Angiotensin-Converting Enzyme (ACE) 2 Overexpression Ameliorates Glomerular Injury in a Rat Model of Diabetic Nephropathy: A Comparison with ACE Inhibition

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The reduced expression of angiotensin-converting enzyme (ACE) 2 in the kidneys of animal models and patients with diabetes suggests ACE2 involvement in diabetic nephrology. To explore the renoprotective effects of *ACE2* overexpression, ACE inhibition (ACEI) or both on diabetic nephropathy and the potential mechanisms involved, 50 Wistar rats were randomly divided into a normal group that received an injection of sodium citrate buffer and a diabetic model group that received an injection of 60 mg/kg streptozotocin. Eight wks after streptozotocin injection, the diabetic rats were divided into no treatment group, adenoviral (Ad)-ACE2 group, Ad–green flurescent protein (GFP) group, ACEI group receiving benazepril and Ad-ACE2 + ACEI group. Four wks after treatment, physical, biochemical, and renal functional and morphological parameters were measured. An experiment in cultured glomerular mesangial cells was performed to examine the effects of ACE2 on cellular proliferation, oxidative stress and collagen IV synthesis. In comparison with the Ad-GFP group, the Ad-ACE2 group exhibited reduced systolic blood pressure, urinary albumin excretion, creatinine clearance, glomeruli sclerosis index and renal malondialdehyde level; downregulated transforming growth factor (TGF)-β1, vascular endothelial growth factor (VEGF) and collagen IV protein expression; and increased renal superoxide dismutase activity. Ad-ACE2 and ACEI had similar effects, whereas combined use of Ad-ACE2 and ACEI offered no additional benefits. *ACE2* transfection attenuated angiotensin (Ang) II–induced glomerular mesangial cell proliferation, oxidative stress and collagen IV protein synthesis. In conclusion, ACE2 exerts a renoprotective effect similar to that of ACEI treatment. Decreased renal Ang II, increased renal Ang-(1–7) levels, and inhibited oxidative stress were the possible mechanisms involved.
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INTRODUCTION

Angiotensin-converting enzyme (ACE) 2, a homolog of ACE and a newly discovered member of the renin-angiotensin system (RAS) (1,2), is expressed predominantly in the endothelium of intrarenal vessels and in renal tubular epithelium (2). In contrast to ACE, which converts inactive decapeptide angiotensin (Ang) I to a vasoconstrictive octopeptide Ang II, ACE2 catalyzes conversion of Ang II to a vasodilative heptapeptide Ang-(1–7) and conversion of Ang I to the inactive nonapeptide Ang-(1–9), thereby functioning effectively as an endogenous ACE inhibitor (3,4).

The tissue-specific expression of ACE2, and its unique cleavage of the key vasoactive peptide Ang II, suggest an essential role of ACE2 in the local RAS of the heart and kidney (2). Recently, the role of ACE2 in the modulation of cardiovascular function has been investigated by deliberate genetic manipulation, including targeted disruption (5,6) and overexpression (7–9), and these studies have consistently demonstrated that ACE2 has beneficial effects of antihypertension, antifibrosis and antiatherosclerosis. Thus, ACE2 may provide a new therapeutic target for the treatment of cardiovascular diseases.

RAS, especially local renal RAS activation, plays an important role in the pathogenesis of diabetic nephropathy, which is clinically characterized by proteinuria and progressive renal insufficiency. Blockade of RAS by ACE inhibition (ACEI) and Ang II receptor antagonists is currently the standard

