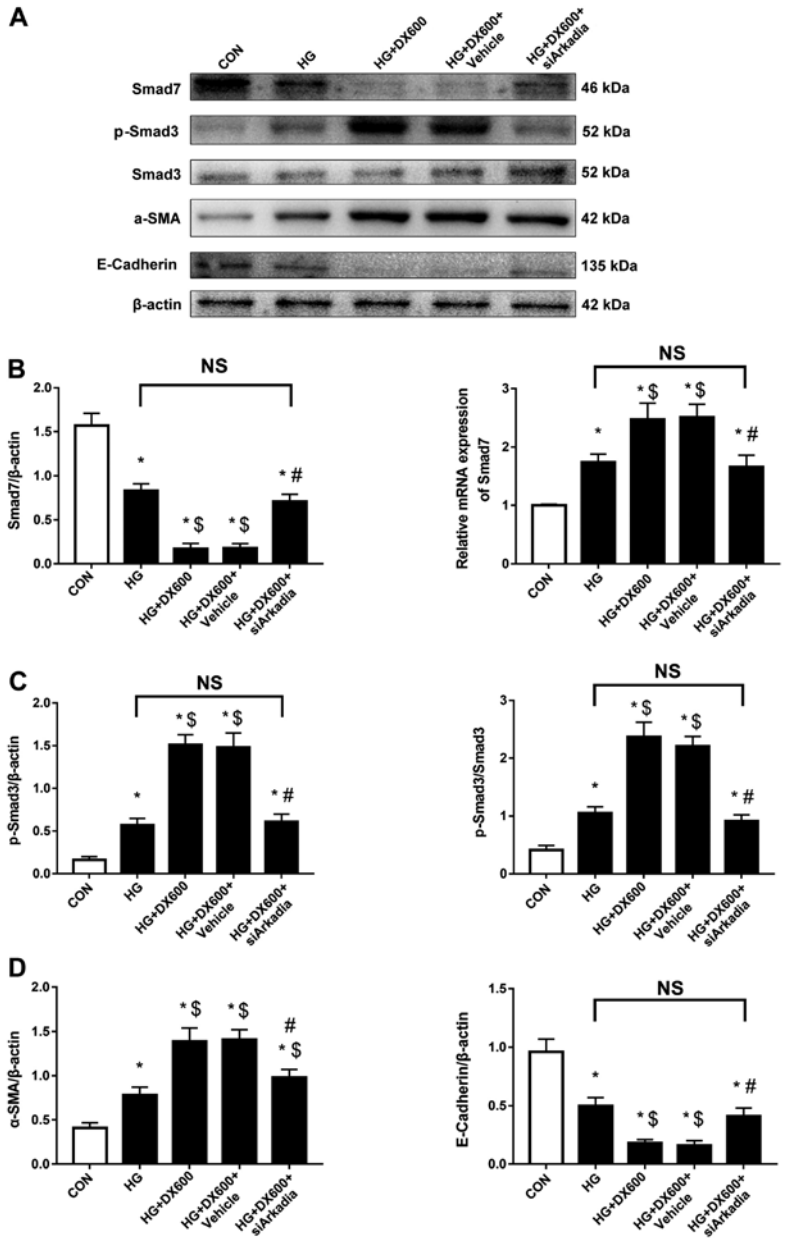

Figure 5. Arkadia knockdown increases Smad7 protein and inhibits activation of the TGF-β/Smad signaling pathway and epithelial to mesenchymal cell transition in HRPTEpiCs. (A) Western blotting demonstrated that Smad7 and E-cadherin protein expression levels were decreased in HRPTEpiCs co-treated with high glucose and DX600, but increased following transfection with siArkadia, while p-Smad3 and α-SMA exhibited the opposite changes. (B) Semi-quantification of Smad7 protein and mRNA expression levels after Arkadia depletion as detected by western blotting and reverse transcription-quantitative PCR, respectively. (C) Semi-quantification of p-Smad3 protein after Arkadia knockdown. (D) Semi-quantification of α-SMA and E-cadherin protein after Arkadia knockdown. Results were normalized to β-actin expression. *P<0.05 compared with control cells; $P<0.05 compared with high glucose-treated cells; #P<0.05 compared with cells co-treated with high glucose and DX600. α-SMA, α-smooth muscle actin; CON, control; HG, high glucose; HRPTEpiCs, human renal proximal tubular epithelial cells; siArkadia, small interfering RNA targeting Arkadia; p-, phosphorylated; Smad, SMAD family member; NS, not significant

