Murine recombinant angiotensin-converting enzyme 2 attenuates kidney injury in experimental Alport syndrome

Eun Hui Bae[1,2,7], Fei Fang[1,7], Vanessa R. Williams[1,7], Ana Konvalinka[1,3], Xiaohua Zhou[1], Vaibhav B. Patel[4], Xuewen Song[5], Rohan John[6], Gavin Y. Oudit[4], York Pei[5,8] and James W. Scholey[1,3,8]

[1]*Department of Medicine and Institute of Medical Science, University of Toronto, Toronto, Canada;* [2]*Department of Internal Medicine, Chonnam National University Medical School, Gwangju, Korea;* [3]*Division of Nephrology, Department of Medicine, University Health Network and University of Toronto, Toronto, Canada;* [4]*Division of Cardiology, Department of Medicine, University of Alberta, Edmonton, Canada;* [5]*Divisions of Nephrology and Genomic Medicine, University Health Network and University of Toronto, Toronto, Canada; and* [6]*Department of Pathology, University Health Network and University of Toronto, Toronto, Canada*

Angiotensin-converting enzyme 2 (ACE2) is a monocarboxypeptidase in the renin-angiotensin system that catalyzes the breakdown of angiotensin II to angiotensin 1-7. We have reported that ACE2 expression in the kidney is reduced in experimental Alport syndrome but the impact of this finding on disease progression has not been studied. Accordingly, we evaluated effects of murine recombinant ACE2 treatment in *Col4a3* knockout mice, a model of Alport syndrome characterized by proteinuria and progressive renal injury. Murine recombinant ACE2 (0.5 mg/kg/day) was administered from four to seven weeks of age via osmotic mini-pump. Pathological changes were attenuated by murine recombinant ACE2 treatment which ameliorated kidney fibrosis as shown by decreased expression of COL1α1 mRNA, less accumulation of extracellular matrix proteins, and inhibition of transforming growth factor-β signaling. Further, increases in proinflammatory cytokine expression, macrophage infiltration, inflammatory signaling pathway activation, and heme oxygenase-1 levels in *Col4a3* knockout mice were also reduced by murine recombinant ACE2 treatment. Lastly, murine recombinant ACE2 influenced the turnover of renal ACE2, as it suppressed the expression of tumor necrosis factor-α converting enzyme, a negative regulator of ACE2. Thus, treatment with exogenous ACE2 alters angiotensin peptide metabolism in the kidneys of *Col4a3* knockout mice and attenuates the progression of Alport syndrome nephropathy.

Kidney International (2017) ■, ■–■; http://dx.doi.org/10.1016/j.kint.2016.12.022

KEYWORDS: Alport syndrome; angiotensin-converting enzyme 2; renal fibrosis; renin-angiotensin system; TNFα-converting enzyme

Correspondence: *Vanessa Williams, University of Toronto, 1 King's College Circle, Room 7326, Toronto, Ontario M5S 1A8, Canada. E-mail: vanessa.williams@mail.utoronto.ca*

[7]These authors contributed equally to this manuscript.

[8]These authors contributed equally to this manuscript as senior authors.

Received 15 April 2016; revised 20 December 2016; accepted 22 December 2016

Kidney fibrosis, associated with renal failure, is known to be the common final stage of progressive renal disease. Alport syndrome (AS) is a hereditary nephropathy characterized by progressive kidney fibrosis.[1–3] Studies in Alport mice suggest that angiotensin inhibition not only has antiproteinuric effects but also suppresses cytokine and collagen production as well as tubulointerstitial fibrosis and inflammation.[4] Inhibitors of the renin-angiotensin system (RAS), including angiotensin-converting enzyme inhibitors and angiotensin II receptor blockers, have been demonstrated to slow kidney disease progression in both experimental and clinical AS.[3,5–8]

Recently we have demonstrated that the RAS activation–associated increase in angiotensin II (Ang II) and decrease in angiotensin-(1–7) (Ang-[1–7]) were caused by decreased intrarenal angiotensin-converting enzyme 2 (ACE2) expression and activity and may play an important pathogenic role in AS.[9] Ang II has been shown to trigger cleavage of ACE2 by tumor necrosis factor-α (TNFα)–converting enzyme (TACE) and its shedding as a soluble form from the membrane in the myocardium.[10] In the AS model, the mechanism of decreased ACE2 expression has not been established, and TACE may play a role in this process.

Loss of ACE2 is associated with age-dependent development of glomerulosclerosis and albuminuria,[11] and exacerbation of diabetic kidney injury in mice.[12] We have recently reported that mice with experimental AS exhibit a loss of ACE2 in the brush border of proximal tubules in association with a rise in intrarenal Ang II and a decrease in Ang-(1–7) levels. We also observed that administration of recombinant ACE2 attenuated these changes in peptide levels.[9] Taken together, these studies suggest that treatment with ACE2 may limit kidney injury in AS. Thus, we treated *Col4a3*$^{-/-}$ mice with murine recombinant ACE2 (mrACE2) after weaning from 4 to 7 weeks of age, before mortality became a significant confounding factor, and studied the effects of mrACE2

on the development of kidney fibrosis, inflammation, and oxidative stress.

RESULTS

Treatment with mrACE2 attenuated morphological changes in experimental AS

At 7 weeks of age, *Col4a3*$^{-/-}$ mice had lower body weights but higher kidney-to-body weight ratios and 24-hour urinary outputs than wild-type mice (Table 1). These parameters were not significantly different between mrACE2- and saline-treated *Col4a3*$^{-/-}$ mice. Plasma ACE2 protein levels decreased in *Col4a3*$^{-/-}$ mice compared with wild-type mice; however, mrACE2 administration increased plasma ACE2 protein (Supplementary Table S1). In a separate set of experiments performed in wild-type mice, we found that treatment with mrACE2 increased plasma ACE2 activity (Supplementary Figure S1). In the 3 groups of mice, there were no significant differences in urinary ACE2 protein levels ($P = 0.15$) (Supplementary Table S1). ACE2 mRNA and protein were decreased in kidneys of *Col4a3*$^{-/-}$ mice (Figure 1a, c, and d). TACE protein was increased and there was a trend toward increased TACE mRNA in kidneys of *Col4a3*$^{-/-}$ mice (Figure 1b, c, and e). ACE2 and TACE activity levels complemented mRNA and protein measurements (Figure 1f and g, Supplementary Figure S2). Accordingly, we have previously shown[9] that treatment with mrACE2 was able to attenuate the increase in Ang II and induce a corresponding elevation of Ang-(1–7) levels in the kidneys of *Col4a3*$^{-/-}$ mice (Supplementary Table S2), and now we show that treatment with mrACE2 partially reversed the changes in renal levels of ACE2 and TACE.

Studies have suggested that increasing ACE2 expression or activity has important effects on organ function. For example, Yamazato and colleagues showed that overexpression of ACE2 in the brain lowered blood pressure in spontaneously hypertensive rats.[13] Therefore, we sought to assess the effect of mrACE2 administration on kidney function and morphology. Heart rate and mean arterial pressure were significantly elevated in *Col4a3*$^{-/-}$ mice compared with wild-type controls (Figure 2a–d). Although the differences did not reach statistical significance, there was a numerical decrease in blood pressure after treatment with mrACE2, suggesting that mrACE2 may have an antihypertensive effect in this model.

Table 1 | Whole animal data

	WT + saline	KO + saline	KO + mrACE2
Body weight (g)	21.23 ± 0.54	18.45 ± 0.70[a]	19.03 ± 0.49[a]
Kidney weight (g)	0.162 ± 0.005	0.166 ± 0.005	0.171 ± 0.008
KW (g)/BW (kg)	7.62 ± 0.09	9.04 ± 0.27[a]	8.97 ± 0.22[a]
Urine output (ml)	0.58 ± 0.16	2.94 ± 0.20[a]	2.25 ± 0.27[a]

BW, body weight; KO, *Col4a3*$^{-/-}$; KW, kidney weight; KW/BW, kidney weight–to–body weight ratio; mrACE2, murine recombinant angiotensin-converting enzyme 2; WT, wild type.
BW and KW were recorded at the time of killing at 7 weeks of age. Urine output was measured for 24 hours the day before killing. For all groups, $n = 8$. Values are presented as mean ± SE.
[a]$P < 0.05$ compared with WT + saline.

Tissue sections were stained with periodic acid–Schiff (PAS) and assessed for glomerular injury (Figure 2e and f). Interestingly, the extent of glomerular involvement was quite variable in *Col4a3*$^{-/-}$ mice with or without mrACE2 treatment. Damage to the tubular compartment could also be seen from PAS-stained sections, and levels of urinary neutrophil gelatinase-associated lipocalin (NGAL) were used to quantify tubular injury. The urinary NGAL excretion rate was significantly higher in *Col4a3*$^{-/-}$ mice, but was reduced by mrACE2 treatment (Figure 2g). The urinary albumin excretion rate was profoundly elevated in *Col4a3*$^{-/-}$ mice and was significantly reduced by mrACE2 treatment (Figure 2h). As expected, values for plasma creatinine tended to increase in *Col4a3*$^{-/-}$ mice, although there was considerable variability in this measurement. Although urinary albumin excretion was reduced by mrACE2 treatment, mean values for plasma creatinine and blood urea nitrogen did not decline (Supplementary Table S3). To further assess the effects of mrACE2 on kidney function, creatinine clearance was calculated (Figure 2i). Treatment with recombinant ACE2 led to a numerical improvement in creatinine clearance that failed to reach statistical significance ($P = 0.07$).

Treatment with mrACE2 ameliorated kidney fibrosis in experimental AS

Kidney cortical expression of collagen type I alpha 1, transforming growth factor beta 1 (TGF-β1), alpha smooth muscle actin (αSMA), and fibronectin was quantified by quantitative polymerase chain reaction. The mRNA levels of these fibrotic markers were higher in *Col4a3*$^{-/-}$ mice than in wild-type mice but showed a decreasing trend after mrACE2 treatment (Figure 3). In agreement with mRNA expression data, picrosirius red staining revealed increased collagen deposition in glomeruli and the tubulointerstitium of *Col4a3*$^{-/-}$ mice, which was attenuated by mrACE2 treatment (Figure 4a and b). Immunohistochemical staining and Western blot for αSMA, a myofibroblast marker, also showed increased positivity of this profibrotic protein in kidney tissue of *Col4a3*$^{-/-}$ mice (Figure 4a, c, and d). Treatment with mrACE2 reduced the level of αSMA in *Col4a3*$^{-/-}$ mice.