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therapy (10) and confers renoprotection in experimental and human diabetic kidney disease (11). However, the antiproteinuric efficacy of these RAS inhibitors depends at least in part on ACE2 (12) and its major product Ang-(1–7) (13–15) in the context of diabetes. The high expression of ACE2 in the normal kidney (2) and the reduced expression of ACE2 in diabetic rats (16) and human kidney diseases (17,18) suggest an ACE2 involvement in diabetic nephrology. To explore the renoprotective effects of ACE2, most investigators have focused on ACE2 inhibition and demonstrated that ACE2 deficiency aggravated glomerular injury in mice (19–21). A recent study found that intraperitoneal injection of human recombinant ACE2 slowed the progression of diabetic nephropathy in diabetic mice, although exogenous proteins may cause immunity reaction after injection, which shortens its half-life (22). In spite of these research efforts, a number of key issues remain unsolved. First, is ACE2 overexpression superior to ACEI in the treatment of diabetic nephrology? Second, is the combined use of ACE2 and ACEI superior to the use of either of the two therapies alone in the treatment of diabetic nephrology? Third, what are the possible mechanisms underlying the therapeutic effects of ACE2 on diabetic nephrology? The present study was carried out to address these critical issues by using a recombinant adenoviral-mediated *ACE2* gene transfer (Ad-ACE2) and/or ACEI in a rat model of diabetic nephrology to compare the effects of the combined therapies (Ad-ACE2 + ACEI) and isolated therapy (Ad-ACE2 or ACEI) on glomerular morphology and function and to explore the signaling pathways mediating these therapeutic effects.

MATERIALS AND METHODS

Preparation of ACE2 Adenovirus Vectors

The murine ACE2 cDNA was amplified by reverse-transcription polymerase chain reaction (RT-PCR) from RNA of a mouse kidney. Recombinant adenoviruses (Ad) carrying the murine ACE2 (Ad-ACE2) or a control transgene EGFP (Ad-EGFP) were prepared with the AdMax system (Microbix Biosystems) using our previously described method (8).

Animal Model

Fifty male Wistar rats, 10 wks old, were obtained from the Animal Center of the Shandong Agriculture Science Academy and were given free access to food and water throughout the study. After an overnight fast, the animals were randomly divided into a normal group (n = 10) that received an intraperitoneal injection of sodium citrate buffer (pH 4.5) and a diabetic model group (n = 50) that received an intraperitoneal injection of 60 mg/kg streptozotocin (STZ) (Sigma Chemical, St. Louis, MO, USA) dissolved in sodium citrate buffer. The diabetic status was confirmed 48 h later by measurement of the tail blood glucose level that was higher than 16.7 mmol/L (23). Eight wks after STZ administration, diabetic rats were further randomly divided into five groups (n = 10 in each group): no treatment group that served as a diabetic control group, Ad-ACE2 group that received an intravenous injection of adenovirus-carried murine ACE2 gene at a dose of 4×10^{10} plaque-forming units (pfu), Ad-GFP group that received an intravenous injection of adenovirus-carried green fluorescent protein at a dose of 4×10^{10} pfu, ACEI group that received benazepril given by intra-gastric intubation at a dose of $10 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ (24); and Ad-ACE2 + ACEI group that received a combined Ad-ACE2 and benazepril treatment as described above. Injection of Ad-GFP or Ad-ACE2 was repeated 2 wks later in the Ad-ACE2, Ad-GFP and Ad-ACE2 + ACEI groups to ensure a sustained effect of gene transfer. None of the rats received insulin treatment during the entire course of experiment. All animals underwent euthanasia at the end of the experiment. The animal care and experimental protocol complied with the Animal Management Rules of the Ministry of Health of the People's Republic of China (document no. 55, 2001) and was approved by the Animal Care Committee of Shandong University.

Blood Pressure Measurement and Sample Collection

Blood pressure was measured with the use of a photoelectric tail-cuff device (Natsume, Tokyo, Japan) 8 and 12 wks after STZ injection, and 24-h urine samples were collected before euthanasia. After an intraperitoneal injection of pentobarbital (50 mg/kg), blood samples were drawn from the left ventricle, and systemic perfusion with normal saline through the left ventricle was performed to wash out blood. The left renal artery and vein were clipped with hemostatic forceps, and the left kidney was quickly removed, decapsulated, weighed, dissected and immediately frozen in liquid nitrogen and stored at –80°C for molecular biological studies. The heart was then perfused with 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4), and the liver and the right kidney were removed, sliced transversely and immersed in 4% paraformaldehyde solution overnight at 4°C for post-fixation.