It is widely accepted that the activation of RAS, particularly the intrarenal RAS, is important for the progression of DN (12). ACE2 is an ACE homolog that counter-regulates the RAS. It exists both as a tissue-bound enzyme, which is highly expressed in the proximal tubule brush border, and as a soluble protein that has enzymatic effects in the circulatory system (13,14). ACE2 expression is downregulated in the kidneys of patients with DN (15). More recently, ACE2 knockout was revealed to be associated with EMT and renal fibrosis in mouse models of obstructive, hypertensive and diabetic nephropathy, which feature α-SMA accumulation and E-cadherin deficiency (3,16,17). In contrast, ACE2 overexpression ameliorated renal fibrosis and albuminuria in diabetic mice (18). ACE2 is generally conceived as a renoprotective factor that works by converting Ang II to Ang(1-7), which has its cellular effects via the G protein-coupled receptor Mas (19,20). The cross talk between renal ACE and ACE2, as well as the balance between the ACE/Ang II/AT1R and ACE2/Ang(1-7)/Mas axes have also been emphasized *in vivo* and *in vitro* (21-24). However, it appears that ACE2 has effects that are far more complex than RAS regulation (3,25). This led to the present investigation to decipher the detailed mechanism by which ACE2 exerts its renoprotective effects in patients with DN and HRPTEpiCs in a high glucose environment.

In the present study, ACE2 expression was downregulated, whereas the expression of Arkadia, an E3 ubiquitin ligase critical for the TGF-β/Smad signaling pathway during EMT (26,27), was upregulated in both diabetic human kidneys and HRPTEpiCs stimulated with high glucose. These results indicated that ACE2 affected EMT by modulating Arkadia expression. Accumulating evidence suggests that Smad7 is a major regulator of the intensity and duration of Smad signals (28-31). In the present study, Smad7 protein expression level was decreased while its mRNA expression level was increased. Therefore, it was hypothesized that the decrease in Smad7 protein expression was caused by greater degradation rather than lower expression. Arkadia has been reported to aggravate organ damage by inducing Smad signals via the degradation of Smad7 in pulmonary fibrosis, atrial fibrillation and aristolochic acid nephropathy (32-34). In fact, inhibition or overexpression of ACE2 caused upregulation and downregulation of Arkadia expression, respectively, which resulted in changes in Smad7 protein and mRNA expression levels in high-glucose-stimulated HRPTEpiCs. Consistently, Arkadia knockdown increased Smad7 protein expression levels and attenuated high-glucose-induced EMT when ACE2 was inhibited. Therefore, it was hypothesized that ACE2 was able to suppress EMT by preventing Arkadia-dependent ubiquitination, which degrades Smad7 protein in high-glucose-stimulated HRPTEpiCs. This process is essential for renal fibrosis in DN and had not been reported in previous studies.

Another finding of the present study was that patients with DN with microalbuminuria displayed a significantly higher serum ACE2 expression level compared with the controls. Few human studies have previously measured circulating ACE2 in the context of DN. In experimental studies, circulating ACE2 activity has been reported to be higher in mouse models of diabetes (35-38). To date, only one study of patients with type I diabetes with vascular complications demonstrated higher ACE2 activity in the circulation (39). In concordance, the present study revealed a high serum ACE2 level in patients with DN with microalbuminuria. Numerous previous studies have linked high circulating ACE2 expression levels with an increased risk of cardiovascular disease, which is common in patients with DN (38,40-42). The increased circulating ACE2 expression level in these patients may stem from cardiovascular injury or occur to compensate for the downregulated renal ACE2. However, this result was difficult to confirm due to the small sample size. Nevertheless, the present findings still suggest a possible diagnostic role for the measurement of circulating ACE2 in DN, but further larger-scale clinical studies are still required to confirm this finding.