We also investigated the effect of mrACE2 on TGF-β signaling in *Col4a3*$^{-/-}$ mice. There were no differences in TGF-β1 tissue levels between the 3 groups of mice, but there was evidence of TGF-β pathway activation in *Col4a3*$^{-/-}$ mice as indicated by higher TGF-β1 levels in urine (Figure 5a–c). Phosphorylation of downstream signal mediators SMAD2 and SMAD3, and expression of SMAD4, were also increased in kidneys of *Col4a3*$^{-/-}$ mice (Figure 5d–g). Recent work has shown that TGF-β can also exert its functions through other noncanonical signal transduction pathways, including the RhoA and mitogen-activated protein kinase (MAPK) pathway.[14,15] We, therefore, investigated extracellular signal–regulated kinase (ERK) and found a trend toward increased activation of ERK in *Col4a3*$^{-/-}$ mice (Figure 5d and h). With mrACE2 administration, levels of urinary TGF-β1, SMAD4,

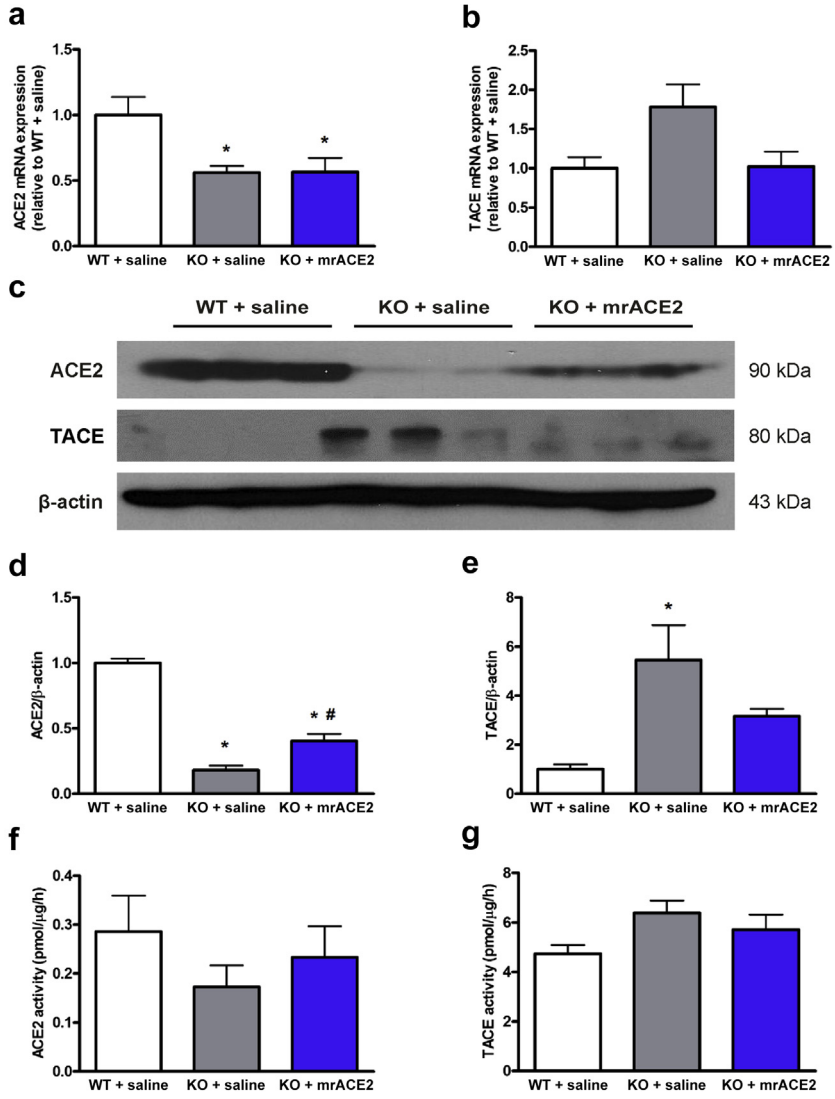

Figure 1 | The loss of angiotensin-converting enzyme 2 (ACE2) and the upregulation of tumor necrosis factor (TNF)-α converting enzyme (TACE) in the kidney were partially reversed by murine recombinant ACE2 (mrACE2) administration. (**a**) mRNA expression of ACE2 and (**b**) TACE were determined by quantitative polymerase chain reaction; $n = 6$ for wild-type (WT) + saline and knockout (KO; *Col4a3*$^{-/-}$) + saline; $n = 5$ for KO + mrACE2. (**c**) Western blot and corresponding densitometry for (**d**) ACE2 and (**e**) TACE are shown. β-actin was used as the loading control; $n = 3$ for each group. (**f**) ACE2 and (**g**) TACE activities from kidney tissue samples using fluorescent substrates, ACE2 inhibitor linear DX-600 (Phoenix Pharmaceuticals, Burlingame, CA), and TACE inhibitor TAPI-2 (Enzo Life Sciences, Farmingdale, NY). Results were calculated as picomoles of the substrate per hour per micrograms of sample protein. For ACE2 activity, $n = 4$ for WT + saline; $n = 5$ for KO + saline; and $n = 3$ for KO + mrACE2. For TACE activity, $n = 5$ for WT + saline; $n = 5$ for KO + saline; and $n = 4$ for KO + mrACE2. Results are presented as mean ± SE. $^{*}P < 0.05$ compared with WT + saline. $^{\#}P < 0.05$ compared with the KO + saline.

and phospho-ERK were diminished compared with untreated *Col4a3*$^{-/-}$ mice.

mrACE2 attenuated renal inflammation and oxidative stress in experimental AS

Transcript levels of proinflammatory cytokines interleukin (IL)-1β, IL-6, TNFα, and monocyte chemoattractant protein-1 were significantly increased in *Col4a3*$^{-/-}$ mice. Administration of mrACE2 suppressed the increase of IL-6 (Figure 6). In addition, the number of infiltrating macrophages in the kidney was substantially higher in *Col4a3*$^{-/-}$ mice, but there was a numerical decline after mrACE2 treatment (Figure 7). JNK and p38 MAPK pathways have been implicated in tissue inflammation by promoting proinflammatory cytokine production and monocyte/macrophage differentiation.[16] Both JNK and p38 MAPK were increasingly phosphorylated in kidneys of *Col4a3*$^{-/-}$ mice, but phosphorylation of JNK was blocked by mrACE2 (Figure 8).

To assess the degree of oxidative stress in the kidney, we measured the level of heme oxygenase-1 (HO-1) in kidney tissue and urine. We previously reported that HO-1 was tightly associated with the effect of Ang II on renal tubular cells.[17] Tissue Western blot and urinary enzyme-linked immunosorbent assay (ELISA) analyses demonstrated that

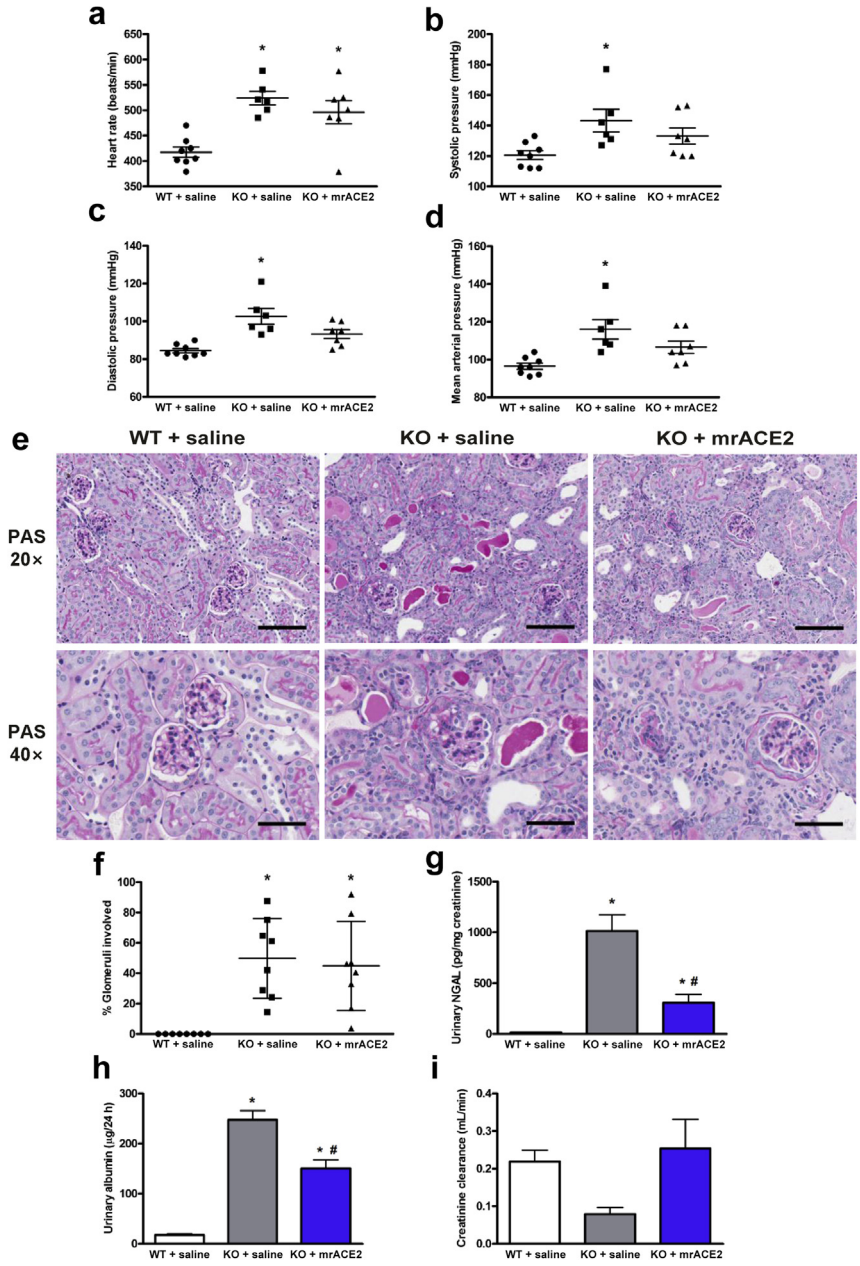

Figure 2 | Murine recombinant angiotensin-converting enzyme 2 (mrACE2) treatment attenuated morphological changes in *Col4a3*$^{-/-}$ (KO) mice. (**a**) Heart rate, (**b**) systolic, (**c**) diastolic blood pressure, and (**d**) mean arterial pressure measurements; $n = 8$ for wild-type (WT) + saline; $n = 6$ for KO + saline; and $n = 7$ for KO + mrACE2. (**e**) Representative images of periodic acid-Schiff (PAS) staining on kidney sections at low (original magnification ×20; bar = 100 μm) and high (original magnification ×40; bar = 50 μm) power magnification. (**f**) Percentage of injured to total glomeruli per kidney section. An average of 100 glomeruli were scored per animal; $n = 8$ per group. (**g**) Urinary neutrophil gelatinase-associated lipocalin excretion was quantified by enzyme-linked immunosorbent assay (ELISA) and normalized to urinary creatinine; $n = 3$ for WT + saline; $n = 6$ for KO + saline; $n = 4$ for KO + mrACE2. (**h**) Urinary albumin excretion was determined by ELISA. The 24-hour albumin excretion was calculated by multiplying the concentration of urinary albumin with total urine volume over a 24-hour period; $n = 5$ for WT + saline; $n = 8$ for KO + saline; and $n = 8$ for KO + mrACE2. (**i**) Creatinine clearance was determined as specified in the Methods section; $n = 4$ for each group. Results are presented as mean ± SE. $^{*}P < 0.05$ compared with WT + saline. $^{\#}P < 0.05$ compared with KO + saline.

HO-1 tissue expression and urinary excretion were significantly increased in 7-week-old *Col4a3*$^{-/-}$ mice compared with age-matched controls, and the increase in urinary HO-1 was attenuated by mrACE2 treatment (Figure 9a–c). Additionally, the urinary excretion rate of nitric oxide metabolites, nitrite and nitrate, rose in *Col4a3*$^{-/-}$ mice and was partially normalized by mrACE2 treatment (Figure 9d).

