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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

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It is known that angiotensin (Ang)-converting enzyme (ACE) 2 catalyzes Ang II to Ang 1–7 to prevent the detrimental effect of Ang II on blood pressure, renal fibrosis, and inflammation. However, mechanisms of renoprotective role of Ace2 remain largely unclear. The present study tested the hypothesis that deficiency of *Ace2* may accelerate intrarenal Ang II-mediated fibrosis and inflammation independent of blood pressure in a model of unilateral ureteral obstructive (UUO) nephropathy induced in *Ace2*$^{+/y}$ and *Ace2*$^{-/y}$ mice. Results showed that both *Ace2*$^{+/y}$ and *Ace2*$^{-/y}$ mice had normal levels of blood pressure and plasma Ang II/Ang 1–7. In contrast, deletion of ACE2 resulted in a fourfold increase in the ratio of intrarenal Ang II/Ang 1–7 in the UUO nephropathy. These changes were associated with the development of more intensive tubulointerstitial fibrosis (α-SMA, collagen I) and inflammation (TNF-α, IL-1β, MCP-1, F4/80$^+$ cells, and CD3$^+$T cells) in *Ace2*$^{-/y}$ mice at day 3 (all $P<0.05$) after UUO, becoming more profound at day 7 (all $P<0.01$). Enhanced renal fibrosis and inflammation in the UUO kidney of *Ace2*$^{-/y}$ mice were largely attributed to a marked increase in the intrarenal Ang II signaling (AT1-ERK1/2 mitogen-activated protein kinase), TGF-β/Smad2/3, and NF-κB signaling pathways. Further studies revealed that enhanced TGF-β/Smad and NF-κB signaling in the UUO kidney of *Ace2*$^{-/y}$ mice was associated with upregulation of an E3 ligase Smurf2 and a loss of renal Smad7. In conclusion, enhanced Ang II-mediated TGF-β/Smad and NF-κB signaling may be the mechanisms by which loss of *Ace2* enhances renal fibrosis and inflammation. Smad7 ubiquitin degradation mediated by Smurf2 may be a central mechanism by which *Ace2*$^{-/y}$ mice promote TGF-β/Smad2/3-mediated renal fibrosis and NF-κB-driven renal inflammation in a mouse model of UUO nephropathy.
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The renin–angiotensin system has been recognized for many years as a critical pathway leading to chronic kidney disease. Renin promotes the production of angiotensin I (Ang I), which is converted to Ang II by Ang I-converting enzyme (ACE) and also possibly by other enzymes.[1] Ang II, as an important active peptide, exerts a variety of biological and pathological effects on several target tissues and organs, including blood vessels, kidney, and heart under normal and disease conditions. ACE 2 is the homolog of ACE but counterbalances the ACE activity via promoting Ang II degradation to the vasodilator peptide Ang 1–7. Ang 1–7 is a biologically active peptide and acts on the Mas receptor to exert the opposite effect on Ang II.[1] Ace2 is highly expressed in the normal kidney, largely by tubular epithelial cells.[2] Increasing evidence shows that Ace2 has an essential role in the cardiovascular and kidney diseases.[1,3,4] Ace2 inhibition also leads to the development of albuminuria in a mouse model of diabetes.[5,6]

Direct evidence for ACE2 in the development of hypertensive kidney disease comes from the *Ace*2 gene knockout mice. Loss of *Ace*2 leads to the late development of glomerulosclerosis by 12 months of the age, and accelerates kidney injury in mouse models of diabetes and Ang II infusion.[7–9] Recent finding that administration of human recombinant

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ACE2 inhibits progression of diabetic kidney disease clearly demonstrated a renoprotective role for ACE2 in the progression of chronic kidney diseases.[10] All these studies suggest a negative regulatory role for ACE2 in blood pressure and Ang II-mediated hypertensive and diabetic nephropathy. The function of ACE2 in non-hypertension and diabetic condition was not yet clear. Furthermore, the mechanisms of ACE2 in protection against Ang II-mediated renal injury remain largely unclear. Thus, the present study aimed to investigate the potential role of Ace2 in chronic kidney disease independent of hypertension and diabetes and explored the renoprotective mechanisms of Ace2 in progressive renal fibrosis and inflammation.

As a mouse model of UUO is a well-established chronic kidney disease model without underlying hypertensive and diabetic conditions, we thus induced the UUO nephropathy in *Ace2*$^{-/y}$ mice and examined the hypothesis that loss of Ace2 may promote intrarenal Ang II-mediated renal fibrosis and inflammation independent of blood pressure. In addition, the mechanisms by which loss of *Ace*2 enhances renal fibrosis and inflammation were investigated.

MATERIALS AND METHODS

Obstructive Kidney Disease Model

Ace2$^{-/y}$ mice (C57BL/6 background) were generated as described previously.[11] A mouse model of UUO nephropathy was induced in the littermate male *Ace2*$^{+/y}$ and *Ace2*$^{-/y}$ mice (20 g body weight, 8 weeks of age) by the left ureteral ligation as described previously.[12] To investigate the role of *Ace*2 in the early and late stages of UUO, groups of eight *Ace2*$^{+/y}$ or *Ace2*$^{-/y}$ mice were killed at day 3 and day 7 after UUO. In addition, six age-matched *Ace2*$^{+/y}$ or *Ace2*$^{-/y}$ mice were received sham operation as control. The experimental procedures were approved by the Animal Experimental Committee at the Chinese University of Hong Kong. Kidney tissue samples were collected at day 3 and day 7 after UUO for histology, immunohistochemistry, western blot, and real-time PCR analyses.

Histology and Immunohistochemistry

Changes in renal morphology were examined in methyl Carnoy's fixed, paraffin-embedded tissue sections (4 μm) using the Masson trichrome staining. Immunohistochemistry was performed on paraffin sections using a microwave-based antigen retrieval technique.[12,13] Antibodies used in this study included rabbit antibodies recognized mouse phospho-Smad2/3, MCP-1, IL-1β, TNF-α, TGF-β1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), collagen I (Southern Tech, Birmingham, AL, USA), α-SMA (Sigma, St Louis, MO, USA), and rat anti-mouse monoclonal antibody to macrophages (F4/80) (Serotec, Oxford, UK) and rabbit polyclonal antibodies to CD3$^+$ T cells (Abcam, Cambridge, UK). All slides (except α-SMA and phospho-Smad2/3 stained sections) were counterstained with hematoxylin.

Quantitation of immunostaining was carried on coded slides as previously described.[12–14] IL-1β, TNF-α, and MCP-1 in the entire cortical tubulointerstitium (a cross-section of the renal cortex) were determined by the quantitative Image-Pro plus software (Media Cybernetics, Bethesda, MD, USA) as previously described.[12–14] The number of F4/80$^+$ cells in the tubulointerstitium was counted under high-power fields ($\times$ 40 objective) by means of a 0.0625-mm^2 graticule fitted in the eyepiece of the microscope and expressed as cells per millimeters squared (mm^2).