Biochemical Measurements

Blood samples were centrifuged at 2,000g for 10 min, and serum was stored in aliquots at –80°C. Urine samples were centrifuged in the same way to remove any suspended particles and stored in aliquots at –80°C. Biochemical parameters including serum glucose level, serum creatinine level and urinary creatinine level were measured, and urinary albumin concentration was determined by radioimmunoassay (Beijing North Institute of Biological Technology, Beijing, China). Renal function was assessed by measurement of creatinine clearance (Ccr).

Measurement of Renal Ang II and Ang-(1–7) Levels

Renal cortex was homogenized in ice-cold buffer containing a protease inhibitor cocktail (Sigma Chemical), and the soluble fraction obtained by centrifugation was stored in aliquots at –80°C.

The protein concentration of each sample was determined using a Bio-Rad protein assay kit and BSA as a standard. Ang II concentrations were measured by radioimmunoassay with a kit purchased from Beijing North Institute of Biological Technology (China), following the manufacturer's instructions. The quantity of Ang-(1–7) in the extract was measured by enzyme-linked immunosorbent assay (ELISA) using a commercially available kit (Uscnlife, Wuhan, China) and a 96-well immunoplate. Briefly, ELISA plates coated with antirat Ang-(1–7) antibody were used to capture Ang-(1–7) from the test samples. The amount of Ang-(1–7) captured in each well was detected using another unique biotinylated Ang-(1–7) antibody followed by streptavidin-horseradish peroxidase to develop the signal absorbance, which was recorded at 405 nm. For each sample, the mean of the triplicates was calculated and the concentration of the intact conjugate was determined by comparison with the standard curve.

Measurement of Renal Malondialdehyde Levels and Superoxide Dismutase Activity

Renal cortex samples (100 mg) were chipped and homogenized in ice-cold isotonic saline, which contained 400 μmol/L EGTA, 20 μmol/L butylated hydroxytoluene and 20 μmol/L deferoxamine. Homogenates were then centrifuged at 10,000*g* for 10 min at 4°C to remove any cell debris. The malondialdehyde (MDA) content and the total superoxide dismutase (SOD) activity were measured by the thiobarbituric acid method and xanthine oxidase method, respectively, with commercially available kits following the manufacturer's instruction (NJBC, Nanjing, China). All measurements were performed in triplicate, and the results were normalized to milligram tissue protein.

Measurement of Renal ACE2 Expression Levels and Activity

ACE2 mRNA expression level was determined by real-time RT-PCR using a sequence detection system (Prism 7500; Applied Biosystems, Foster City, CA, USA). Briefly, kidney cortex was snap-frozen in liquid nitrogen, and RNA was extracted using TRIZOL Reagent (Invitrogen, Carlsbad, CA, USA). Total RNA (1 μg) was reversely transcribed. The forward and reverse primers of the murine ACE2 gene were 5′-ACCCTTCTTA CATCAGCCCTACTG-3′ and 5′-TGTCC AAAACCTACCCCACATAT-3′, respectively. β-Actin was used as an internal control with the forward and reverse primers being 5′-GAAGTGTGAC GTTGACAT-3′ and 5′-ACATCTGCTG GAAGGTG-3′, respectively. The data were analyzed by the $2^{-\Delta\Delta CT}$ method.

ACE2 protein expression level in the renal cortex was detected with an ELISA kit (Uscnlife, Wuhan, China), and experiments were performed in triplicate for each data. A microplate-based fluorometric method was applied to determine the renal ACE2 activity as described previously (25). A reagent, 7-Mca-YVADAPK(Dnp) (R&D Systems, Minneapolis, USA), which is cleaved by ACE2, was used as a fluorogenic substrate. Ten 10 μg total protein extracts were incubated with 1.0 μmol/L 7-Mca-YVADAPK(Dnp) in a final volume of 100 μL reaction buffer at room temperature. EDTA (1 mmol/L) and mouse ACE2 (25 ng) (R&D Systems, Minneapolis, MN, USA) were designed as negative and positive controls, respectively. Fluorescence kinetics was measured for 4 h by use of Varioskan Flash (Thermo Scientific, Worcester, MA, USA) at an excitation wavelength of 320 nm and an emission wavelength of 400 nm. ACE2 activity was defined as the difference in fluorescence with or without the ACE2 inhibitor DX600 (1 μmol/L, Phoenix Pharmaceuticals, Belmont, CA, USA). Data were calculated from triplicate wells and presented as fluorescence unit per hour and normalized to milligram tissue protein.