Dysregulation of ACE2 was associated with alteration of TACE expression

TACE, also known as ADAM17, is a sheddase that is capable of proteolytically cleaving and releasing membrane-bound proteins.[18,19] In the heart, TACE has been shown to mediate ACE2 shedding after Ang II stimulation and to contribute to cardiac injury.[10] We, therefore, sought to

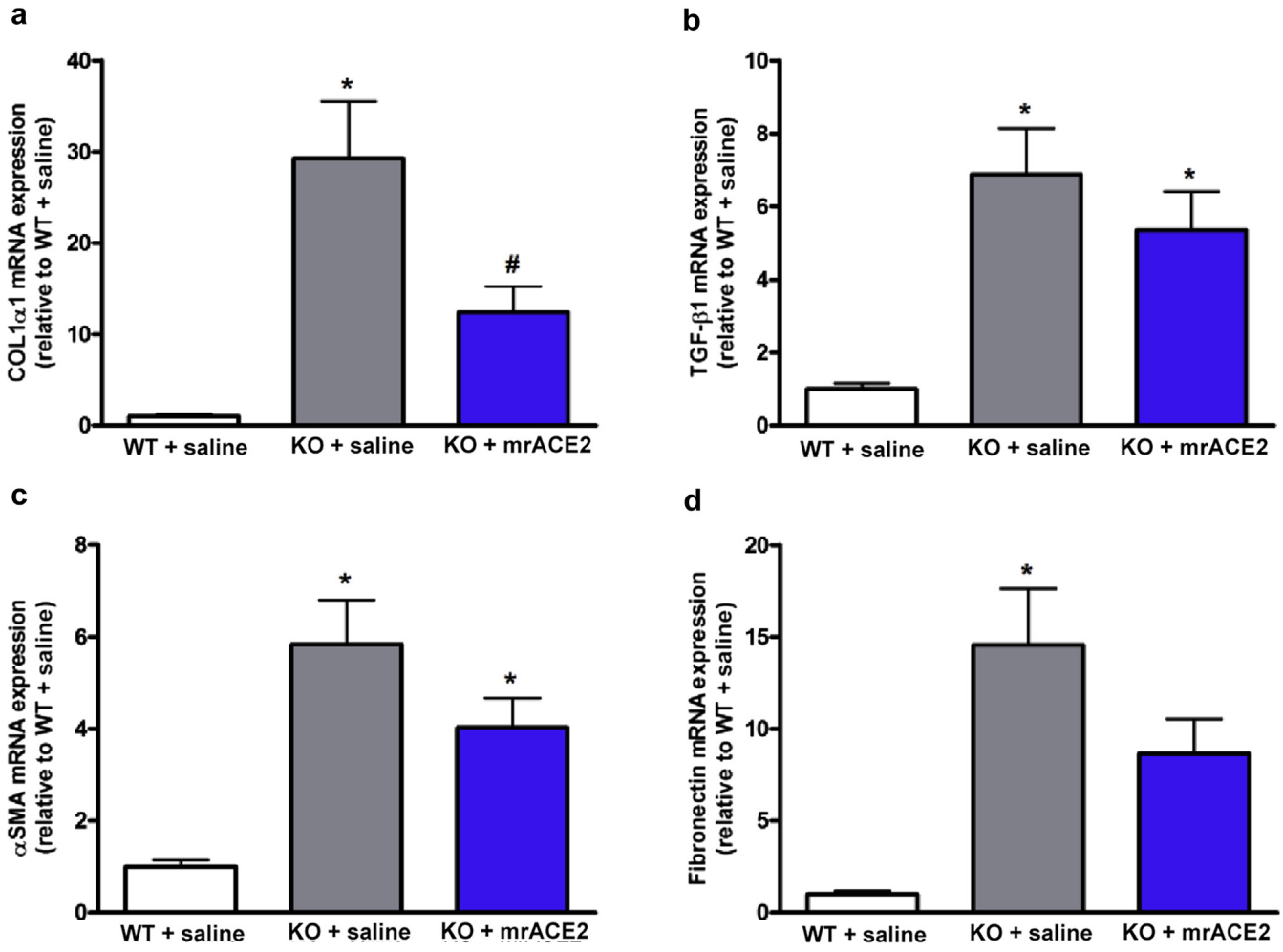

Figure 3 | The mRNA expression of profibrotic markers (a) collagen type I alpha 1 (COL1α1), (b) transforming growth factor beta 1 (TGF-β1), (c) αSMA, and (d) fibronectin was determined by quantitative polymerase chain reaction. Total mRNA was isolated from frozen kidney cortical tissue; $n = 6$ for each group. Results are presented as mean ± SE. KO, *Col4a3*$^{-/-}$; mrACE2, murine recombinant angiotensin-converting enzyme 2; WT, wild type. *$P < 0.05$ compared with WT + saline. $^{\#}P < 0.05$ compared with KO + saline.

determine whether TACE could potentially be involved in the loss of ACE2 in the kidney. Compared with control mice, Ang II-infused mice exhibited lower ACE2 protein and activity but higher TACE protein and activity in the kidney (Figure 10a–d). The mean value for urinary ACE2 activity was numerically greater in Ang II-infused wild-type mice compared with saline-infused mice. The difference did not reach statistical significance ($P = 0.09$) (Supplementary Figure S3). ACE2 and TACE were colocalized in kidney cells of control animals. The immunofluorescent signal for ACE2 was weaker while TACE was stronger in kidneys of Ang II-infused mice (Figure 10e).

Combining mrACE2 and ACE inhibition ameliorated urinary albumin excretion in experimental AS

Blockade of the RAS has been shown to attenuate injury observed in experimental AS,[7,8] and current guidelines recommend ACE inhibition or angiotensin II receptor blocker treatment in humans with AS. Accordingly we sought to define the impact of combining mrACE2 and ACE inhibition on urinary albumin excretion in experimental AS. ACE inhibition led to a dramatic decline in the extent of urinary albumin excretion. The addition of mrACE2 led to a numerical reduction that failed to reach statistical significance in urinary albumin loss as compared with ramipril monotherapy, suggesting that mrACE2 may supplement standard RAS inhibition therapy (Figure 11).

DISCUSSION

The RAS plays a central role in the pathophysiology of many kidney diseases, and suppression of the ACE/Ang II/AT_1 receptor axis limits progression of chronic kidney disease (CKD).[20] The ACE2/Ang-(1–7)/Mas receptor axis plays a role as a counter-regulatory axis of the RAS, where ACE2 serves as an endogenous negative regulator by reducing Ang II levels and increasing the generation of Ang-(1–7).[21] ACE2 has been shown to be an important determinant of kidney diseases in a number of experimental studies.[22] We have previously

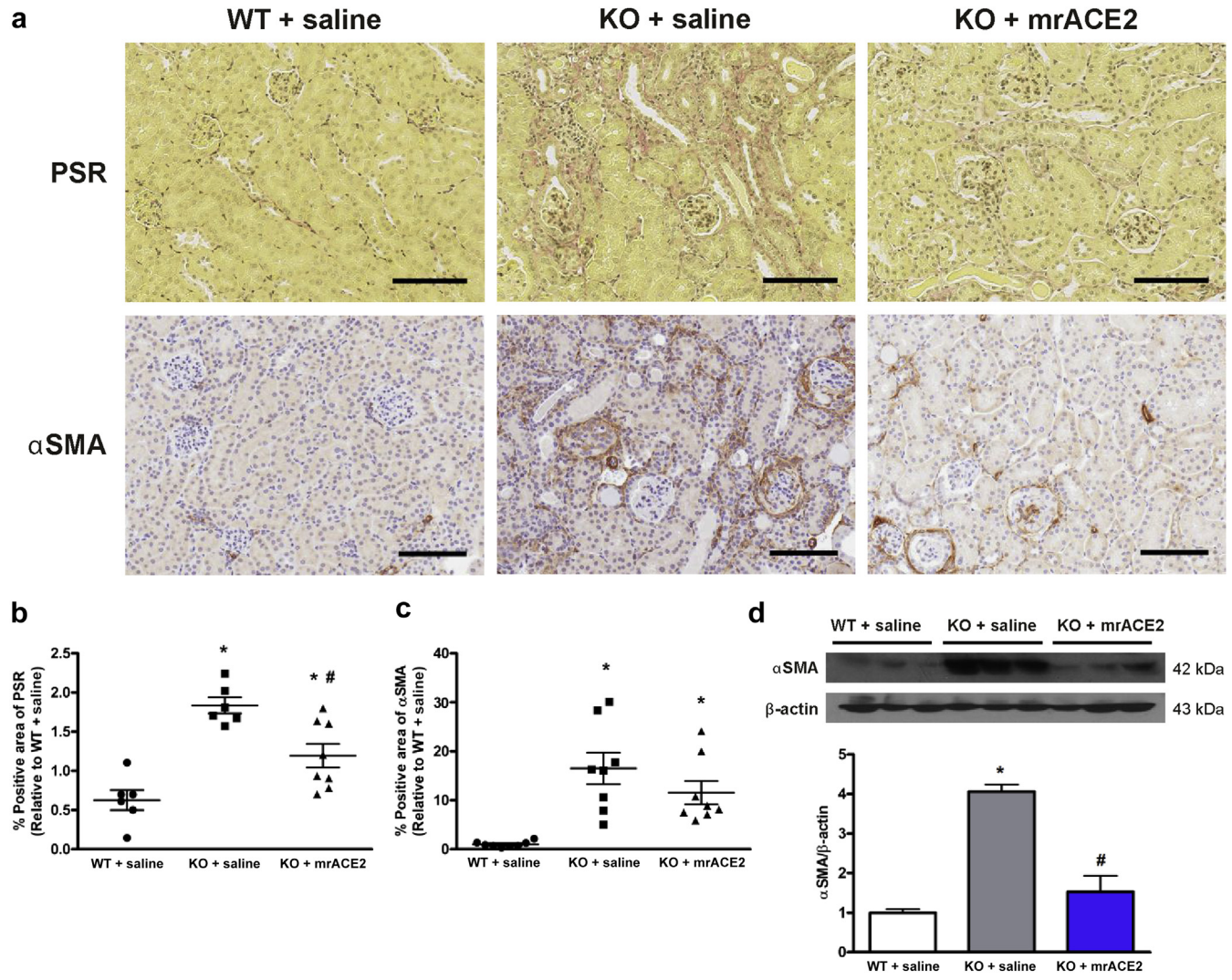

Figure 4 | (a) Kidney sections were stained for picrosirius red (PSR) and αSMA, and (b,c) the levels of positive staining were quantified with the positive pixel count algorithm. For PSR, $n = 6$ for WT + saline and KO + saline, and $n = 8$ for KO + mrACE2. For αSMA, $n = 8$ for each group. Bar = 100 μm. (**d**) Tissue αSMA levels were also determined by Western blot; $n = 3$ for each group. Results are shown as mean ± SE. KO, *Col4a3*$^{-/-}$; mrACE2, murine recombinant angiotensin-converting enzyme 2; WT, wild type. *$P < 0.05$ compared with WT + saline. $^{\#}P < 0.05$ compared with KO + saline.

reported that ACE2 protein and activity decline, while intrarenal Ang II levels are increased in *Col4a3*$^{-/-}$ mice.[9]

Our first major observation was that mrACE2 lowered the urinary albumin excretion rate in *Col4a3*$^{-/-}$ mice. This reduction in urinary albumin was associated with a numerical increase in creatinine clearance compared with untreated mice, but the difference did not reach statistical significance. *Col4a3*$^{-/-}$ mice also exhibited a significant increase in blood pressure, but treatment with mrACE2 did not lead to a significant decrease although values tended to be lower. These effects were accompanied by reductions in kidney Ang II levels and a rise in Ang-(1–7) levels, which we have previously reported.[9] It is tempting to speculate that a reduction in kidney Ang II levels contributed, at least in part, to the effect on blood pressure. In support of this notion, it has been reported that increased renal angiotensinogen expression in transgenic mice is sufficient to lead to an Ang II–dependent elevation in blood pressure.[23]

To better understand ACE2-mediated attenuation of kidney injury in AS, we compared the degree of fibrosis, inflammation, and oxidative stress in kidneys of AS mice with or without mrACE2 treatment. Although the disease originates in glomeruli, the degree of tubulointerstitial injury has been shown to better correlate with CKD progression and functional decline in glomerular filtration rate.[24] Accordingly, we performed our analyses in samples of whole kidney cortex, which predominantly represents the tubulointerstitial compartment.