Measurement of Blood Pressure and Ang II and Ang 1–7

Blood pressure was measured by tail-cuff method using the CODA non-invasive blood pressure system (Kent Scientific, Torrington, CT, USA) in conscious mice according to the manufacturer's instruction. Both plasma and intrarenal Ang II and Ang 1–7 were measured using commercially available enzyme immunoassay kits (Peninsula Laboratories, San Carlos, CA, USA). Briefly, the kidney tissue was weighed, homogenized in 1 ml methanol on ice. Then, the samples were centrifuged at 12 000 *g* at 4°C for 10 min and the supernatant was collected and dried by evaporation. The dried samples were then reconstituted with EIA buffer. Blood plasma was collected from mice, and an equal amount of buffer A was added. The mixture was centrifuged at 17 000 *g* for 20 min at 4°C and the supernatant was passed through the pre-treated C-18 SEP-COLUMN. The elute peptide was freezer dried and was dissolved in EIA buffer. Concentrations of Ang II and Ang 1–7 were measured by the ELISA kits following the manufacturer's instructions.

Western Blot Analysis

Renal tissues were collected by carefully removing the renal pelvis and medullar tissues, and were frozen at −80°C freezer for western blot analysis as previously described.[12–14] Briefly, after the protein was transferred onto a nitrocellulose membrane, the membrane was incubated overnight with primary antibodies against phospho-Smad3, phospho-IκBα (ser32), phospho-NF-κB/p65 (ser276), ERK1/2 (Cell Signaling Technology, MA, USA); IκBα, NF-κB/p65, phospho-ERK1/2, Smad2/3, Smad7, ACE2, Smurf2, Ang II receptor-1 (AT1) (Santa Cruz Biotech); α-SMA (Sigma), collagen I (Southern Tech), and GAPDH (Chemicon, Temecula, CA, USA) followed by the LI-COR IRDye 800-labeled secondary antibodies (Rockland Immunochemicals, Gilbertsville, PA, USA) in dark for 1 h at room temperature. Signals were scanned and visualized by Odyssey Infrared Imaging System (LiCor, Lincoln, NE, USA). The ratio of the protein interested was subjected to GAPDH and was densitometrically analyzed by the Image J software (NIH, Bethesda, MD, USA).

Real-Time PCR

Renal mRNA expression was quantitatively analyzed by real-time PCR with primers against mouse mRNA of IL-1β, TNF-α, MCP-1, TGF-β1, Smad7, collagen I, α-SMA, and GAPDH as

described previously,[12–14] whereas the primers for AT1 and *Ace2* were described below: AT1: forward 5′-TGACTTT GGCCACCAGCAT-3′, reverse 5′-CCATTGTCCACCCGAT GAA-3′; *Ace2*: forward 5′-ACCCTTCTTACATCAGCCCT ACTG -3′, reverse 5′-TGTCCAAAACCTACCCCACATAT-3′. The reaction specificity was confirmed by melting curve analysis. The housekeeping gene *GAPDH* was used as an internal standard and the ratio of the mRNA examined to the GAPDH was calculated.

Statistical Analyses

Data obtained from this study were expressed as the mean ± s.e.m. Statistical analyses were performed using one-way ANOVA, followed by Newman–Keuls Post Test from Prism 5.0 GraphPad Software (San Diego, CA, USA).

RESULTS

$Ace2^{-/y}$ Mice Promote Renal Fibrosis and Inflammation Independent of Blood Pressure in the UUO Nephropathy

Western blot analysis showed that *Ace2* protein was not detectable in $Ace2^{-/y}$ mice, but was increased in the diseased kidney of $Ace2^{+/y}$ mice (Figure 1a and c). Both $Ace2^{+/y}$ and $Ace2^{-/y}$ mice had similar levels of blood pressure under sham-control (126 ± 0.5 mm Hg in $Ace2^{+/y}$ mice *vs* 122 ± 5.8 mm Hg in $Ace2^{-/y}$ mice) or UUO disease conditions over 7 days (125.8 ± 2 mm Hg in $Ace2^{+/y}$ mice *vs* 121 ± 5.5 mm Hg in $Ace2^{-/y}$ mice). Histologically, Masson trichrome staining showed no detectable abnormalities in normal or sham-operation $Ace2^{-/y}$ mice compared with $Ace2^{+/y}$ mice (Figure 1b and d). However, 3 days after UUO, $Ace2^{-/y}$ mice developed more intensive tubulointerstitial damage, including tubular atrophy, interstitial extracellular matrix accumulation, which became much more profound at day 7 after UUO (Figure 1b and d). Immunohistochemically, compared to the $Ace2^{+/y}$ mice, tubulointerstitial fibrosis was enhanced in $Ace2^{-/y}$ mice as demonstrated by more abundant collagen I (Figure 1e) and α-SMA (Figure 1i) accumulation in the fibrotic area of tubulointerstitium. Quantitative real-time PCR and western blot analyses further confirmed these findings that disrupted *Ace2* largely enhanced collagen I and α-SMA mRNA and protein expression in the UUO kidney when compared with $Ace2^{+/y}$ mice (Figure 1f–h and j–l).

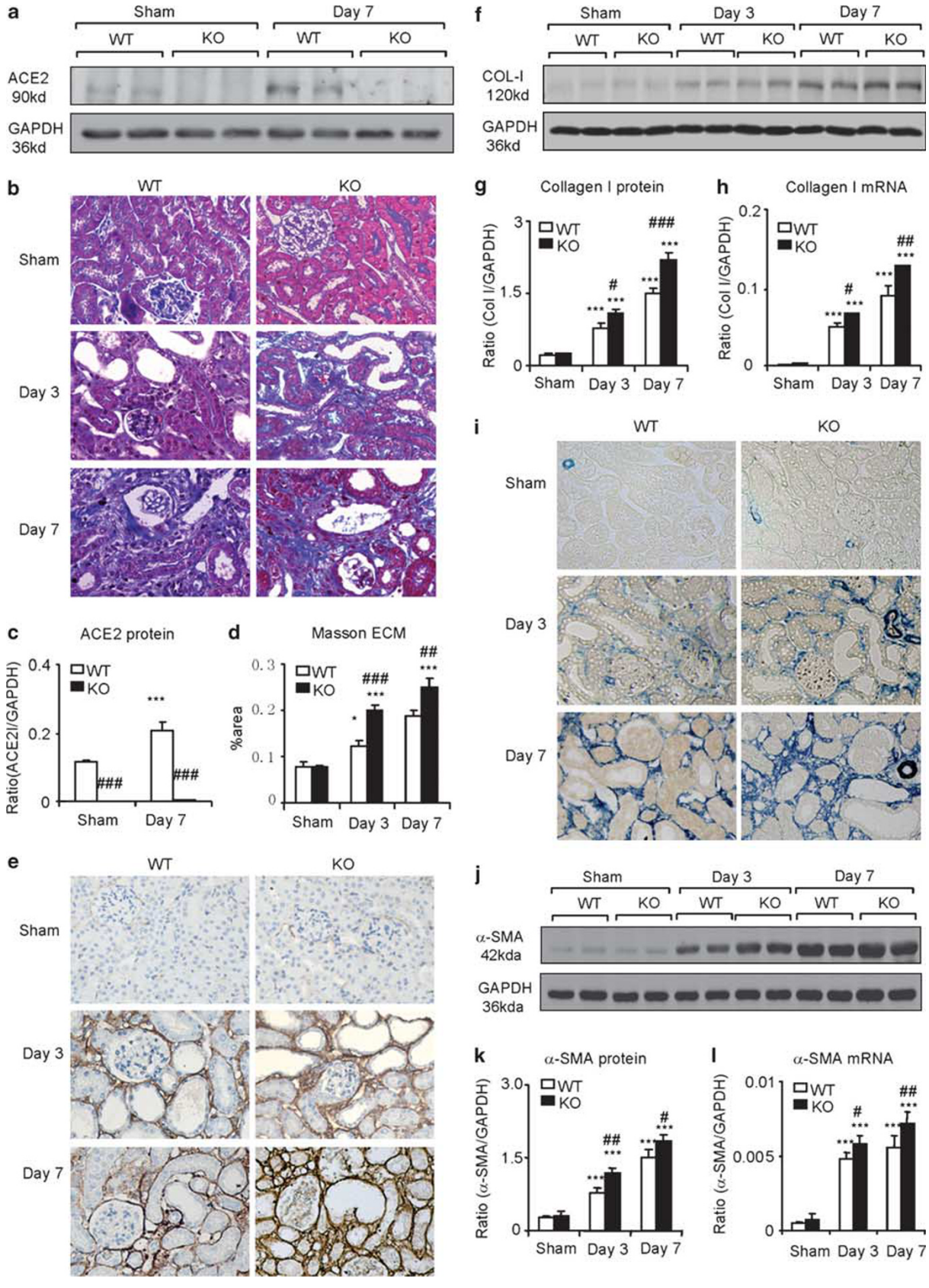
As inflammation is a critical process in the development of UUO, we next examined whether disruption of *Ace2* gene influences renal inflammation in the UUO kidney. As shown in Figure 2, immunohistochemistry and real-time PCR revealed that compared with $Ace2^{+/y}$ mice, mice deficient for *Ace2* exhibited a substantial increase in renal inflammation as demonstrated by a remarkable upregulation of pro-inflammatory cytokines (TNF-α, IL-1β) and chemokine MCP-1. Enhanced expression of renal TNF-α, IL-1β, and MCP-1 in $Ace2^{-/y}$ mice was accompanied by a significant increase in $CD3^+$ T cells and $F4/80^+$ cells infiltrating the tubulointerstitium (Figure 3).