Histopathological Analysis

Cryosections (10 μm) of the liver and kidney tissues were prepared for GFP fluorescence visualization. The remaining renal tissues were embedded in paraffin and cut into 3-μm sections for periodic acid Schiff staining to assess basement membrane changes. The glomeruli sclerosis index (GSI), measured as level 0, 1, 2, 3 and 4, corresponds respectively to 0%, 1% to 25%, 26% to 50%, 51% to 75% and 76% to 100% of increased extracellular matrix deposition per glomerulus as described previously (26).

Immunohistochemistry Analysis

Sections (3 μm) were deparaffinized in xylene and rehydrated through graded alcohols. Antigen retrieval was performed in 0.01 mol/L citrate buffer (pH 6.0) in a microwave. Endogenous peroxidase activity was blocked with 3% H_2O_2 and then incubated with 5% normal goat serum. Sections were then incubated overnight in a humidified chamber at 4°C with primary antibodies diluted in PBS. Thereafter, sections were washed extensively with PBS and incubated with a secondary antibody at room temperature for 30 min by use of the UltraVision One horseradish peroxidase polymer detection system (NeoMarkers, Fremont, CA, USA). After a thorough rinse, the final detection step involved use of 3,3′-diaminobenzidine plus chromogen. Sections were lightly counterstained with hematoxylin, dehydrated and covered. Primary antibodies and dilutions used were 1:300 for rabbit polyclonal ACE2 antibody, 1:400 for rabbit polyclonal ACE antibody, 1:200 for mouse monoclonal transforming growth factor (TGF)-β1 antibody, 1:400 for monoclonal type IV collagen antibody, 1:400 for mouse monoclonal PCNA antibody, 1:500 for mouse monoclonal vascular endothelial growth factor (VEGF) antibody and 1:500 for rabbit polyclonal nephrin antibody (Abcam, Cambridge, UK). Negative controls replaced primary antibody with normal rabbit IgG (NeoMarkers, Fremont, USA) or mouse isotype control antibody (Abcam). All morphological analyses and cell counting were performed on blinded slides. The intensity of positive staining area was measured in