Tissue fibrosis is the final common pathway of CKD progression and leads to the irreversible loss of kidney function.[25] Transcript levels of several profibrotic markers, including collagen type I alpha 1, TGF-β1, αSMA, and

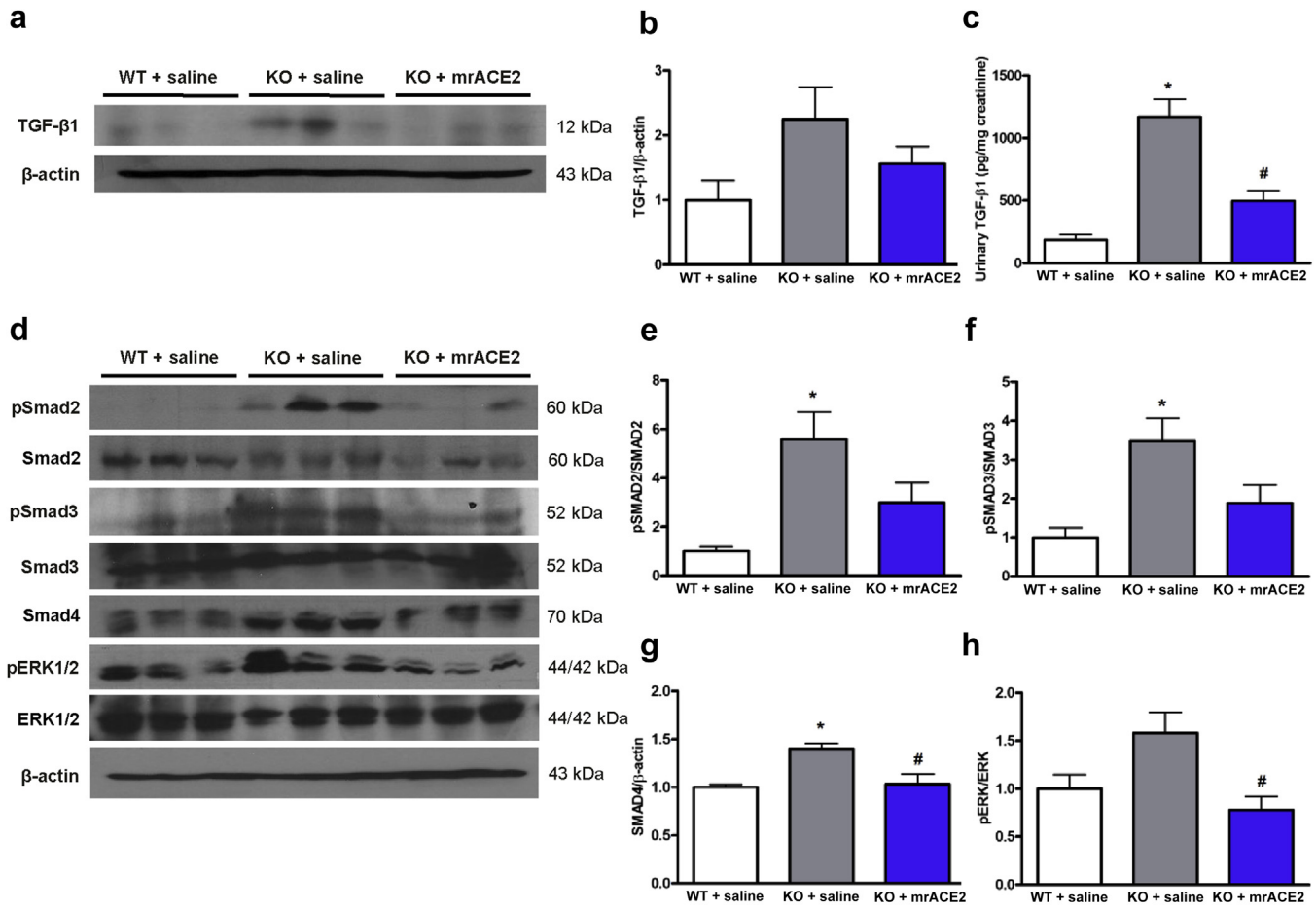

Figure 5 | Activation of the transforming growth factor beta (TGF-β) signal transduction pathway was attenuated by murine recombinant angiotensin-converting enzyme 2 (mrACE2) treatment. (**a,b**) Representative images and corresponding densitometry of renal tissue TGF-β1 determined by Western blot; $n = 3$ for each group. (**c**) Urinary TGF-β1 was measured by enzyme-linked immunosorbent assay and normalized to urinary creatinine; $n = 7$ for each group. (**d–h**) Representative images and corresponding densitometry of tissue (**e**) phospho- and total-SMAD2, (**f**) phospho- and total-SMAD3, (**g**) SMAD4, and (**h**) phospho- and total–extracellular signal–regulated kinase (ERK) determined by Western blot. β-actin was used as the loading control; $n = 3$ for each group. Results are presented as mean ± SE. KO, *Col4a3*$^{-/-}$; WT, wild type. *$P < 0.05$ compared with WT + saline. $^{\#}P < 0.05$ compared with KO + saline.

fibronectin, tended to be suppressed with mrACE2 infusion. The corresponding protein expression levels of fibrillar collagen, αSMA, and TGF-β1 also showed a decrease after mrACE2 treatment. It is well-understood that the TGF-β signal transduction pathway critically mediates tissue fibrosis,[25] and TGF-β levels are higher in patients with AS as well as in *Col4a3*$^{-/-}$ mice[26] compared with healthy individuals and wild-type mice, respectively. Blockade of TGF-β with a soluble fusion protein of the type II receptor prevented thickening of the glomerular basement membrane in mice with AS.[27] Furthermore, blockade of TGF-β and integrin α1β1 preserved the podocyte population in glomeruli and maintained kidney function in mice with AS.[27] We confirmed that biological activity of the TGF-β signaling pathway was also increased in 7 week-old *Col4a3*$^{-/-}$ mice compared with wild-type mice and mrACE2 treatment tended to inhibit these increases. In addition, noncanonical signaling downstream of TGF-β through mediator ERK1/2 was inhibited in kidneys of *Col4a3*$^{-/-}$ mice after mrACE2 treatment. Taken together, these findings suggest that treatment with mrACE2 attenuated kidney injury at least in part by reducing TGF-β pathway activation and subsequent tissue fibrosis.

We then investigated the impact of mrACE2 on kidney inflammation and oxidative stress. The rationale for these analyses was based on previous studies suggesting that intrarenal inflammation exacerbated the severity of kidney disease in AS.[28] In addition, TNFα has been implicated in podocyte apoptosis,[29] and lymphocyte infiltration has been associated with interstitial inflammation and fibrosis in experimental AS.[30,31] These studies suggest that inflammation is an important pathological process in progression of CKD in experimental AS. We observed that treatment with mrACE2 led to a decline in mRNA levels of IL-6 and a numerical reduction in macrophage infiltration in the kidney. Notably, MAPK signal transduction cascades have important yet complex roles in mediating inflammation.[16] For example, the activation of JNK and p38 MAPK has been shown to promote the secretion of cytokines and chemokines and the

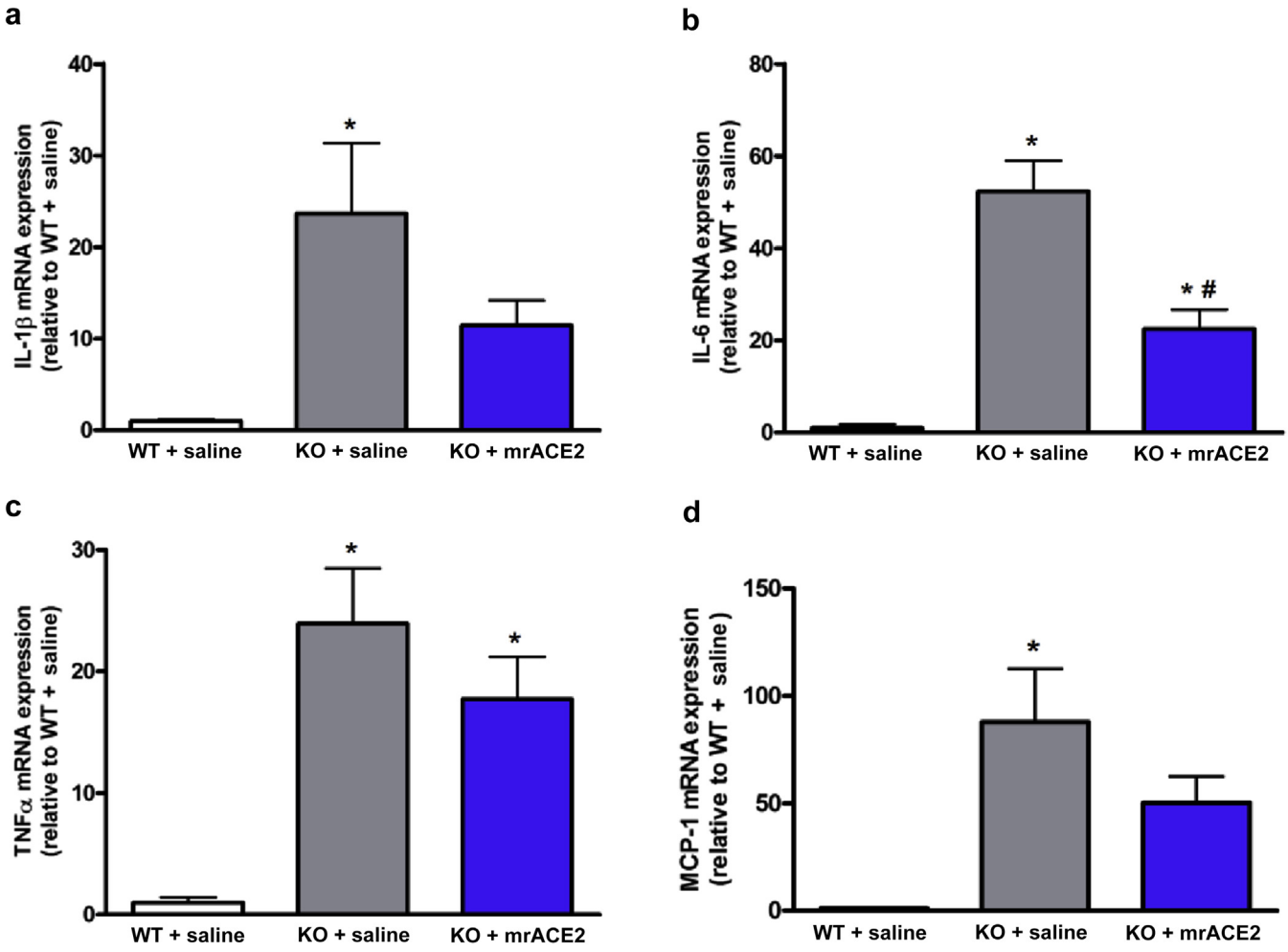

Figure 6 | Transcript levels of proinflammatory markers (a) interleukin (IL)-1β, (b) IL-6, (c) TNFα, and (d) monocyte chemoattractant protein-1 (MCP-1) were determined by quantitative polymerase chain reaction. Total mRNA was isolated from frozen kidney cortical tissue; $n = 6$ for each group. Results are presented as mean ± SE. KO, *Col4a3*$^{-/-}$; mrACE2, murine recombinant angiotensin-converting enzyme 2; WT, wild type. *$P < 0.05$ compared with WT + saline. $^{\#}P < 0.05$ compared with KO + saline.

maturation of macrophages into a proinflammatory phenotype.[16,32] Importantly, the RAS has been shown to activate MAPK signal transduction cascades.[33] We found that administration of mrACE2 led to a significant reduction of JNK phosphorylation and a trend toward reduction of p38 MAPK phosphorylation in *Col4a3*$^{-/-}$ mice.