Enhanced Ang II, TGF-β/Smad, and NF-κB Signaling Pathways Are Key Mechanisms by which $Ace2^{-/y}$ Mice Promote Renal Fibrosis and Inflammation in the UUO Nephropathy

It has been shown that Ang II is capable of activating the Smads to mediate fibrosis via both AT1-MAP kinase-crosstalk and TGF-β1-dependent pathways.[15–17] We thus investigated whether enhanced renal fibrosis in the UUO kidney of $Ace2^{-/y}$ mice is associated with an increase in Ang II-mediated TGF-β/Smad pathway. As shown in Figure 4a and b, significant higher levels of intrarenal Ang II and Ang 1–7 were detected in $Ace2^{+/y}$ mice after UUO. Deletion of *Ace2* resulted in doubling the intrarenal Ang II in the UUO kidney, while levels of intrarenal Ang 1–7 remained low (Figure 4a and b). Interestingly, the imbalance between Ang II generation and degradation occurred only locally in the UUO kidney, as plasma levels of Ang II and Ang 1–7 remained normal in $Ace2^{+/y}$ and $Ace2^{-/y}$ mice throughout the 7-day disease course (Figure 4c and d). Western blot and real-time PCR analyses detected that increased intrarenal Ang II in $Ace2^{-/y}$ mice was associated with a marked activation of the intrarenal Ang II signaling pathway, including upregulation of AT1 and activation of ERK1/2 mitogen-activated protein kinase (Figure 4e–h). Further study revealed that enhanced Ang II signaling in the UUO kidney of $Ace2^{-/y}$ mice was accompanied by a marked increase in renal TGF-β1 expression (Figure 5a, c and d) and higher levels of Smad2/3 phosphorylation and phospho-Smad2/3 nuclear translocation when compared with $Ace2^{+/y}$ mice (Figure 5b and e–g).

We then determined the mechanism by which deletion of *Ace2* promotes renal inflammation in the UUO kidney by

Figure 1 Mice lacking *Ace2* (KO) are promoted histological damage and renal fibrosis in the UUO nephropathy. (**a**) Western blot analysis of *Ace2* expression in the kidney. (**b**) Masson's trichrome staining. (**c**) Semi-quantitative analysis of *Ace2* protein expression detected by western blotting. (**d**) Semi-quantitative analysis of matrix protein accumulation detected by Masson's trichrome staining. (**e**) Immunohistochemical staining of collagen I. (**f**) Renal collagen I expression detected by western blot. (**g**) Semi-quantitative analysis of collagen I detected by western blotting. (**h**) Collagen I mRNA expression detected by real-time PCR. (**i**) Immunohistochemical staining of α-SMA expression. (**j**) α-SMA expression detected by western blot. (**k**) Semi-quantitative analysis of α-SMA protein expression by western blotting. (**l**) α-SMA mRNA expression detected by real-time PCR. Each bar represents mean ± s.e.m. for at least six mice. $^*P<0.05$, $^{***}P<0.001$ compared with sham-operation mice. $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ when compared with time-matched $Ace2^{+/y}$ (WT) mice with UUO. Magnification × 200.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examining the NF-κB signaling pathway. As shown in Figure 6, western blot analysis revealed that compared to *Ace2*$^{+/y}$ mice, enhanced activation of the NF-κB pathway such as an increase in the levels of phospho-IκBα and phospho-NF-κB/p65 was accompanied by degradation of IκBα and NF-κB/p65 in the UUO kidney of *Ace2*$^{-/y}$ mice (Figure 6).