In conclusion, the present study has demonstrated that intrarenal ACE2 had a protective effect in DN. ACE2 suppressed Arkadia-dependent ubiquitin degradation of Smad7, which may be an essential mechanism by which TGF-β/Smad-mediated EMT was ameliorated in high-glucose-stimulated HRPTEpiCs.

Acknowledgements

Not applicable.

Funding

This study was supported by the Beijing Municipal Administration of Hospitals Clinical Medicine Development of Special Funding Support, Grant/Award no. ZYLX201824 and the National Natural Science Foundation of China, Grant/Award no. 81570660.

Availability of data and materials

The datasets used and analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

WL and ZC provided the concept and designed the study. ZC and XC performed the experiments, and the results were then interpreted by ZC, ZD and YB. ZC prepared the figures and drafted the manuscript. ZC, ZD and WL edited and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All procedures were approved by the Ethics Committee of Beijing Friendship Hospital, Capital Medical University. Each participant provided their written informed consent before being included in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Giacco F, Du X, D'Agati VD, Milne R, Sui G, Geoffrion M and Brownlee M: Knockdown of glyoxalase 1 mimics diabetic nephropathy in nondiabetic mice. Diabetes 63: 291-299, 2014.
2. Rahimi Z: The role of renin angiotensin aldosterone system genes in diabetic nephropathy. Can J Diabetes 40: 178-183, 2016.
3. Culver S, Li C and Siragy HM: Intrarenal angiotensin-converting enzyme: The old and the new. Curr Hypertens Rep 19: 80, 2017.
4. Batlle D, Wysocki J, Soler MJ and Ranganath K: Angiotensin-converting enzyme 2: Enhancing the degradation of angiotensin II as a potential therapy for diabetic nephropathy. Kidney Int 81: 520-528, 2012.
5. Chen L, Yang T, Lu DW, Zhao H, Feng YL, Chen H, Chen DQ, Vaziri ND and Zhao YY: Central role of dysregulation of TGF-β/Smad in CKD progression and potential targets of its treatment. Biomed Pharmacother 101: 670-681, 2018.
6. Meng XM, Tang PM, Li J and Lan HY: TGF-β/Smad signaling in renal fibrosis. Front Physiol 6: 82, 2015.
7. Liu FY, Li XZ, Peng YM, Liu H and Liu YH: Arkadia regulates TGF-β signaling during renal tubular epithelial to mesenchymal cell transition. Kidney Int 73: 588-594, 2008.
8. Chen X, Wu Y, Diao Z, Han X, Li D, Ruan X and Liu W: C1q/tumor necrosis factor-related protein-3 improves renal fibrosis via inhibiting notch signaling pathways. J Cell Physiol 234: 22352-22364, 2019.
9. Li X, Diao Z, Ding J, Liu R, Wang L, Huang W and Liu W: The downregulation of SnoN expression in human renal proximal tubule epithelial cells under high-glucose conditions is mediated by an increase in Smurf2 expression through TGF-β1 signaling. Int J Mol Med 37: 415-422, 2016.