RAS activation can also lead to increased oxidative stress, which in turn contributes to the progression of CKD.[34] In this regard, HO-1 has been shown to be upregulated during oxidative stress and to function as an indicator of increased Ang II activity in the kidney.[17,35] HO-1 was increased in kidneys and urine of *Col4a3*$^{-/-}$ mice. Treatment with mrACE2 led to a numerical decrease of HO-1 expression in the kidney and significant lowering of HO-1 in urine. In addition, the urinary excretion rate of nitric oxide metabolites was also increased in untreated *Col4a3*$^{-/-}$ mice and lowered by mrACE2 treatment. These findings suggest that mrACE2, by reducing tissue Ang II levels and increasing tissue Ang-(1–7) levels,[9] attenuated oxidative stress in the kidneys.

The rationale for treating *Col4a3*$^{-/-}$ mice with mrACE2 was based on our observation that this model of chronic kidney injury was associated with a reduction in kidney ACE2 expression.[9] A number of mechanisms may be responsible for the decrease in tissue ACE2. Increasing permselectivity defects associated with glomerular injury in these mice leads to a progressive rise in the urinary albumin excretion rate. Albumin uptake by proximal tubule cells is associated with oxidative stress,[36,37] and oxidative stress is a determinant of angiotensinogen expression in the kidney.[37,38] In this regard, we have seen evidence of oxidative stress in *Col4a3*$^{-/-}$ mice compared with age-matched wild-type controls as demonstrated by a significant rise in the urinary HO-1 excretion rate, and we have reported that angiotensinogen expression also increases in the kidney,[9] which is sufficient to activate the kidney RAS and increase blood pressure in an Ang II-dependent manner.[23]

Increased Ang II levels in the kidney will not only increase oxidative stress[39] but may also contribute to the shedding of

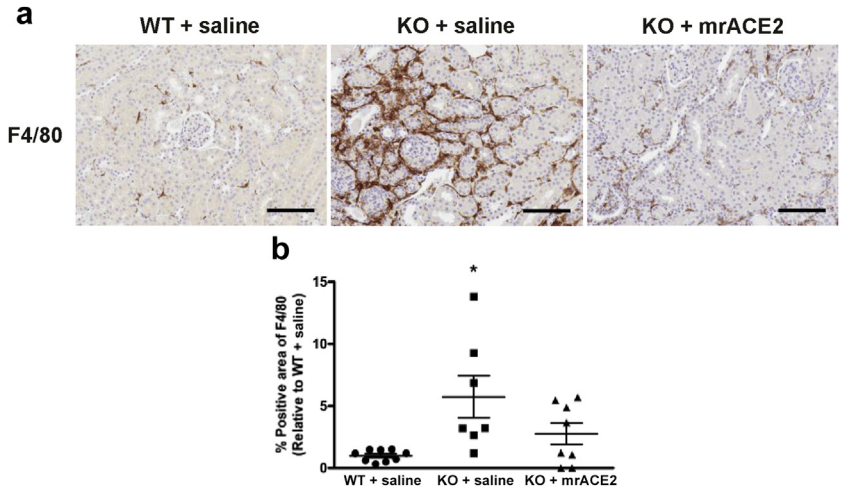

Figure 7 | Treatment with murine recombinant angiotensin-converting enzyme 2 (mrACE2) tended to reduce macrophage infiltration in kidneys of *Col4a3*$^{-/-}$ (KO) mice. (**a**) Representative images and (**b**) computerized quantification of F4/80 staining in kidney sections; $n = 9$ for wild type (WT) + saline; $n = 7$ for KO + saline; $n = 8$ for KO + mrACE2. Bar = 100 μm. Results are shown as mean ± SE. *$P < 0.05$ compared with WT + saline.

ACE2.[40] This has been observed in cultured cells[41] and in the heart, where Ang II infusion has been shown to promote TACE expression and activity.[10] Our experiments with Ang II-infused mice support the hypothesis that this mechanism is also important in the kidney. Ang II infusion also increased TACE expression and activity, whereas ACE2 expression and activity levels were diminished in kidneys of Ang II-infused mice. Ang II infusion also disrupted the colocalization of ACE2 and TACE in the brush borders of renal proximal tubules.

Interestingly we did not see an increase in the urinary excretion rate of ACE2 in our *Col4a3*$^{-/-}$ mice when injury was established at 7 weeks of age. This may be due in part to a decline in ACE2 expression at the mRNA level, independent

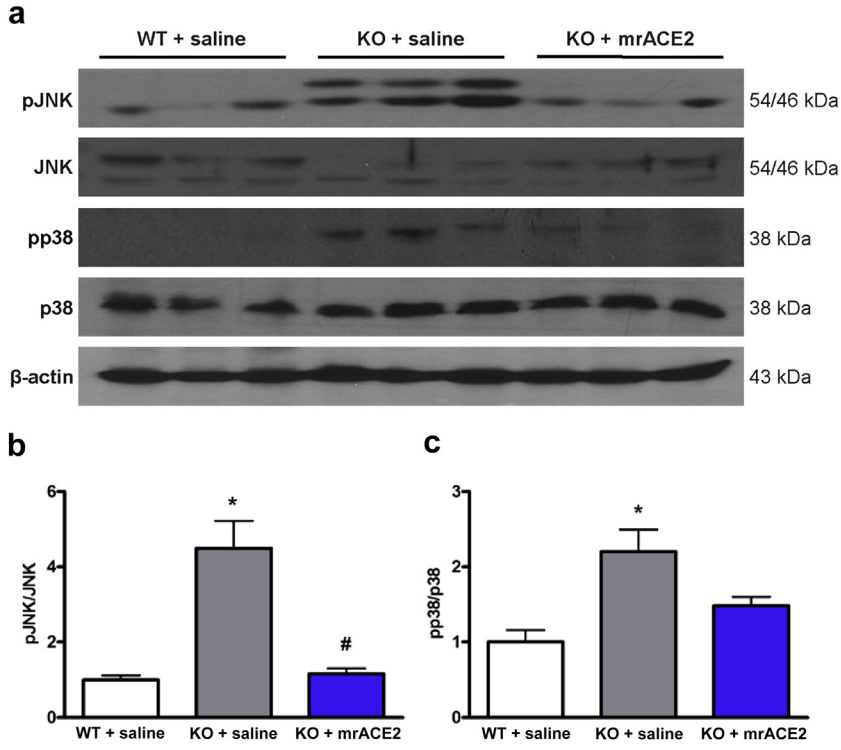

Figure 8 | Murine recombinant angiotensin-converting enzyme 2 (mrACE2) suppressed the activation of JNK. (**a**) Representative images and (**b,c**) corresponding densitometries for tissue phospho- and total-JNK and p38 MAPK determined by Western blot. β-actin was used as the loading control; $n = 3$ for each group. Results are presented as mean ± SE. KO, *Col4a3*$^{-/-}$; WT, wild type. *$P < 0.05$ compared with WT + saline. $^{\#}P < 0.05$ compared with KO + saline.

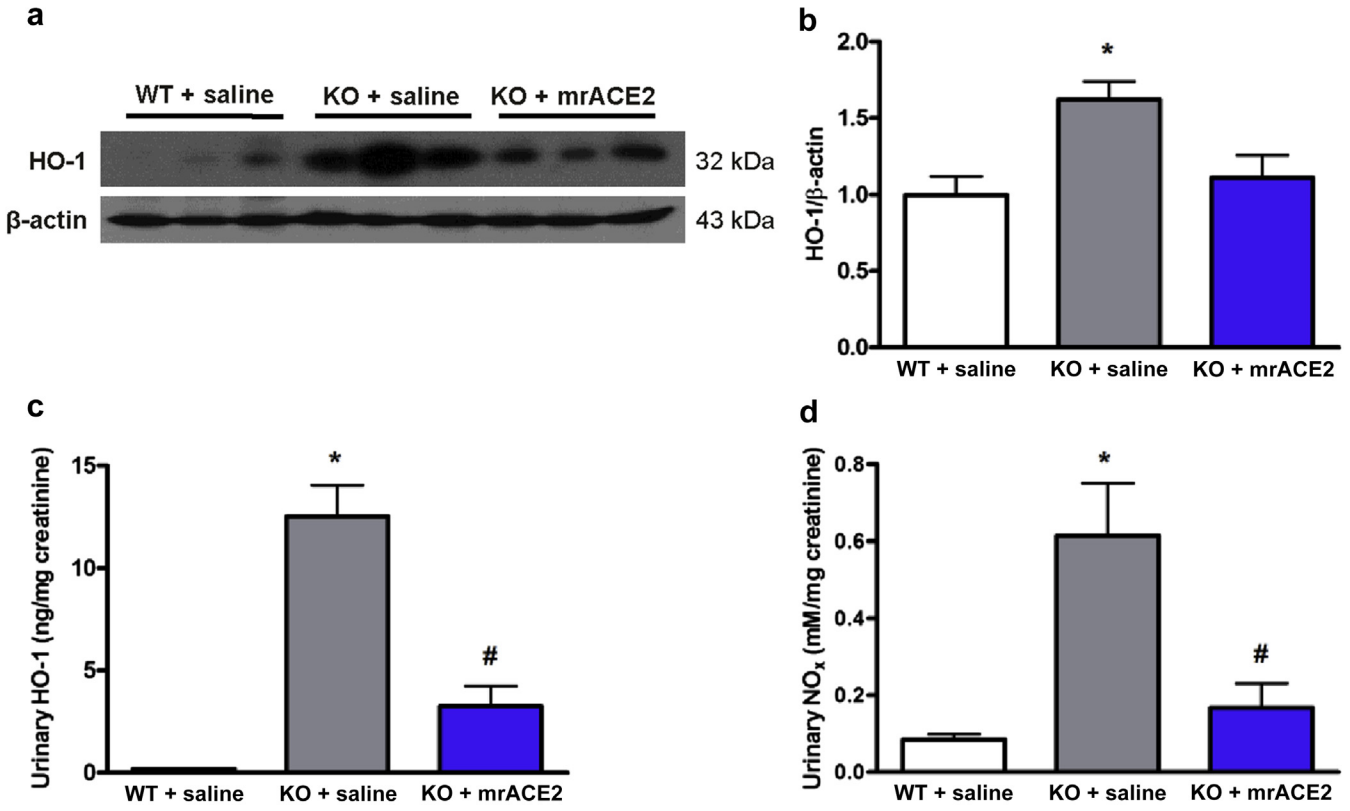

Figure 9| Treatment with murine recombinant angiotensin-converting enzyme 2 (mrACE2) ameliorated oxidative stress in kidneys of *Col4a3*$^{-/-}$ (KO) mice. (**a,b**) Representative images and corresponding densitometry for tissue heme oxygenase-1 (HO-1) determined by Western blot. β-actin was used as the loading control; $n = 3$ for each group. (**c**) Urinary HO-1 levels were determined by enzyme-linked immunosorbent assay and normalized to the urinary creatinine concentration; $n = 7$ for each group. (**d**) Urinary nitrite and nitrate levels were measured by a colorimetric assay and normalized to the urinary creatinine concentration; $n = 5$ for wild type (WT) + saline; $n = 7$ for KO + saline and KO + mrACE2. Results are presented as mean ± SE. $^{*}P < 0.05$ compared with WT + saline. $^{\#}P < 0.05$ compared with KO + saline.

of the shedding of the mature protein. It has been proposed that Ang II negatively regulates ACE2 levels *in vitro* in an AT_1 receptor–dependent manner.[42] Taken together, these findings suggest that the reason for the decrease in kidney ACE2 we observed is multifactorial and likely a consequence of both increased shedding and decreased expression. In this regard, there may be a positive feedback loop contributing to the progression of chronic kidney injury in *Col4a3*$^{-/-}$ mice, whereby decreases in kidney ACE2 activity augment tissue Ang II levels, oxidative stress, and the subsequent shedding of ACE2. Moreover, mrACE2 treatment increased ACE2 expression in association with a decrease in TACE expression in kidneys of *Col4a3*$^{-/-}$ mice. These findings suggest that mrACE2 reduced kidney tissue Ang II levels, leading to a reduction in TACE expression and activity, and are consistent with a role for TACE in kidney disease.[43,44]

There are some limitations to our study. We did not establish the dominant mechanism(s) responsible for the loss of ACE2, and further work will be necessary to determine the role of oxidative stress and Ang II on TACE activity. We used creatinine clearance as a surrogate marker for GFR. This is a reasonable approach in the rat, but there is less data in the mouse,[45] and we recognize that creatinine clearance measurements may overestimate murine GFR.[46] Additionally, while alkaline methods of creatinine measurement have been modified to minimize measurement of interfering substances, some interferences have been reported.[47] We only observed a trend toward improved creatinine clearance values in *Col4a3*$^{-/-}$ mice treated with mrACE2. Treatment did not lead to a significant effect on plasma creatinine levels in our studies likely due to variability in the measurements. In this model, blood urea nitrogen and serum creatinine levels are quite variable. Significant increases in both blood urea nitrogen and serum creatinine levels have been reported at 10 weeks of age when kidney injury is more severe.[48] In other reports, blood urea nitrogen or serum creatinine levels were not significantly different between *Col4a3*$^{-/-}$ and wild-type mice at approximately 7 weeks of age.[7,8,49,50] Although plasma ACE2 activity was significantly increased with mrACE2 administration, this increase was modest and may be limiting the effect of mrACE2 therapy. Another limitation of the current study may be based on the timing of our intervention. Recent studies by Wysocki *et al.*[51] suggest that systemic ACE2 may only be filtered when there is significant impairment of glomerular permselectivity. We started treatment at 4 weeks of age, when there is only a modest increase in the urinary albumin excretion rate,[9] which may limit the direct intrarenal effects of mrACE2 treatment.