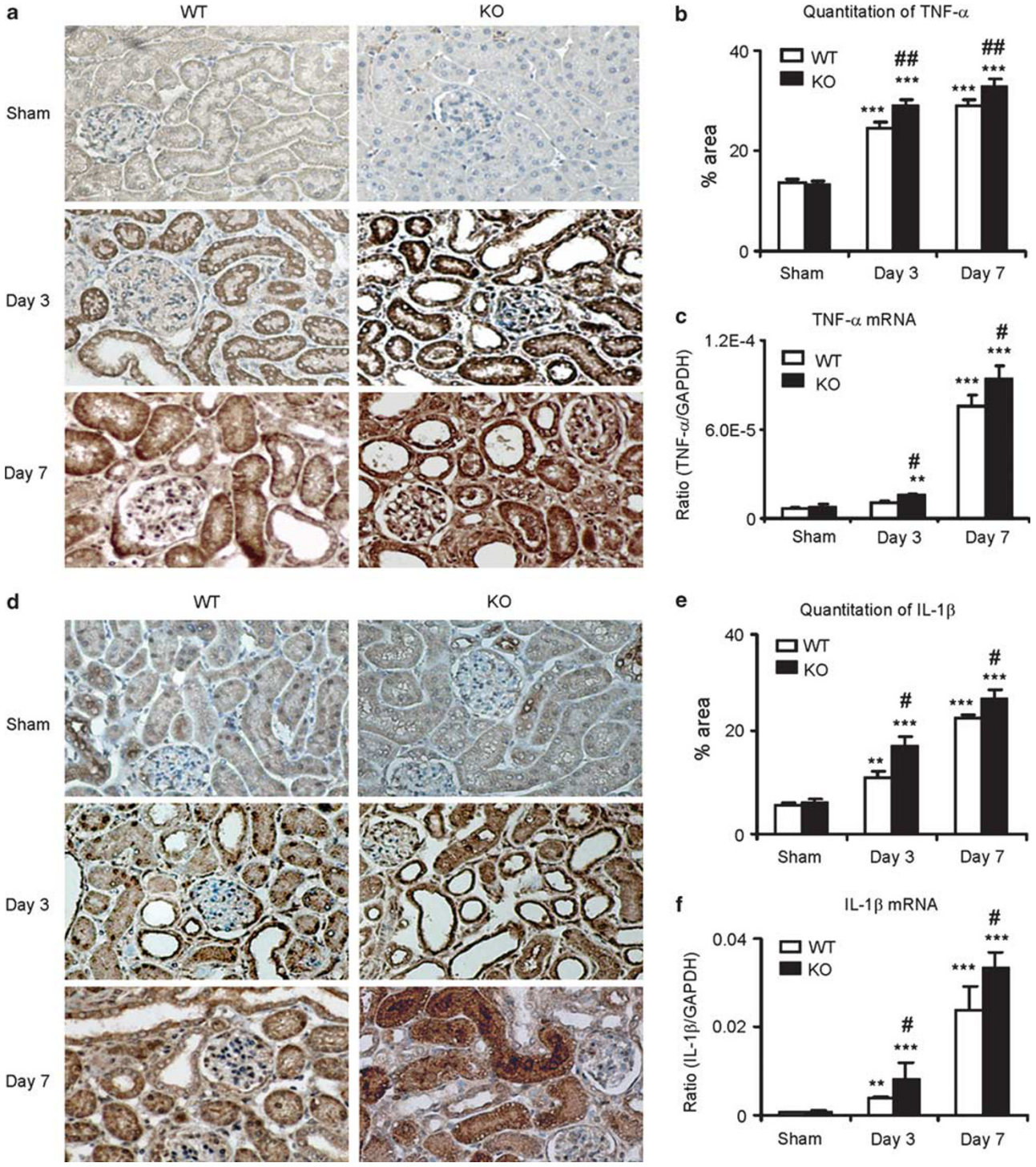

Figure 2 Mice lacking *Ace2* (KO) are promoted renal inflammation in the UUO nephropathy. (**a**) Immunohistochemical staining of TNF-α expression. (**b**) Semi-quantitative analysis of TNF-α immunostaining. (**c**) TNF-α mRNA expression detected by real-time PCR. (**d**) Immunohistochemical staining of IL-1β. (**e**) Semi-quantitative analysis of IL-1β immunostaining. (**f**) IL-1β mRNA expression detected by real-time PCR. (**g**) Immunohistochemical staining of MCP-1 expression; (**h**) Semi-quantitative analysis of MCP-1 immunostaining. (**i**) MCP-1 mRNA expression detected by real-time PCR. Each bar represents mean ± s.e.m. for at least six mice. $^{**}P<0.01$, $^{***}P<0.001$ compared with sham-operation mice. $^{\#}P<0.05$, $^{\#\#}P<0.01$, when compared with time-matched *Ace2*$^{+/y}$ (WT) mice with UUO. Magnification × 200.

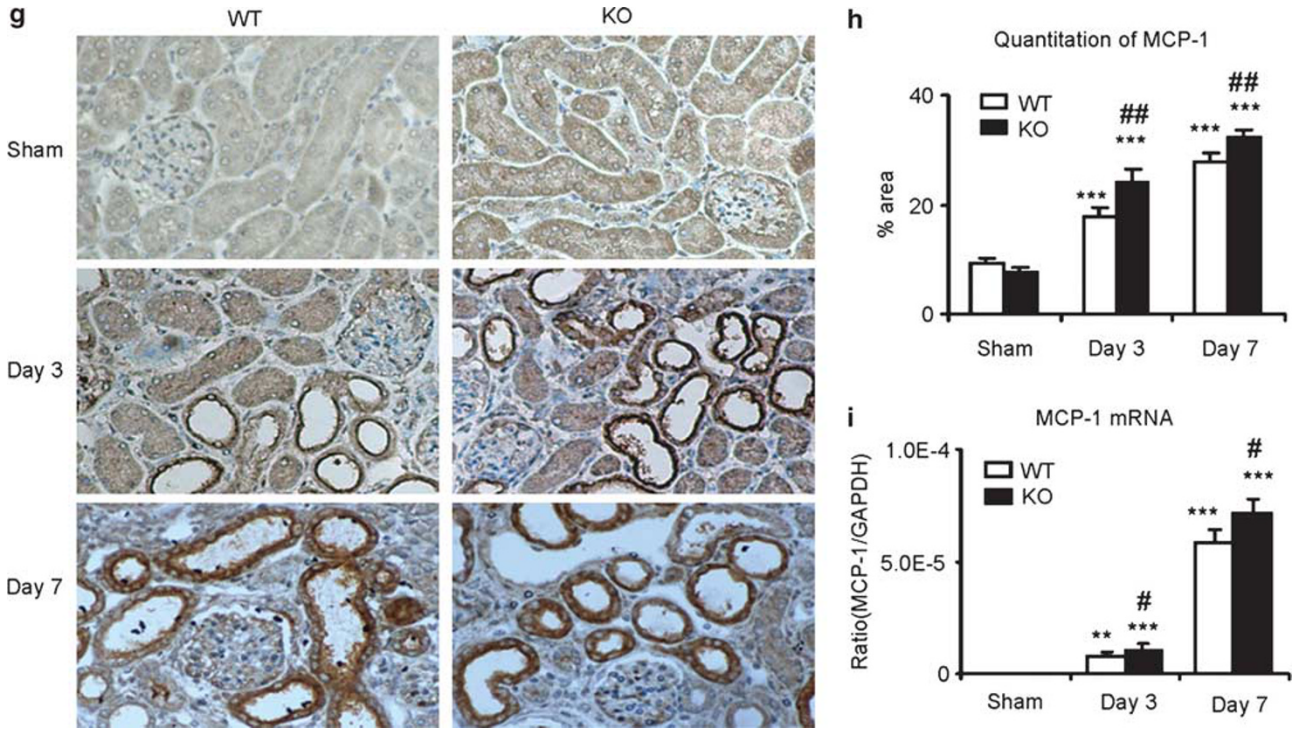

Figure 2 Continued.

Loss of Renal Smad7 Is an Underlying Mechanism Required for the Promotion of TGF-β/Smad-Mediated Renal Fibrosis and NF-κB-Driven Renal Inflammation in the UUO Nephropathy in Ace2$^{-/y}$ Mice

We next examined whether enhanced TGF-β/Smad and NF-κB signaling in *Ace2*$^{-/y}$ mice is associated with a loss of renal Smad7 via the Smurf2-ubiquitin degradation mechanism. As shown in Figure 7, western blot analysis detected that Smurf2 expression was significantly increased in the UUO kidney of *Ace2*$^{+/y}$ mice, which was further increased in the *Ace2*$^{-/y}$ mice (Figure 7a and b). Importantly, an increase in Smurf2 expression in the UUO kidney was associated with a reduction of renal Smad7 protein in *Ace2*$^{+/y}$ mice, which became more profound in mice lacking *Ace2* (Figure 7a and c). Interestingly, in contrast to Smad7 protein, real-time PCR detected that Smad7 mRNA expression was significantly increased in the UUO kidney of *Ace2*$^{+/y}$ mice, which became higher in *Ace2*$^{-/y}$ mice (Figure 7d). An increase in Smad7 mRNA but loss of Smad7 protein along with upregulation of Smurf2 suggests that the Smurf2-dependent ubiquitin degradation of renal Smad7 occurs within the UUO kidney.