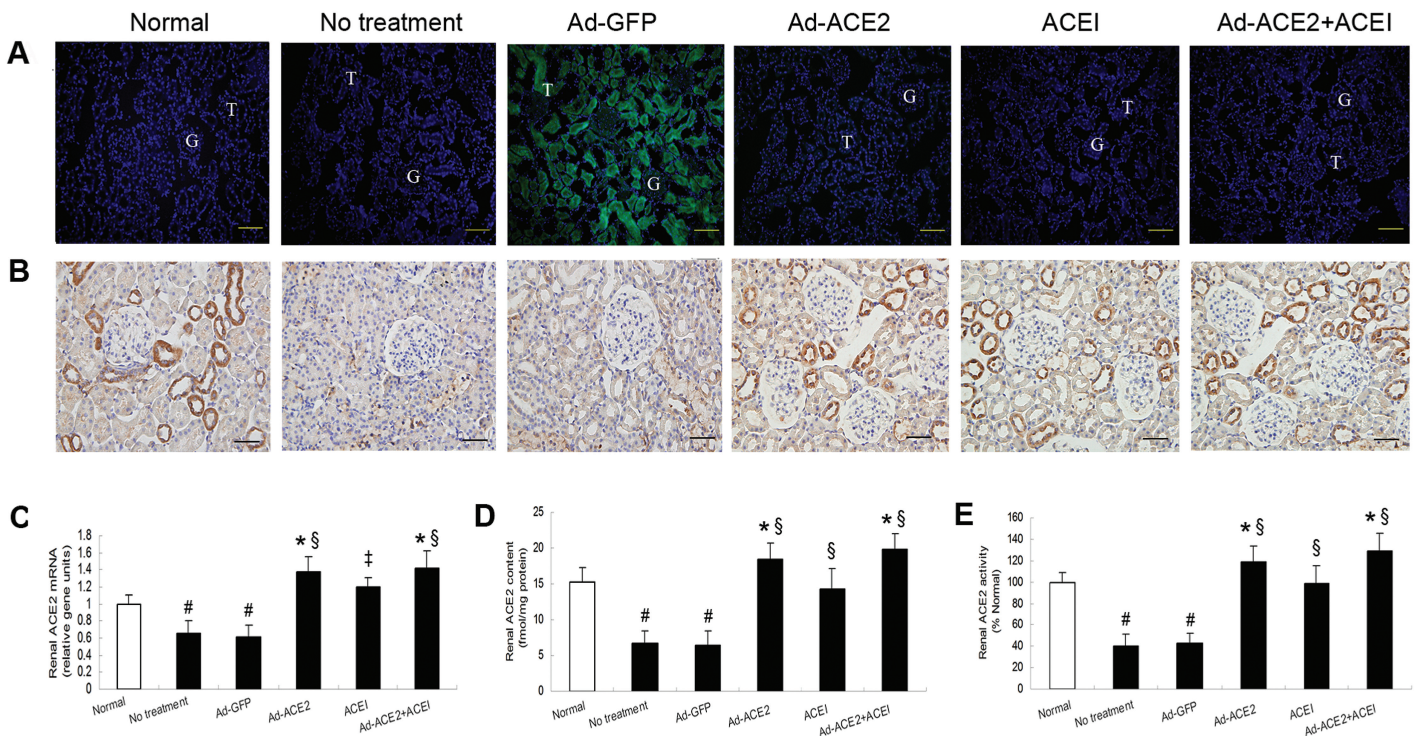

Figure 1. Efficiency of gene transfer 4 wks after gene delivery. (A) Fluorescence microscopic images of frozen sections of the kidneys in the normal, no treatment, Ad-GFP, Ad-ACE2, ACE1 and Ad-ACE2 + ACEI groups, respectively (bar = 50 μm). G indicates glomeruli and T indicates tubules. (B) Immunochemical staining showing the localization of ACE2 protein in cortical sections from each group. Weak ACE2 staining was observed in tubules from a rat in the no treatment and Ad-GFP groups, whereas marked ACE2 staining was depicted in both tubules and glomeruli from rats in the normal, Ad-ACE2, ACEI and Ad-ACE2 + ACEI groups (bar = 50 μm). (C, D, E) Quantitative analysis of ACE2 mRNA levels, protein levels and activities. $^{*}P < 0.05$, $^{\#}P < 0.01$ versus normal group; $^{\ddagger}P < 0.05$, $^{\S}P < 0.01$ versus no treatment group.

at least 20 high-power fields of the renal cortex.

Cell Culture and Gene Transfer

To examine the effects of *ACE2* overexpression on glomerular mesangial cells (GMCs) after Ang II stimulation, HBZY-1 cells, a rat GMC line purchased from the China Center for Type Culture Collection, were cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin. Cells were grown to ~50% confluence and then transfected with Ad-ACE2 or Ad-GFP at a multiplicity of infection of 20. Twenty-four h later, cells were stimulated with Ang II (100 nmol/L, Sigma Chemical) for 24 h. The preincubation time and the doses of adenovirus and Ang II were determined on the basis of our preliminary studies. At the end of the incubation, cells were typsinized and counted using a hemocytometer, and the cell cycle was assessed by flow cytomerical analysis. Reactive oxygen production was determined using the fluorescent probe dihydroethidium (DHE, 1 μmol/L, biyotime, Shanghai, China), which indicates hydroxyethidium derived from reaction with superoxide products. Fluorescent images were obtained using an inverted fluorescence microscope (Olympus, Tokyo, Japan). Type IV collagen level in the medium was detected by ELISA (Uscnlife, Wuhan, China).