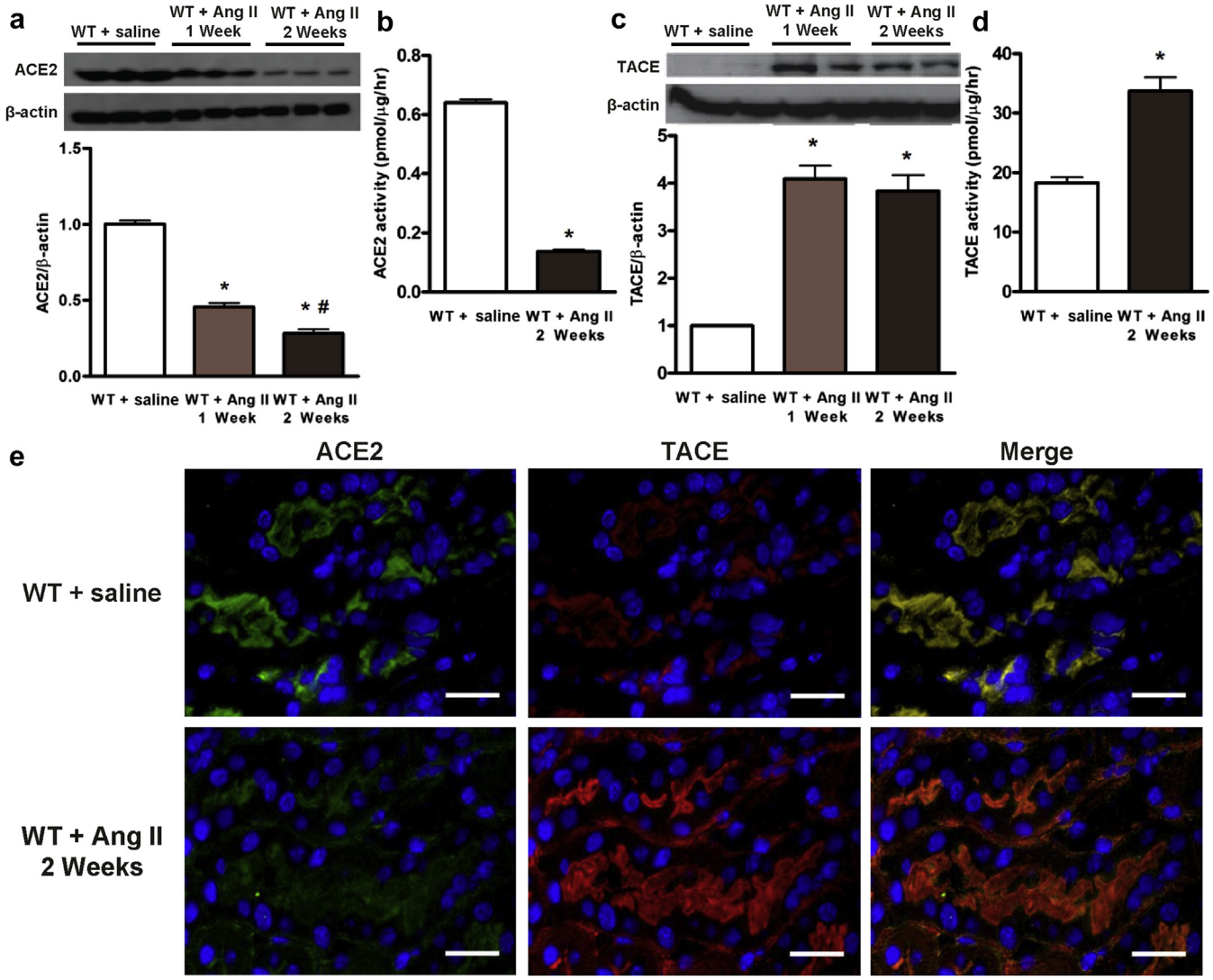

Figure 10 | Angiotensin II (Ang II) perfusion suppressed the expression and activity of angiotensin-converting enzyme 2 (ACE2) but enhanced the expression and activity of TNFα-converting enzyme (TACE). Tissue expression of (**a**) ACE2 and (**c**) TACE were determined by Western blot. β-actin was used as the loading control; $n = 3$ for each group. Activities of (**b**) ACE2 and (**d**) TACE were determined using fluorescent substrates, ACE2 inhibitor linear DX-600 (Phoenix Pharmaceuticals, Burlingame, CA), and TACE inhibitor TAPI-2 (Enzo Life Sciences, Farmingdale, NY). Results were calculated as picomoles of substrate per hour per micrograms of sample protein; $n = 8$ for each group. (**e**) Representative immunofluorescence images of ACE2 and TACE; $n = 4$ for each group. Bar = 25 μm. Results are presented as mean ± SE. $^{*}P < 0.05$ compared with wild type (WT) + saline. $^{\#}P < 0.05$ compared with WT + Ang II 1 week.

Finally, we did not aim to compare mrACE2 with ACE inhibition or determine the effect of rmACE2 on lifespan in *Col4a3*$^{-/-}$ mice. The combined effect of mrACE2 and ramipril, an ACE inhibitor, led to a trend toward further decline in the urinary albumin excretion rate, but the difference did not reach statistical significance. Further studies will be necessary to determine whether combination treatment provides any additional benefit in terms of renal injury and lifespan.

In summary, we found that ACE2 was markedly downregulated in mice with experimental AS, and treatment with exogenous mrACE2 attenuated progression of CKD. Treatment with mrACE2 limited development of fibrosis, inflammation, and oxidative stress in kidneys of *Col4a3*$^{-/-}$ mice. These beneficial effects of mrACE2 may be due in part to the attenuation of renal TACE expression and activity. Taken together, our findings suggest that Ang II plays an important role in the progression of experimental AS and rACE2 may have important therapeutic implications in humans with AS.

METHODS

A detailed Supplementary Methods section, Supplementary Figures S1–S3, and Supplementary Tables S1–S3 are available in the online-only Supplementary Material.

Generation and characterization of mrACE2

mrACE2 was generously provided by Dr. Manfred Schuster (Apeiron Biologics, Vienna, Austria). Briefly, the extracellular domain of

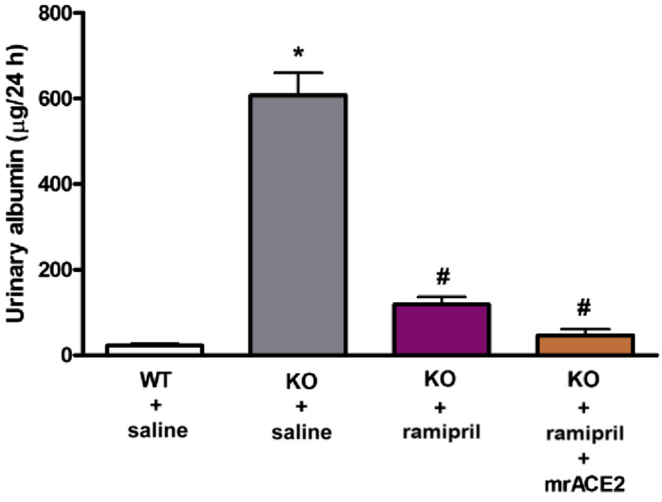

Figure 11 | Murine recombinant angiotensin-converting enzyme 2 (mrACE2) and ramipril dual therapy may result in better preservation of kidney function than does ramipril monotherapy. *Col4a3*$^{-/-}$ (KO) mice were treated with ramipril or a combination of both ramipril and mrACE2 for a duration of 3 weeks. Urinary albumin excretion was measured over a 24-hour time period; $n = 4$ for wild type (WT) + saline; $n = 7$ for KO + saline; $n = 3$ for KO + ramipril; $n = 5$ for KO + ramipril + mrACE2. Results are presented as mean ± SE. $^{*}P < 0.05$ compared with WT + saline. $^{\#}P < 0.05$ compared with KO + saline.

murine ACE2 (amino acid residues 1 to 740) was expressed recombinantly in Chinese hamster ovary cells under serum-free conditions in a chemically defined medium. The expression product was purified to homogeneity by applying the definitive production process. The quality of mrACE2 was tested by size exclusion high-performance liquid chromatography with the use of aMapPac-SEC1, 5 mm, 300 Å at a flow rate of 0.25 ml/minute. Integration of the signal monitored at 214 nm indicated a purity > 99%, and the signal monitored at 280 nm also indicated a purity > 99%.

Experimental animals and protocols

All protocols were approved by the University of Toronto Faculty of Medicine Animal Care Committee according to the Regulations of the Animals for Research Act in Ontario and the Guidelines of the Canadian Council on Animal Care, and the Animal Care and Use Committee at the University of Alberta.

Wild-type and *Col4a3*$^{-/-}$ mice on a congenic 129X1/SvJ background were purchased from the Jackson Laboratory (Bar Harbor, ME), housed at the Division of Comparative Medicine (University of Toronto, Toronto, Canada), and fed standard mouse chow with free access to water. Only male mice were used in this study. The genotype of *Col4a3*$^{-/-}$ mice was verified by tail-clip genotyping using the following primers: common 5'-CCA GGC TTA AAG GGA AAT CC-3', wild type reverse 5'-TGC TCT CTC AAA TGC ACC AG-3', mutant reverse 5'-GCT ATC AGG ACA TAG CGT TGG-3'. Male C57BL/6 mice were used for Ang II infusion experiments.

For mrACE2 treatment studies, the following groups of mice were studied beginning at 4 weeks of age for a period of 3 weeks via subcutaneously implanted micro-osmotic pump (model 1004; Alzet Osmotic Pumps, Cupertino, CA): (i) *Col4a3*$^{-/-}$ mice that received mrACE2 at a dose of 0.5 mg/kg/day, (ii) *Col4a3*$^{-/-}$ mice that received saline, and (iii) wild-type littermate controls that received saline. For Ang II infusion experiments, micro-osmotic pumps (model 1002, Alzet Osmotic Pumps) were subcutaneously implanted in wild-type mice to continuously infuse Ang II (1.5 mg/kg/day) or saline for 1 or 2 weeks. For the ACE inhibitor treatment experiment, ramipril (Sigma-Aldrich, St. Louis, MO) was added to the drinking water of *Col4a3*$^{-/-}$ mice starting at 4 weeks of age for a period of 3 weeks. Ramipril remained stable in water for > 4 days at room temperature, and water bottles were replaced twice per week. Daily fluid intake was measured during the 3-week treatment period to ensure a daily dose of 10 mg/kg/day of ramipril was consumed. In the dual treatment group, *Col4a3*$^{-/-}$ mice were administered mrACE2 and ramipril at the same time via micro-osmotic pump and drinking water, respectively.