DISCUSSION

The present study provided the evidence that loss of *Ace2* enhanced renal fibrosis and inflammation in a mouse model of UUO nephropathy. Results from this study also delineated a critical role of Ace2 in negatively regulating endogenous Ang II-mediated progressive renal injury independent of systemic hypertension. Thus, Ace2 is an essential regulator in maintaining the balance between Ang II generation and Ang II degradation locally within the kidney regardless of systemic disease conditions. Furthermore, the present study also found that enhanced TGF-β/Smad and NF-κB signaling pathways were mechanisms by which *Ace2*$^{-/y}$ mice promote renal fibrosis and inflammation, which was attributed to a loss of renal Smad7 mediated by a mechanism of Ang II-induced Smurf2-dependent ubiquitin-degradation pathway.

Many studies have shown that the expression of Ace2 within the kidney varies with disease conditions. In hypertensive nephropathy, we found that renal ACE2 is largely reduced along with a marked upregulation of ACE and AT1 receptor in patients with hypertension.[2] This is consistent with the findings in animal models of hypertension,[18,19] although renal ACE2 expression and activity is unaltered during established hypertension in adult SHRSP and TGR(mREN2)27 rats.[20] Similarly, in diabetic nephropathy, while upregulation of *Ace2* is noted in the diabetic kidney in mice[8,10,21,22] and in humans,[23] downregulation of *Aces* is also reported.[24–26] The functional importance of Ace2 is now established by recent findings that loss of *Ace2* promotes but administration of recombinant human ACE2 inhibits exogenous Ang II-induced hypertensive nephropathy and streptozotocin-induced diabetic nephropathy.[8–11,27] All these findings imply that *Ace2* may protect against progressive renal injury in diseases associated with hypertension and diabetes. In the present study, we found that disruption of *Ace2* largely enhanced intrarenal Ang II levels (a twofold

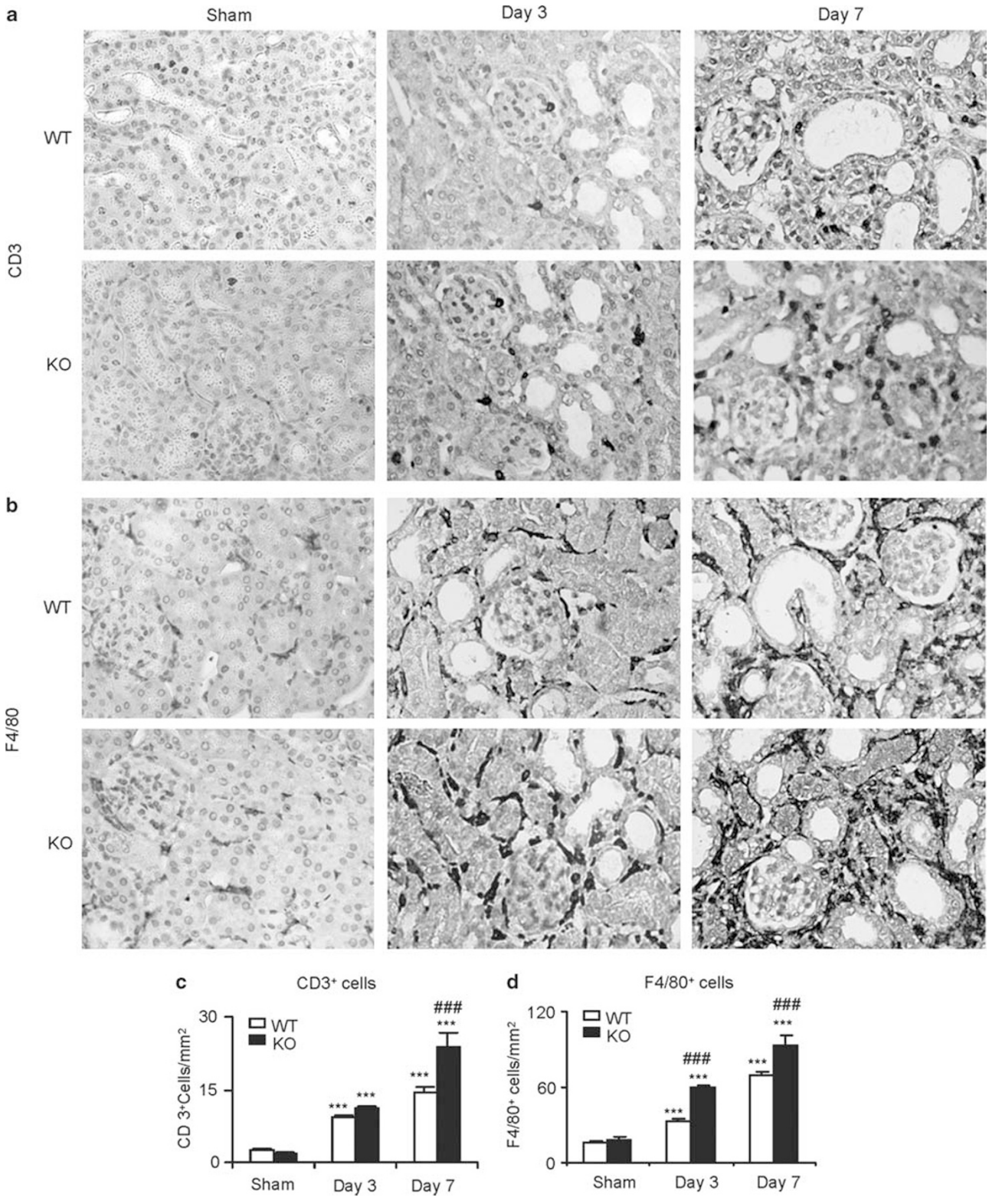

Figure 3 Mice lacking *Ace2* (KO) are promoted CD3$^+$ T-cell and F4/80$^+$ cell infiltration in the UUO nephropathy. (**a**) Immunohistochemical staining of CD3$^+$ T cells. (**b**) Immunohistochemical staining of F4/80$^+$ cell. (**c**) Quantitative analysis of CD3$^+$ T cells. (**d**) Immunohistochemical staining of F4/80$^+$ cell. CD3$^+$ T-cell and F4/80$^+$ cell infiltration in the tubulointerstitium are significantly increased in *Ace2* $^{-/y}$ (KO) mice at day 3 after UUO, which becomes maximal at day 7. Each bar represents mean ± s.e.m. for at least six mice. $^{***}P<0.001$ compared with sham-operation mice. $^{###}P<0.001$ when compared with time-matched *Ace2* $^{+/y}$ (WT) mice with UUO. Magnification × 200.