Statistical Analysis

Statistical analysis was performed by SPSS for Windows, v11.0 (SPSS, Chicago, IL, USA). All data were expressed as mean ± SD. One-way analysis of variance was applied to analyze the difference among different animal groups. $P < 0.05$ was considered statistically significant.

All supplementary materials are available online at www.molmed.org.

RESULTS

Gene Transfer Efficiency

Four wks after gene transfer, fluorescence microscopy revealed that the reporter gene GFP was expressed in the majority of the liver cells (supplemental Figure 1) and in at least 65% of the cortex tubular epithelial cells in the Ad-GFP group (Figure 1A). By comparison, glomeruli showed much less GFP-positive cells. Compared with the normal group, *ACE2* expression was downregulated in the no treatment group (Figure 1B), a finding consistent with previous reports (18). In the Ad-GFP group, expression of the endogenous ACE2 was detectable mainly in tubules but was weak in glomeruli as shown by

Table 1. Biochemical and renal function measurements in six groups of rats.

Parameters	Normal group (n = 10)	No treatment group (n = 10)	Ad-GFP group (n = 10)	Ad-ACE2 group (n = 10)	ACEI group (n = 10)	Ad-ACE2 + ACEI group (n = 10)
Blood glucose (mmol/L)	8.92 ± 0.48	28.94 ± 1.60	29.65 ± 1.42	29.07 ± 1.51	29.84 ± 1.61	29.70 ± 1.25
Body weight (g)	450.39 ± 9.36	259.65 ± 18.96[b]	261.43 ± 21.34[b]	295.56 ± 21.89[a,c]	287.24 ± 20.18[a,c]	286.60 ± 21.95[a]
KW/BW (g/kg)	3.41 ± 0.34	5.47 ± 0.46[b]	5.42 ± 0.51[b]	4.46 ± 0.42[a,c]	4.73 ± 0.37[a,c]	4.72 ± 0.31[a,c]
Urine volume (ml/24 h)	17.10 ± 1.57	140.19 ± 10.38[b]	133.86 ± 16.58[b]	62.68 ± 15.67[a,c]	58.33 ± 8.45[a,c]	53.91 ± 7.67[a,c]

KW/BW, kidney weight–to–body weight ratio.
[a]$P < 0.05$, [b]$P < 0.01$ versus normal group.
[c]$P < 0.05$ for no treatment group.

immunostaining (Figure 1B). Four wks after gene transfer, the ACE2 protein expression depicted by immunochemical staining was significantly increased in tubules and glomeruli compared with the no treatment group (Figure 1B). In addition, increased ACE2 expression and activity were confirmed by real-time PCR, ELISA and enzymatic activity analysis, respectively, in the renal cortex 4 wks after *ACE2* gene transfer. Compared with the Ad-GFP group, *ACE2* mRNA level, protein level and activity in the renal cortex of the Ad-ACE2 group were increased by 2.26-, 2.28- and 2.50-fold, respectively (Figure 1C, D, E). These results demonstrated a high efficiency of *ACE2* gene transfer in diabetic rats.

Blood Glucose and Blood Pressure Levels

Twelve wks after STZ injection, serum blood glucose level was significantly higher in the five diabetic groups than in the normal group but did not differ among the five diabetic groups (Table 1). Eight wks after STZ injection and before treatment, the systolic blood pressure (SBP) showed no significant difference among the six groups of rats. Twelve wks after STZ injection, SBP was significantly higher in the no treatment group and Ad-GFP group than in the normal group but was normalized in the Ad-ACE2, ACEI and Ad-ACE2 + ACEI groups (Figure 2A).