Whole animal data, mean arterial pressure, urinary albumin excretion rate, and creatinine clearance

Body weight and kidney weight were recorded at killing. Blood pressure and heart rate were measured at 7 weeks of age after 3 weeks of mrACE2 treatment via catheterization of the right common carotid artery using a mouse single-pressure probe (Millar, Inc., Houston, TX) as previously described.[52] Briefly, mice were anesthetized using 3% isoflurane and the carotid artery was catheterized. Blood pressure was allowed to stabilize for 3 minutes with 1% isoflurane. Blood pressure measurement was performed for 1 minute. Mean arterial pressure was calculated using the following formula: **mean arterial pressure = diastolic blood pressure + 1/3 pulse pressure**. Twenty-four-hour urine samples were collected from 7-week-old animals the day before killing and used to measure urinary albumin excretion rates with the Albuwell ELISA kit (Exocell, Philadelphia, PA). Creatinine clearance (CrCl) was calculated using the following equation:

$$\mathrm{CrCl} = \mathrm{U_{Cr}}/\mathrm{P_{Cr}} \times \mathrm{U_V}/\mathrm{t}$$

where $\mathrm{U_{Cr}}$ is the 24-hour urine specimen creatinine concentration in mg/dl, $\mathrm{P_{Cr}}$ is the plasma creatinine concentration in mg/dl, $\mathrm{U_V}$ is the 24-hour urine specimen volume in ml, and t is the duration of urine collection in minutes. Values were expressed as ml of cleared creatinine per minute (ml/minute). Blood samples were collected from the carotid artery with Microvette 500 LH tubes (Sarstedt Inc., Montreal, Canada) at the time of killing at 7 weeks of age. Plasma was isolated by centrifuging blood samples at 2000 *g* for 5 minutes at room temperature, and stored at –80°C until use. Plasma creatinine assessments were performed using a kinetic modification of the Jaffe procedure on a Beckman Coulter AU480 chemistry analyzer at the Clinical Phenotyping Facility at The Centre for Phenogenomics (Toronto, Canada). Urine creatinine concentrations were determined using The Creatinine Companion kit (Exocell) according to the protocol provided by the manufacturer, an adaptation of the alkaline picrate acid method.

Urinary NGAL, TGF-β1, and HO-1 ELISAs

Mouse urine samples were centrifuged for 5 minutes at 8000 *g* immediately after collection and then stored at –80°C. Samples were then thawed overnight at 4°C, and then centrifuged for 20 minutes at 1000 *g* to remove any pellets before use. Levels of NGAL were determined with a commercial ELISA (R&D Systems, Minneapolis, MN) according to the protocol provided by the manufacturer. A 1:20 sample dilution was performed for NGAL measurement. Urinary TGF-β1 levels were measured using the Quantikine TGF-β1 kit (R&D Systems) according to the manufacturer's protocol. The

Mouse HO-1 ELISA kit (Wuhan USCN Business Co., Wuhan, China) was used to measure urine HO-1 protein levels. Urine samples were diluted 2 times and assayed as per manufacturer's instructions. Results for the urinary protein assays were normalized to urinary creatinine.

Histology, immunohistochemistry, and immunofluorescence

The right kidney was harvested and transversely sectioned into 3 approximately equal portions. The 2 polar portions were snap-frozen, and the middle portion was placed into 10% neutral buffered formalin (Sigma-Aldrich) for histology and immunohistochemistry analyses. Fixed kidney tissue was paraffin-embedded, sectioned, stained, and scanned. Three-μm PAS-stained sections were used to assess histopathological injury. Picrosirius red staining was used to assess the degree of tissue fibrosis. Primary antibodies against αSMA and F4/80 were purchased from Abcam (Cambridge, MA) and AbD Serotec (Raleigh, NC), respectively. Immunofluorescence for ACE2 and TACE was performed as previously published.[10] Briefly, 5-μm cryosections were first incubated with primary antibodies against ACE2 (Abcam) and TACE (EMD Millipore, Temecula, CA) overnight at 4°C, then incubated with Alexa Fluor 488 conjugated donkey anti-goat and Alexa Fluor 594 conjugated donkey anti-rabbit secondary antibodies (Invitrogen, Eugene, OR) at 37°C for 1 hour in a humidified chamber. Nuclei were counterstained with DAPI (Invitrogen). Kidney sections were visualized with the OlympusIX81 microscope and analyzed with ImageJ software.

Quantitative polymerase chain reaction

RNA was extracted from snap-frozen kidney tissue using the RNeasy Mini kit (Qiagen Canada, Mississauga, Canada). Isolated RNA was reverse transcribed into cDNA with the QuantiTech Reverse Transcription kit (Qiagen), which was subsequently used in quantitative polymerase chain reaction (run on ABI Prism 7900, Applied Biosystems, Foster City, CA). Specific mouse Taqman Assay primer sets (Life Technologies, Burlington, Canada) were purchased for the following genes: *Col1a1*, Mm00801666_g1; *Tgfb* (TGF-β1), Mm00441726_m1; *Acta2* (αSMA), Mm01204962_gH; *Fn1* (fibronectin), Mm00692666_m1; *Il1b*, Mm01336189_m1; *Il6*, Mm99999064_m1; *Tnfa* (TNFα), Mm00443260_g1; *Ccl2* (MCP-1), Mm00441242_m1. Mouse primer sets (Bioneer Corporation, Daejeon, Korea) were purchased for the following genes: *Ace2* (forward: 5'-GGA TAC CTA CCC TTC CTA CAT CAGC-3', reverse 5'-CTA CCC CAC ATA TCA CCA AGC A-3'), and *Adam17* (TACE) (forward 5'-AAG TGC AAG GCT GGG AAA TG-3', reverse 5'- CAC ACG GGC CAG AAA GGT T-3').

Western blot

Mouse kidney tissue was washed with ice-cold phosphate-buffered saline 2 times. Renal tissue (50 μl/mg) was placed in modified radioimmunoprecipitation assay buffer (150 mM sodium chloride, 50 mM Tris-HCl [pH 7.4], 1 mM EDTA, 1% v/v Triton-X 100, 1% w/v sodium deoxycholic acid, 0.1% v/v SDS). Tissues were then sonicated 2 times for 10 seconds, and incubated on ice for 30 minutes. Following centrifugation at 18,000 *g* for 10 minutes, the supernatant was transferred to a tube with loading buffer and boiled for 5 minutes. Proteins in tissue lysates were separated by 6% to 12% SDS-PAGE gel, blotted onto a polyvinylidene diflouride membrane, and detected with an enhanced chemiluminescence system kit (EMD Millipore). Densitometry was calculated by Scion Image software (Scion Corp, Frederick, MD). The following primary antibodies were purchased from Cell Signaling Technology (Danvers, MA): phospho- and total-SMAD2, phospho- and total-SMAD3, SMAD4, phospho- and total-ERK1/2, phospho- and total-JNK, phospho- and total-p38 MAPK. The other primary antibodies were purchased as specified: αSMA (Sigma-Aldrich), TGF-β1 (Santa Cruz Biotechnology, Dallas, TX), heme oxygenase-1 (HO-1) (Abcam), ACE2 (Abcam), TACE (EMD Millipore), β–actin (Sigma-Aldrich).

ACE2 and TACE activity

ACE2 and TACE activities were determined from kidney tissue samples using fluorescent substrates as previously published.[43] Briefly, tissue lysates containing 25 μg of protein were incubated with 20 μM fluorogenic substrates in 100 μl of assay buffer, and fluorescence was read at room temperature with an excitation wavelength of 320 nm and an emission wavelength of 405 nm. Substrates used were Mca-Y-V-A-D-A-P-K(Dnp)-OH (R&D Systems) for ACE2 and Mca-K-P-L-G-L-Dpa-A-R-NH_2 for TACE (R&D Systems). Compositions for assay buffers were as follows: ACE2, 1 M NaCl, 75 mM Tris-HCl, and 5 mM ZnCl2 at pH 6.5; TACE, 50 mM Tris-HCl at pH 9.0. ACE2 inhibitor DX-600 Linear (1 μM or 30 μM, Phoenix Pharmaceuticals, Burlingame, CA) and TACE inhibitor TAPI-2 (20 μM, Enzo Life Sciences, Farmingdale, NY) were used to assess specificity, and 7-methoxycoumarin-PL-OH (Bachem Americas, Torrance, CA) was used as the calibration standard.

Colorimetric assay of nitrite and nitrate

Measurement of urinary nitrite and nitrate content was based on the Griess method using a colorimetric nitric oxide assay kit (Oxford Biochemical, Oxford, MI) according to the protocol provided by the manufacturer.

Statistical analysis

Unless specified otherwise, results are expressed as mean ± SE. Two-tailed Student's *t*-tests were used for comparisons between 2 groups. A nonparametric Mann-Whitney test was used to compare non-normally distributed data. One-way analysis of variance was used for comparison of three groups or more. All statistical analyses were performed with the GraphPad Prism software (GraphPad Software, La Jolla, CA). A *P* value of less than 0.05 was considered significant.

DISCLOSURE

All the authors declared no competing interests.

AUTHOR CONTRIBUTIONS

All authors contributed to the experimental conception and design, acquisition of data, or analysis and interpretation of data. All authors participated in drafting of the manuscript or revising it critically for important intellectual content and gave approval to the final version of the manuscript.

ACKNOWLEDGMENTS

We gratefully acknowledge the technical expertise for heart rate and mean arterial pressure measurements provided by Hangjun Zhang and Scott Heximer in the Department of Physiology at the University of Toronto. The mrACE2 was provided by Apeiron Biologics (Vienna, Austria). This work was supported by operating grants from the Canadian Institutes of Health Research (CIHR), the Kidney Foundation of Canada (KFoC), and the Korean Government Research Foundation Grant (NRF-2016R1C1B2011883) and Chonnam National University Hospital Biomedical Research Institute Grant (CRI 16018-1). FF and VRW were supported by funding from the Ontario Graduate Student

Program. AK was supported by a research fellowship from the KFoC and the Clinician Scientist Training Program at the University of Toronto. JWS holds a CIHR/AMGEN Chair in Kidney Research at the University of Toronto. The funders had no role in study design, data collection, analysis, or interpretation, the decision to publish, or preparation of the manuscript.

This work has been presented in abstract form at ASN Kidney Week 2015 and the 52nd ERA-EDTA Congress.

SUPPLEMENTARY MATERIAL

Supplementary Methods

Figure S1. Plasma angiotensin-converting enzyme 2 (ACE2) activity was increased in wild-type (WT) mice treated with murine recombinant ACE2 (mrACE2). Plasma ACE2 activity was determined using a fluorescent substrate and ACE2 inhibitor, linear DX-600 (30 μM), in wild-type mice that were given daily i.p. injections of mrACE2. Results are presented as mean ± SE; $n = 4$ per group. *$P < 0.05$ compared with WT + saline.

Figure S2. Mean values for kidney angiotensin-converting enzyme 2 (ACE2) activity were similar in the 3 groups of mice. ACE2 activity from kidney tissue samples was determined using a fluorescent substrate and ACE2 inhibitor, linear DX-600 (30 μM). Results are presented as mean ± SE; $n = 3$ for WT + saline; $n = 6$ for KO + saline; $n = 3$ for KO + mrACE2. KO, knockout; mrACE2, murine recombinant ACE2; WT, wild type.