10. Wang LY, Diao ZL, Zheng JF, Wu YR, Zhang QD and Liu WH: Apelin attenuates TGF-β1-induced epithelial to mesenchymal transition via activation of PKC-ε in human renal tubular epithelial cells. Peptides 96: 44-52, 2017.
11. Yu SM and Bonventre JV: Acute kidney injury and progression of diabetic kidney disease. Adv Chronic Kidney Dis 25: 166-180, 2018.
12. Williams VR and Scholey JW: Angiotensin-converting enzyme 2 and renal disease. Curr Opin Nephrol Hypertens 27: 35-41, 2018.
13. Koka V, Huang XR, Chung AC, Wang W, Truong LD and Lan HY: Angiotensin II up-regulates angiotensin I-converting enzyme (ACE), but down-regulates ACE2 via the AT1-ERK/p38 MAP kinase pathway. Am J Pathol 172: 1174-1183, 2008.
14. Wysocki J, Ye M, Khattab AM, Fogo A, Martin A, David NV, Kanwar Y, Osborn M and Batlle D: Angiotensin-converting enzyme 2 amplification limited to the circulation does not protect mice from development of diabetic nephropathy. Kidney Int 91: 1336-1346, 2017.
15. Reich HN, Oudit GY, Penninger JM, Scholey JW and Herzenberg AM: Decreased glomerular and tubular expression of ACE2 in patients with type 2 diabetes and kidney disease. Kidney Int 74: 1610-1616, 2008.
16. Liu Z, Huang XR, Chen HY, Penninger JM and Lan HY: Loss of angiotensin-converting enzyme 2 enhances TGF-β/Smad-mediated renal fibrosis and NF-κB-driven renal inflammation in a mouse model of obstructive nephropathy. Lab Invest 92: 650-661, 2012.
17. Liu Z, Huang XR, Chen HY, Fung E, Liu J and Lan HY: Deletion of angiotensin-converting enzyme-2 promotes hypertensive nephropathy by targeting Smad7 for ubiquitin degradation. Hypertension 70: 822-830, 2017.
18. Lo CS, Shi Y, Chang SY, Abdo S, Chenier I, Filep JG, Ingelfinger JR, Zhang SL and Chan JS: Overexpression of heterogeneous nuclear ribonucleoprotein F stimulates renal Ace-2 gene expression and prevents TGF-β1-induced kidney injury in a mouse model of diabetes. Diabetologia 58: 2443-2454, 2015.
19. Santos RA, Simoes e Silva AC, Maric C, Silva DM, Machado RP, de Buhr I, Heringer-Walther S, Pinheiro SV, Lopes MT, Bader M, *et al*: Angiotensin-(1-7) is an endogenous ligand for the G protein-coupled receptor Mas. Proc Natl Acad Sci USA 100: 8258-8263, 2003.
20. Simões E Silva AC, Teixeira MM and Teixeira MM: ACE inhibition, ACE2 and angiotensin-(1-7) axis in kidney and cardiac inflammation and fibrosis. Pharmacol Res 107: 154-162, 2016.
21. Oudit GY, Liu GC, Zhong J, Basu R, Chow FL, Zhou J, Loibner H, Janzek E, Schuster M, Penninger JM, *et al*: Human recombinant ACE2 reduces the progression of diabetic nephropathy. Diabetes 59: 529-538, 2010.
22. Márquez E, Riera M, Pascual J and Soler MJ: Albumin inhibits the insulin-mediated ACE2 increase in cultured podocytes. Am J Physiol Renal Physiol 306: F1327-F1334, 2014.
23. Callera GE, Antunes TT, Correa JW, Moorman D, Gutsol A, He Y, Cat AN, Briones AM, Montezano AC, Burns KD, *et al*: Differential renal effects of candesartan at high and ultra-high doses in diabetic mice-potential role of the ACE2/AT2R/Mas axis. Biosci Rep 36: 36, 2016.
24. Lin M, Gao P, Zhao T, He L, Li M, Li Y, Shui H and Wu X: Calcitriol regulates angiotensin-converting enzyme and angiotensin converting-enzyme 2 in diabetic kidney disease. Mol Biol Rep 43: 397-406, 2016.
25. Jin HY, Chen LJ, Zhang ZZ, Xu YL, Song B, Xu R, Oudit GY, Gao PJ, Zhu DL and Zhong JC: Deletion of angiotensin-converting enzyme 2 exacerbates renal inflammation and injury in apolipoprotein E-deficient mice through modulation of the nephrin and TNF-alpha-TNFRSF1A signaling. J Transl Med 13: 255, 2015.
26. Koinuma D, Shinozaki M, Komuro A, Goto K, Saitoh M, Hanyu A, Ebina M, Nukiwa T, Miyazawa K, Imamura T, *et al*: Arkadia amplifies TGF-beta superfamily signalling through degradation of Smad7. EMBO J 22: 6458-6470, 2003.