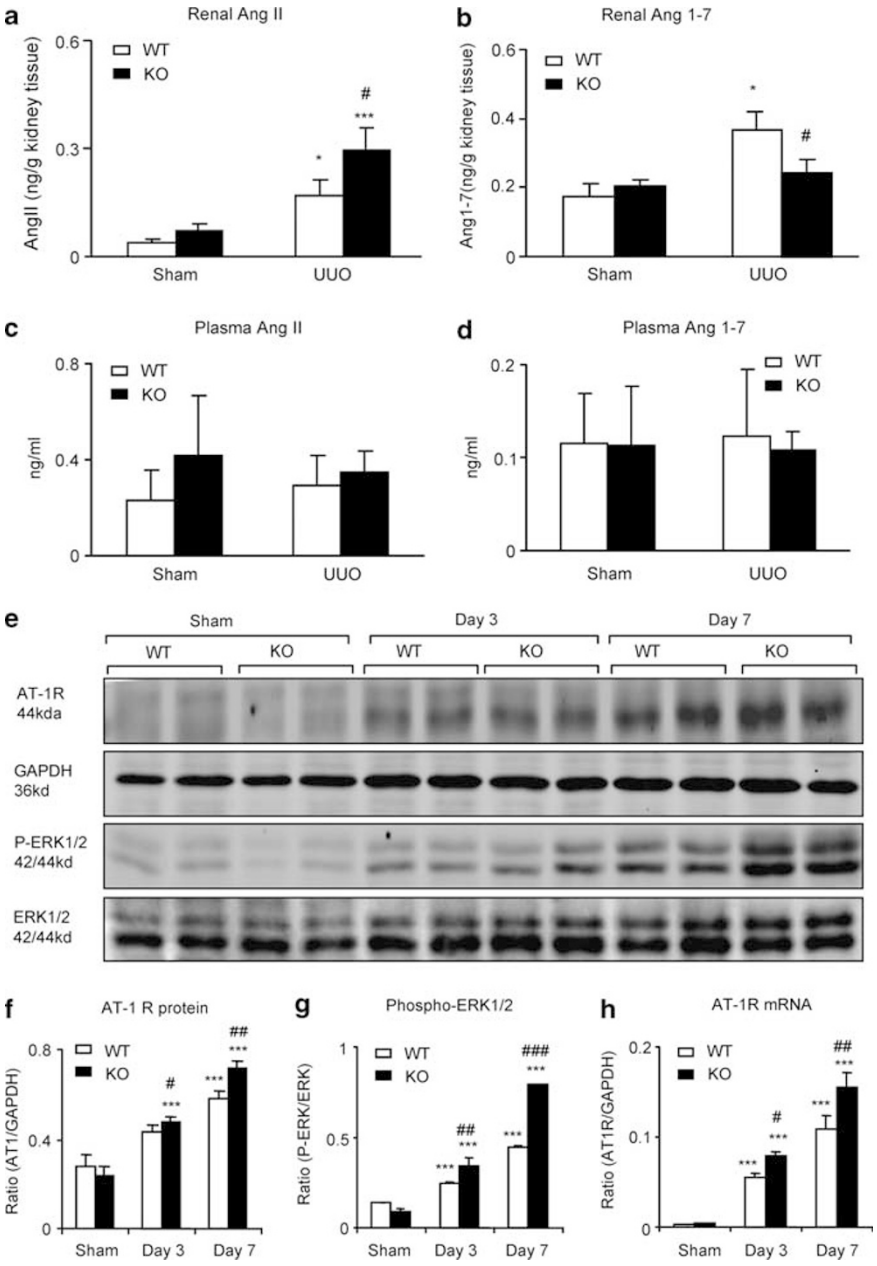

Figure 4 Mice lacking *Ace2* (KO) are promoted intrarenal Ang II and Ang II signaling in the UUO nephropathy. (**a**, **b**) Intrarenal Ang II and Ang 1–7 concentrations measured by ELISA. (**c**, **d**) Plasma levels of Ang II and Ang 1–7 measured by ELISA. (**e**) Western blot analysis of AT1 and phospho-ERK1/2. (**f**, **g**) Semi-quantitative analysis of AT1 protein and phospho-ERK1/2 protein by western blots. (**h**) AT1 mRNA expression detected by real-time PCR. Results show that deletion of *Ace2* largely enhances intrarenal, but not circulating, Ang II concentrations, which results in the enhancement of AT1-dependent intrarenal Ang II signaling. Each bar represents mean ± s.e.m. for at least six mice. $^{*}P<0.05$, $^{***}P<0.001$ compared with sham-operation mice. $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ when compared with time-matched $Ace2^{+/y}$ (WT) mice with UUO.

increase), which was accompanied by much more severe renal fibrosis and inflammation, demonstrating a renoprotective role for *Ace2* in obstructive nephropathy.

The finding of *Ace2* as a critical regulator in maintaining the balance between endogenous Ang II generation and degradation locally in a mouse model of UUO nephropathy is clinically relevant. It implies that Ace2 not only functions to systemically regulate hypertensive renal injury but also acts as a local regulator for renal protection against the endogenous Ang II-mediated injury independent of hypertension. Indeed, the present study revealed that the local, but not systemic, Ang II generation and degradation was critically regulated by *Ace2* as evidenced by the finding that disruption of *Ace2* impaired the ACE2/Ang 1–7 axis but enhanced largely the ACE/Ang II/AT1-dependent Ang II generation in $Ace2^{-/y}$ mice (Ang II *vs* Ang 1–7 = 4:1). This finding was consistent with the