Figure S3. The mean value for urinary angiotensin-converting enzyme 2 (ACE2) activity was numerically greater in angiotensin II (Ang II)-infused wild-type (WT) mice compared with saline-infused mice. The difference did not reach statistical significance ($P = 0.09$). Urinary ACE2 activity was determined using a fluorescent substrate and ACE2 inhibitor, linear DX-600 (30 μM). Results are presented as mean ± SE; $n = 4$ per group.

Table S1. Plasma and urine ACE2 protein levels.

Table S2. Kidney Ang II and Ang-(1–7) peptide levels. Adapted with permission from Adapted Bae EH, Konvalinka A, Fang F, *et al.* Characterization of the intrarenal renin-angiotensin system in experimental alport syndrome. *Am J Pathol*. 2015;185:1423–1435.

Table S3. Kidney functional panel plasma biochemistry.

Supplementary material is linked to the online version of the paper at www.kidney-international.org.

REFERENCES

1. Alport AC. Hereditary familial congenital haemorrhagic nephritis. *Br Med J*. 1927;1:504–506.
2. Flinter FA, Cameron JS, Chantler C, et al. Genetics of classic Alport's syndrome. *Lancet*. 1988;2:1005–1007.
3. Savige J, Gregory M, Gross O, et al. Expert guidelines for the management of Alport syndrome and thin basement membrane nephropathy. *J Am Soc Nephrol*. 2013;24:364–375.
4. Gross O, Kashtan CE. Treatment of Alport syndrome: beyond animal models. *Kidney Int*. 2009;76:599–603.
5. Remuzzi A, Fassi A, Bertani T, et al. ACE inhibition induces regression of proteinuria and halts progression of renal damage in a genetic model of progressive nephropathy. *Am J Kidney Dis*. 1999;34:626–632.
6. Webb NJ, Shahinfar S, Wells TG, et al. Losartan and enalapril are comparable in reducing proteinuria in children with Alport syndrome. *Pediatr Nephrol*. 2013;28:737–743.
7. Gross O, Beirowski B, Koepke M-L, et al. Preemptive ramipril therapy delays renal failure and reduces renal fibrosis in COL4A3-knockout mice with Alport syndrome. *Kidney Int*. 2003;63:438–446.
8. Gross O, Schulze-Lohoff E, Koepke M-L, et al. Antifibrotic, nephroprotective potential of ACE inhibitor vs AT1 antagonist in a murine model of renal fibrosis. *Nephrol Dial Transplant*. 2004;19:1716–1723.
9. Bae EH, Konvalinka A, Fang F, et al. Characterization of the Intrarenal Renin-Angiotensin System in Experimental Alport Syndrome. *Am J Pathol*. 2015;185:1423–1435.
10. Patel VB, Clarke N, Wang Z, et al. Angiotensin II induced proteolytic cleavage of myocardial ACE2 is mediated by TACE/ADAM-17: a positive feedback mechanism in the RAS. *J Mol Cell Cardiol*. 2014;66:167–176.
11. Oudit GY, Herzenberg AM, Kassiri Z, et al. Loss of angiotensin-converting enzyme-2 leads to the late development of angiotensin ii-dependent glomerulosclerosis. *Am J Pathol*. 2006;168:1808–1820.
12. Wong DW, Oudit GY, Reich H, et al. Loss of angiotensin-converting enzyme-2 (Ace2) accelerates diabetic kidney injury. *Am J Pathol*. 2007;171:438–451.
13. Yamazato M, Yamazato Y, Sun C, et al. Overexpression of angiotensin-converting enzyme 2 in the rostral ventrolateral medulla causes long-term decrease in blood pressure in the spontaneously hypertensive rats. *Hypertension*. 2007;49:926–931.
14. Derynck R, Zhang YE. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature*. 2003;425:577–584.
15. Lee MK, Pardoux C, Hall MC, et al. TGF-beta activates Erk MAP kinase signalling through direct phosphorylation of ShcA. *EMBO J*. 2007;26:3957–3967.
16. Arthur JSC, Ley SC. Mitogen-activated protein kinases in innate immunity. *Nat Rev Immunol*. 2013;13:679–692.
17. Konvalinka A, Zhou J, Dimitromanolakis A, et al. Determination of an angiotensin II-regulated proteome in primary human kidney cells by stable isotope labeling of amino acids in cell culture (SILAC). *J Biol Chem*. 2013;288:24834–24847.
18. Murphy G. Regulation of the proteolytic disintegrin metalloproteinases, the 'Sheddases'. *Semin Cell Dev Biol*. 2009;20:138–145.
19. Xu P, Derynck R. Direct activation of TACE-mediated ectodomain shedding by p38 MAP kinase regulates EGF receptor-dependent cell proliferation. *Mol Cell*. 2010;37:551–566.
20. Lambers Heerspink HJ, de Borst MH, Bakker SJL, et al. Improving the efficacy of RAAS blockade in patients with chronic kidney disease. *Nat Rev Nephrol*. 2013;9:112–121.
21. Batlle D, Wysocki J, Soler MJ, et al. Angiotensin-converting enzyme 2: enhancing the degradation of angiotensin II as a potential therapy for diabetic nephropathy. *Kidney Int*. 2012;81:520–528.
22. Varagic J, Ahmad S, Nagata S, et al. ACE2: angiotensin II/angiotensin-(1–7) balance in cardiac and renal injury. *Curr Hypertens Rep*. 2014;16:420.
23. Sachetelli S, Liu Q, Zhang SL, et al. RAS blockade decreases blood pressure and proteinuria in transgenic mice overexpressing rat angiotensinogen gene in the kidney. *Kidney Int*. 2006;69:1016–1023.
24. Hodgkins KS, Schnaper HW. Tubulointerstitial injury and the progression of chronic kidney disease. *Pediatr Nephrol*. 2012;27:901–909.
25. Eddy AA. Progression in chronic kidney disease. *Adv Chronic Kidney Dis*. 2005;12:353–365.
26. Sayers R, Kalluri R, Rodgers KD, et al. Role for transforming growth factor-beta1 in alport renal disease progression. *Kidney Int*. 1999;56:1662–1673.
27. Cosgrove D, Rodgers K, Meehan D, et al. Integrin alpha1beta1 and transforming growth factor-beta1 play distinct roles in alport glomerular pathogenesis and serve as dual targets for metabolic therapy. *Am J Pathol*. 2000;157:1649–1659.
28. Ryu M, Kulkarni OP, Radomska E, et al. Bacterial CpG-DNA accelerates Alport glomerulosclerosis by inducing an M1 macrophage phenotype and tumor necrosis factor-alpha-mediated podocyte loss. *Kidney Int*. 2011;79:189–198.
29. Ryu M, Mulay SR, Miosge N, et al. Tumour necrosis factor-alpha drives Alport glomerulosclerosis in mice by promoting podocyte apoptosis. *J Pathol*. 2012;226:120–131.
30. Jedlicka J, Soleiman A, Draganovici D, et al. Interstitial inflammation in Alport syndrome. *Hum Pathol*. 2010;41:582–593.
31. LeBleu VS, Sugimoto H, Miller CA, et al. Lymphocytes are dispensable for glomerulonephritis but required for renal interstitial fibrosis in matrix defect-induced Alport renal disease. *Lab Invest*. 2008;88:284–292.
32. Huang P, Han J, Hui L. MAPK signaling in inflammation-associated cancer development. *Protein Cell*. 2010;1:218–226.
33. Oudit GY KZ, Patel MP, Chappell M, Butany J. Angiotensin II-mediated oxidative stress and inflammation mediate the age-dependent cardiomyopathy in ACE2 null mice. *Cardiovasc Res*. 2007;75:29–39.
34. Nistala R, Wei Y, Sowers JR, et al. Renin-angiotensin-aldosterone system-mediated redox effects in chronic kidney disease. *Transl Res*. 2009;153:102–113.
35. Agarwal A, Nick HS. Renal response to tissue injury: lessons from heme oxygenase-1 gene ablation and expression. *J Am Soc Nephrol*. 2000;11:965–973.

36. Shalamanova L, McArdle F, Amara AB, et al. Albumin overload induces adaptive responses in human proximal tubular cells through oxidative stress but not via angiotensin II type 1 receptor. *Am J Physiol Renal Physiol*. 2007;292:F1846–F1857.
37. Hsieh T-J, Zhang S-L, Filep JG, et al. High glucose stimulates angiotensinogen gene expression via reactive oxygen species generation in rat kidney proximal tubular cells. *Endocrinology*. 2002;143:2975–2985.
38. Brezniceanu ML, Liu F, Wei CC, et al. Catalase overexpression attenuates angiotensinogen expression and apoptosis in diabetic mice. *Kidney Int*. 2007;71:912–923.
39. Zhong J, Guo D, Chen CB, et al. Prevention of angiotensin II–mediated renal oxidative stress, inflammation, and fibrosis by angiotensin-converting enzyme 2. *Hypertension*. 2011;57:314–322.
40. Iwata M, Silva Enciso JE, Greenberg BH. Selective and specific regulation of ectodomain shedding of angiotensin-converting enzyme 2 by tumor necrosis factor alpha-converting enzyme. *Am J Physiol Cell Physiol*. 2009;297:C1318–C1329.
41. Xiao F, Zimpelmann J, Agaybi S, et al. Characterization of angiotensin-converting enzyme 2 ectodomain shedding from mouse proximal tubular cells. *PLoS One*. 2014;9:e85958.
42. Deshotels MR, Xia H, Sriramula S, et al. Angiotensin II mediates angiotensin converting enzyme type 2 internalization and degradation through an angiotensin ii type i receptor–dependent mechanism. *Hypertension*. 2014;64:1368–1375.
43. Kassiri Z, Oudit GY, Sanchez O, et al. Combination of tumor necrosis factor-alpha ablation and matrix metalloproteinase inhibition prevents heart failure after pressure overload in tissue inhibitor of metalloproteinase-3 knock-out mice. *Circ Res*. 2005;97:380–390.
44. Kassiri Z, Oudit GY, Kandalam V, et al. Loss of TIMP3 enhances interstitial nephritis and fibrosis. *J Am Soc Nephrol*. 2009;20:1223–1235.
45. Zager RA. Exogenous creatinine clearance accurately assesses filtration failure in rat experimental nephropathies. *Am J Kidney Dis*. 1987;10:427–430.
46. Eisner C, Faulhaber-Walter R, Wang Y, et al. Major contribution of tubular secretion to creatinine clearance in mice. *Kidney Int*. 2010;77:519–526.
47. Young DS, Chemistry AAfC. *Effects of Drugs on Clinical Laboratory Tests. 5th ed*. Washington, DC: AACC Press; 2000.
48. Tanaka M, Asada M, Higashi AY, et al. Loss of the BMP antagonist USAG-1 ameliorates disease in a mouse model of the progressive hereditary kidney disease Alport syndrome. *J Clin Invest*. 2010;120:768–777.
49. Beirowski B, Weber M, Gross O. Chronic renal failure and shortened lifespan in col4a3+/- mice: an animal model for thin basement membrane nephropathy. *J Am Soc Nephrol*. 2006;17:1986–1994.
50. Katayama K, Kawano M, Naito I, et al. Irradiation prolongs survival of alport mice. *J Am Soc Nephrol*. 2008;19:1692–1700.
51. Wysocki J, Ye M, Khattab AM, et al. Angiotensin-converting enzyme 2 amplification limited to the circulation does not protect mice from development of diabetic nephropathy [e-pub ahead of print]. *Kidney Int*. http://dx.doi.org/10.1016/j.kint.2016.09.032.
52. Lopes J, Adiguzel E, Gu S, et al. Type VIII collagen mediates vessel wall remodeling after arterial injury and fibrous cap formation in atherosclerosis. *Am J Pathol*. 2013;182:2241–2253.