27. Birkou M, Chasapis CT, Marousis KD, Loutsidou AK, Bentrop D, Lelli M, Herrmann T, Carthy JM, Episkopou V and Spyroulias GA: A Residue Specific Insight into the Arkadia E3 Ubiquitin Ligase Activity and Conformational Plasticity. J Mol Biol 429: 2373-2386, 2017.
28. Chen HY, Huang XR, Wang W, Li JH, Heuchel RL, Chung AC and Lan HY: The protective role of Smad7 in diabetic kidney disease: Mechanism and therapeutic potential. Diabetes 60: 590-601, 2011.
29. Henique C and Tharaux PL: Targeting signaling pathways in glomerular diseases. Curr Opin Nephrol Hypertens 21: 417-427, 2012.
30. Lai JY, Luo J, O'Connor C, Jing X, Nair V, Ju W, Randolph A, Ben-Dov IZ, Matar RN, Briskin D, *et al*: MicroRNA-21 in glomerular injury. J Am Soc Nephrol 26: 805-816, 2015.
31. Luo M, Tan X, Mu L, Luo Y, Li R, Deng X, Chen N, Ren M, Li Y, Wang L, *et al*: MiRNA-21 mediates the antiangiogenic activity of metformin through targeting PTEN and SMAD7 expression and PI3K/AKT pathway. Sci Rep 7: 43427, 2017.
32. He X, Gao X, Peng L, Wang S, Zhu Y, Ma H, Lin J and Duan DD: Atrial fibrillation induces myocardial fibrosis through angiotensin II type 1 receptor-specific Arkadia-mediated downregulation of Smad7. Circ Res 108: 164-175, 2011.
33. Tian Y, Liao F, Wu G, Chang D, Yang Y, Dong X, Zhang Z, Zhang Y and Wu G: Ubiquitination and regulation of Smad7 in the TGF-β1/Smad signaling of aristolochic acid nephropathy. Toxicol Mech Methods 25: 645-652, 2015.
34. Elkouris M, Kontaki H, Stavropoulos A, Antonoglou A, Nikolaou KC, Samiotaki M, Szantai E, Saviolaki D, Brown PJ, Sideras P, *et al*: SET9-Mediated Regulation of TGF-β Signaling Links Protein Methylation to Pulmonary Fibrosis. Cell Rep 15: 2733-2744, 2016.
35. Tikellis C, Bialkowski K, Pete J, Sheehy K, Su Q, Johnston C, Cooper ME and Thomas MC: ACE2 deficiency modifies renoprotection afforded by ACE inhibition in experimental diabetes. Diabetes 57: 1018-1025, 2008.
36. Yamaleyeva LM, Gilliam-Davis S, Almeida I, Brosnihan KB, Lindsey SH and Chappell MC: Differential regulation of circulating and renal ACE2 and ACE in hypertensive mRen2.Lewis rats with early-onset diabetes. Am J Physiol Renal Physiol 302: F1374-F1384, 2012.
37. Riera M, Anguiano L, Clotet S, Roca-Ho H, Rebull M, Pascual J and Soler MJ: Paricalcitol modulates ACE2 shedding and renal ADAM17 in NOD mice beyond proteinuria. Am J Physiol Renal Physiol 310: F534-F546, 2016.
38. Anguiano L, Riera M, Pascual J and Soler MJ: Circulating ACE2 in cardiovascular and kidney diseases. Curr Med Chem 24: 3231-3241, 2017.
39. Soro-Paavonen A, Gordin D, Forsblom C, Rosengard-Barlund M, Waden J, Thorn L, Sandholm N, Thomas MC and Groop PH; FinnDiane Study Group: Circulating ACE2 activity is increased in patients with type 1 diabetes and vascular complications. J Hypertens 30: 375-383, 2012.
40. Anguiano L, Riera M, Pascual J, Valdivielso JM, Barrios C, Betriu A, Mojal S, Fernández E and Soler MJ; NEFRONA study: Circulating angiotensin-converting enzyme 2 activity in patients with chronic kidney disease without previous history of cardiovascular disease. Nephrol Dial Transplant 30: 1176-1185, 2015.
41. Anguiano L, Riera M, Pascual J, Valdivielso JM, Barrios C, Betriu A, Clotet S, Mojal S, Fernández E and Soler MJ; Investigators from the NEFRONA Study: Circulating angiotensin converting enzyme 2 activity as a biomarker of silent atherosclerosis in patients with chronic kidney disease. Atherosclerosis 253: 135-143, 2016.
42. Chen YY, Zhang P, Zhou XM, Liu D, Zhong JC, Zhang CJ, Jin LJ and Yu HM: Relationship between genetic variants of ACE2 gene and circulating levels of ACE2 and its metabolites. J Clin Pharm Ther 43: 189-195, 2018.