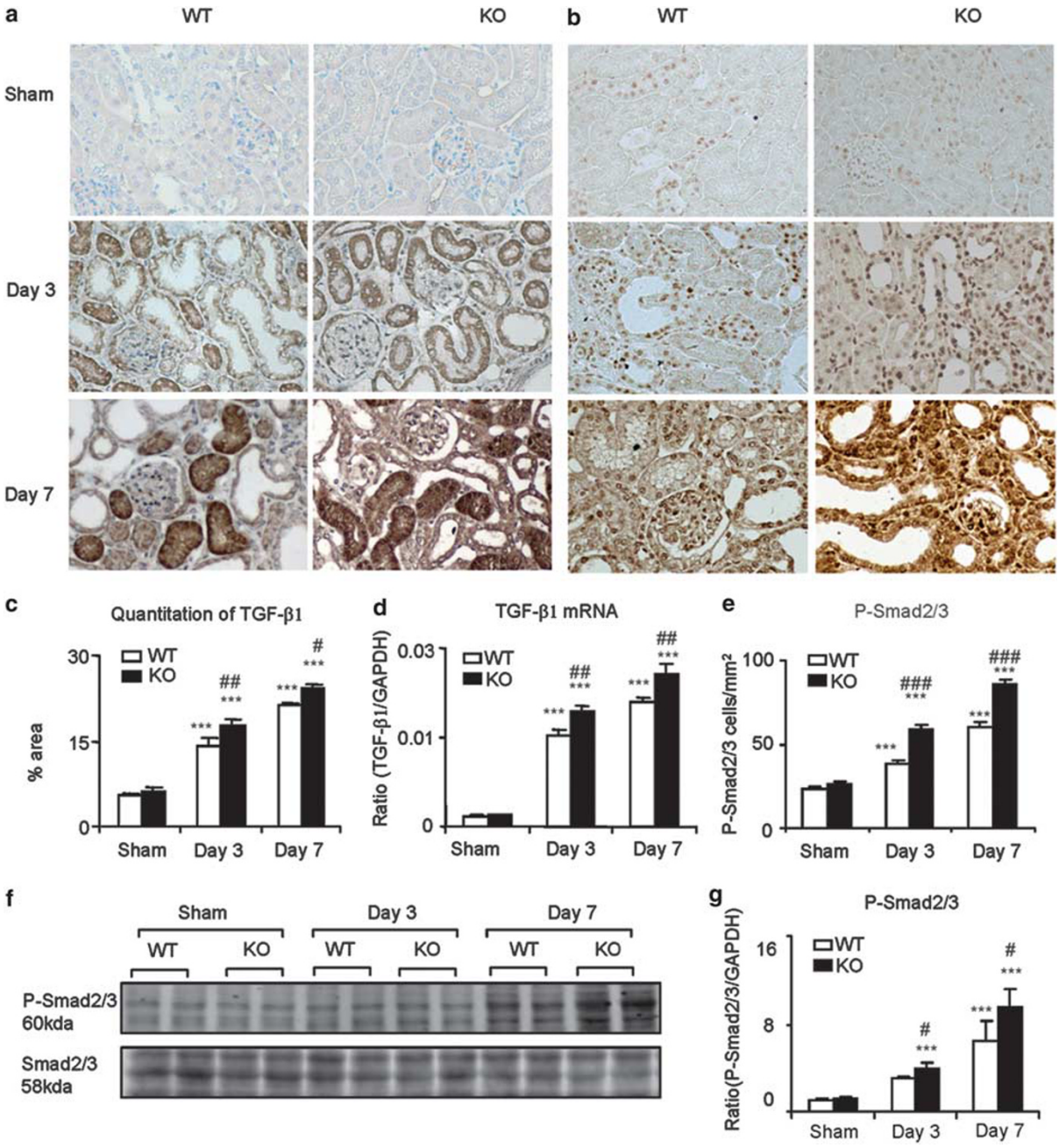

Figure 5 Disrupted *Ace2* enhances TGF-β/Smad signaling in the UUO nephropathy. (**a**) Immunohistochemical staining of TGF-β1 expression. (**b**) Immunohistochemical staining of phospho-Smad2/3 nuclear translocation. (**c**) Semi-quantitative analysis of TGF-β1 immunostaining. (**d**) TGF-β1 mRNA expression detected by real-time PCR. (**e**) Semi-quantitative analysis of phospho-Smad2/3 nuclear translocation. (**f**) Phosphorylation of Smad2/3 (P-Smad2/3) detected by western blots. (**g**) Semi-quantitative analysis of phosphorylation of Smad2/3 (P-Smad2/3) by western blotting. Results show that, compared to *Ace2*$^{+/y}$ (WT) mice, disrupted *Ace2* (KO) largely enhances TGF-β1 mRNA expression and protein, resulting in a further increase in Smad2/3 signaling as demonstrated by its nuclear translocation and phosphorylation. Each bar represents mean ± s.e.m. for at least six mice. $^{***}P<0.001$ compared with sham-operation mice. $^{\#}P<0.05$, $^{\#\#}P<0.01$ when compared with time-matched *Ace2*$^{+/y}$ (WT) mice with UUO. Magnification × 200.

observation in both animals and patients with diabetes or IgA nephropathy.[21–26,28] Thus, Ace2 is an important counter-regulator for systemic hypertension and locally in determining the disease progression by balancing the intrarenal Ang II generation and degradation pathways.

Although ACE2 is a critical enzyme to degrade Ang I and Ang II to Ang 1–7, a number of alternative enzymes, including neprilysin, prolyl-endopeptidase, and thimet oligopeptidase, as well as ACE, are also capable of converting the Ang I and Ang II to Ang 1–7.[4,29,30] Thus, the regulation

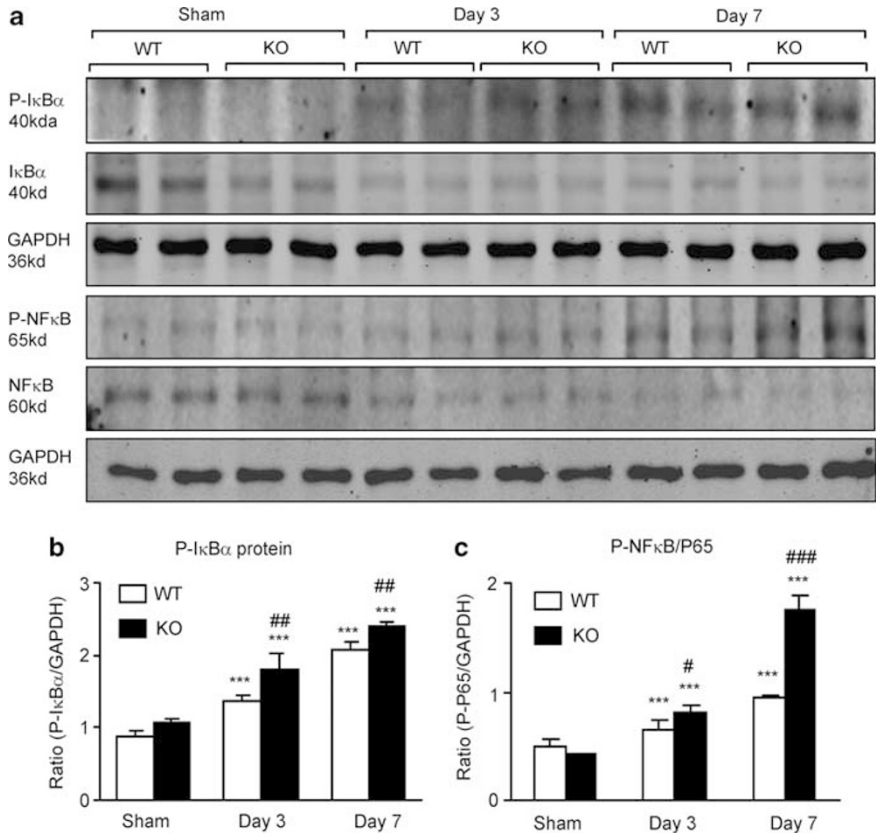

Figure 6 Disrupted *Ace2* enhances NF-κB signaling in the UUO nephropathy. (**a**) Representative western blots. (**b**) Semi-quantitative analysis of phospho-IκBα. (**c**) Semi-quantitative analysis of phospho-NF-κB/p65. Results show that compared to *Ace2*$^{+/y}$ (WT) mice, deletion of *Ace2* (KO) largely enhances phosphorylation of both IκBα and NF-κB/p65. Each bar represents mean ± s.e.m. for at least six mice. $^{***}P<0.001$ compared with sham-operation mice. $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ when compared with time-matched *Ace2*$^{+/y}$ (WT) mice with UUO.

of Ang 1–7 generation is under control by the multiple enzymes systems. It is possible that these Ang 1–7-generating enzymes may compensate each other to maintain the steady-state levels of Ang II and Ang 1–7 both locally and systemically, despite Ace2 as a major pathway for Ang 1–7 generation. This compensatory mechanism may explain our finding that the *Ace2*$^{-/y}$ and *Ace*$^{+/y}$ mice have similar levels of plasma Ang II and Ang 1–7 in both normal and the UUO conditions. It could be also true that because obstructive nephropathy was induced in the left kidney only, this leaves the opposite right kidney normal and enables to overcome the functional loss from the left nephropathy. In contrast, the balance between Ang II and Ang 1–7 was altered locally within the UUO kidney, which was much more profound in *Ace2*$^{-/y}$ mice, resulting in a fourfold increase in the ratio of intrarenal Ang II/Ang 1–7. This finding suggested that the compensatory mechanism locally in the UUO kidney was lost when the *Ace2* pathway is disrupted. Therefore, the local *vs* systemically regulatory networking for both Ang II and Ang 1–7-generating pathways within the body may be attributed to the discrepancy in the levels of Ang II and Ang 1–7 between the plasma and the intrarenal tissue, although the precise mechanisms remain unknown.

A significant finding in the present study was the identification that enhanced TGF-β/Smad-mediated renal fibrosis and NF-κB-driven renal inflammation were key mechanisms by which loss of *Ace*2 enhanced progressive renal injury in a mouse model of UUO nephropathy. We and other investigators have previously demonstrated that Ang II is able to activate the TGF-β/Smad signaling via both TGF-β-dependent and -independent (through the ERK/p38 mitogen-activated protein kinase crosstalk pathway) mechanisms in vascular and renal cells.[15–17] In the present study, we also found that loss of *Ace*2 largely increased intrarenal Ang II signaling via the AT1-mediated activation of ERK1/2 mitogen-activated protein kinase pathway. This was associated with enhanced TGF-β/Smad signaling (Smad2/3 phosphorylation and nuclear translocation) and progressive tubulointerstitial fibrosis in *Ace2*$^{-/y}$ mice when compared with *Ace2*$^{+/y}$ mice. Furthermore, in the context of renal inflammation, we also found that deletion of *Ace*2 promoted Ang II-stimulated NF-κB-dependent renal inflammation such as upregulation of IL-1β, TNF-α, MCP-1, and increased

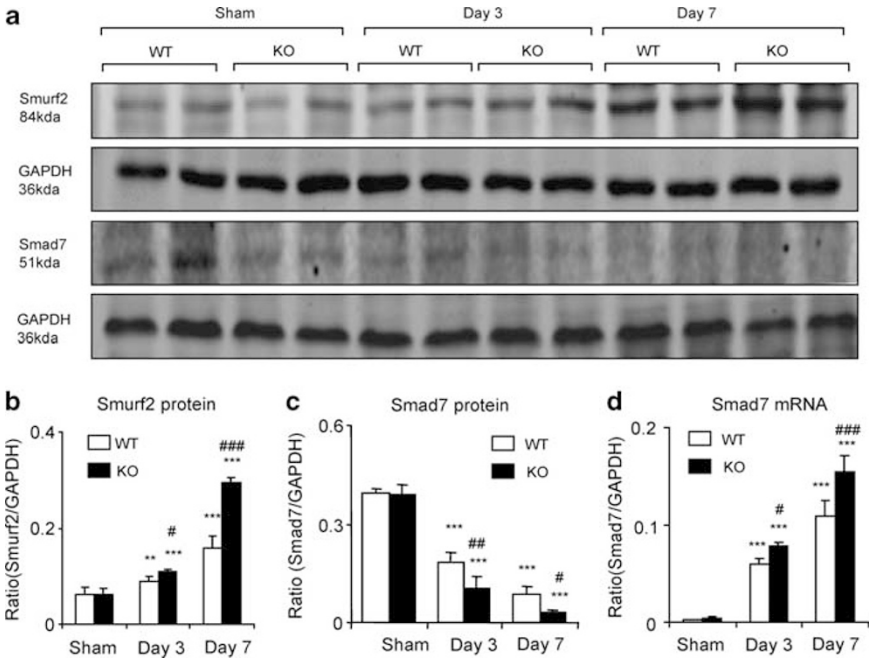

Figure 7 Disrupted *Ace2* enhances Smurf2-mediated degradation of renal Smad7 protein in the UUO nephropathy. (**a**) Representative western blots for renal Smurf2 and Smad7 expression. (**b**) Semi-quantitative analysis of Smurf2 protein. (**c**) Semi-quantitative analysis of Smad7 protein. (**d**) Real-time PCR detection of renal Smad7 mRNA. Results show that compared to $Ace2^{+/y}$ (WT), disrupted *Ace2* (KO) largely enhances Smurf2 expression, which results in renal Smad7 protein degradation. Note that renal Smad7 mRNA is upregulated in the UUO nephropathy in both $Ace2^{+/y}$ and $Ace2^{-/y}$ mice. Each bar represents mean ± s.e.m. for at least six mice. $^{**}P<0.01$, $^{***}P<0.001$ compared with sham-operation mice. $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ when compared with time-matched $Ace2^{+/y}$ (WT) mice with UUO.

macrophage and T-cell infiltration in the UUO nephropathy. This was consistent with the known role of Ang II to activate NF-κB-mediated renal inflammation.[31,32] It is also possible that the development of intrarenal hypoxia within the UUO kidney may also be attributed to the development of renal inflammation.[33] Prevention of Ang II-mediated renal oxidative stress, inflammation, and fibrosis by ACE2 supports the notion that loss of *Ace2* promoted renal inflammation via the oxidative stress mechanism.[9]

More importantly, we also found that increased Ang II-induced Smurf2-dependent ubiquitin degradation of renal Smad7 may be the underlying mechanism required for promotion of TGF-β/Smad-mediated renal fibrosis and NF-κB-driven renal inflammation in the UUO nephropathy in $Ace2^{-/y}$ mice. We have previously shown that Smad7 is an integrated regulator that negatively regulates TGF-β/Smad-mediated renal fibrosis via its negative feedback-loop and NF-κB-dependent renal inflammation by the induction of IκBα, an inhibitor of NF-κB.[34] Indeed, Smad7 acts as an adaptor protein that binds and recruits Smurf2, an E3 ubiquitin ligase, to the TGF-β1 receptor complex to promote its degradation through the proteasomal pathway.[35] At the same time, ubiquitin-degradation of Smad7 occurs simultaneously.[35] We have recently shown that Ang II can induce Smurf2 to degrade renal Smad7 in tubular epithelial cells *in vitro*.[36] Once Smad7 is degraded, activation of Smad2/3 and renal fibrosis are enhanced. This is clearly demonstrated by the findings that Ang II activates Smad3 to induce epithelial-myofibroblast transition and renal fibrosis via Smurf2-dependent ubiquitin degradation of Smad7,[36] and that upregulation of renal Smurf2 degrades renal Smad7 to promote TGF-β/Smad-dependent renal fibrosis in the UUO nephropathy.[37] In addition, we also found that Smad7 is a negative regulator of NF-κB signaling.[34] Overexpression of Smad7 is capable of inducing IκBα expression and preventing IκBα from phosphorylation and activation of NF-κB signaling, thereby inhibiting renal inflammation *in vivo* and *in vitro*.[38,39] In contrast, deletion of Smad7 promotes NF-κB-dependent renal inflammation in a mouse model of UUO.[12] Taken together, loss of Smad7 promotes, while overexpression of Smad7 inhibits, TGF-β/Smad-mediated progressive renal fibrosis and NF-κB-driven renal inflammation as evidenced in a number of kidney disease models including UUO nephropathy,[12,37,40] hypertension-associated remnant kidney disease,[38,41] diabetic nephropathy,[14] and immunologically-mediated glomerulonephritis.[42] Therefore, loss of *Ace*2 promoted Ang II-induced Smurf2-dependent ubiquitin degradation of renal Smad7 may be another essential mechanism by which $Ace2^{-/y}$ mice were promoted TGF-β/Smad-mediated renal fibrosis and NF-κB-driven renal inflammation in a mouse model of UUO nephropathy.

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Author contributions. ZL conceived all the experiments, analyzed data and drafted the manuscript. XRH and HYC conceived the generation of *Ace2*$^{+/y}$ and *Ace2*$^{-/y}$ mice, data analysis, and manuscript editing. JMP provided *Ace2*$^{-/y}$ mice and manuscript editing. HYL was responsible for the experimental design, data interpretation, and writing up manuscript for publication.

DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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