Renal Function

Twelve wks after STZ injection, the body weight of the rats in the five diabetic groups was reduced in comparison with the normal group. On the contrary, the ratio of kidney-to-body weight was significantly higher in all five diabetic groups than in the normal group, with the value of kidney weight–to–body weight ratio being lower in the Ad-ACE2, ACEI and Ad-ACE2 + ACEI groups than in the no treatment group (Table 1). The Ccr was higher in the no

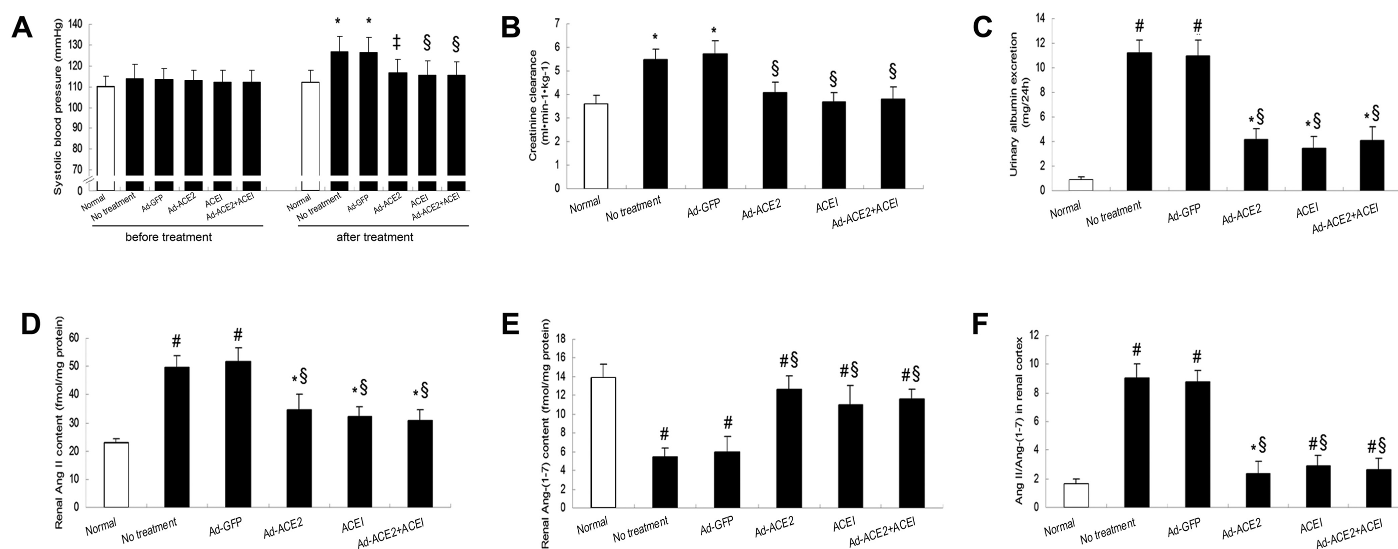

Figure 2. Systolic blood pressure (A), creatinine clearance (B), urinary albumin excretion (C), levels of Ang II (D) and Ang-(1–7) (E), and ratio of Ang II to Ang-(1–7) (F) in six groups of rats. SBP was measured before (8 wks after STZ injection) and after (12 wks after STZ injection) treatment. *$P < 0.05$, #$P < 0.01$ versus normal group; ‡$P < 0.05$, §$P < 0.01$ versus no treatment group.

treatment and Ad-GFP group than in the normal group but was normalized in the Ad-ACE2, ACEI and Ad-ACE2 + ACEI groups (Figure 2B). The urinary albumin excretion was increased by six-fold in the no treatment and Ad-GFP group but was substantially lowered in the Ad-ACE2, ACEI and Ad-ACE2 + ACEI groups compared with the normal group (Figure 2C).

Renal Ang II and Ang-(1–7) Levels

Four wks after gene transfer or ACEI treatment, Ang II level in the renal cortex was significantly higher in all five diabetic groups than in the normal group and tended to be lower in the Ad-ACE2, ACEI and Ad-ACE2 + ACEI groups than in the no treatment and Ad-GFP groups (Figure 2D). The Ang-(1–7) level in the renal cortex was lower in the Ad-ACE2, ACEI and Ad-ACE2 + ACEI groups than in the normal group but was higher than in the no treatment and Ad-GFP groups (Figure 2E). Although the ratio of Ang II/ Ang-(1–7) was still higher in the diabetic groups than in the normal group, it was substantially lower in the Ad-ACE2, ACEI and Ad-ACE2 + ACEI groups than in the no treatment group (Figure 2F).

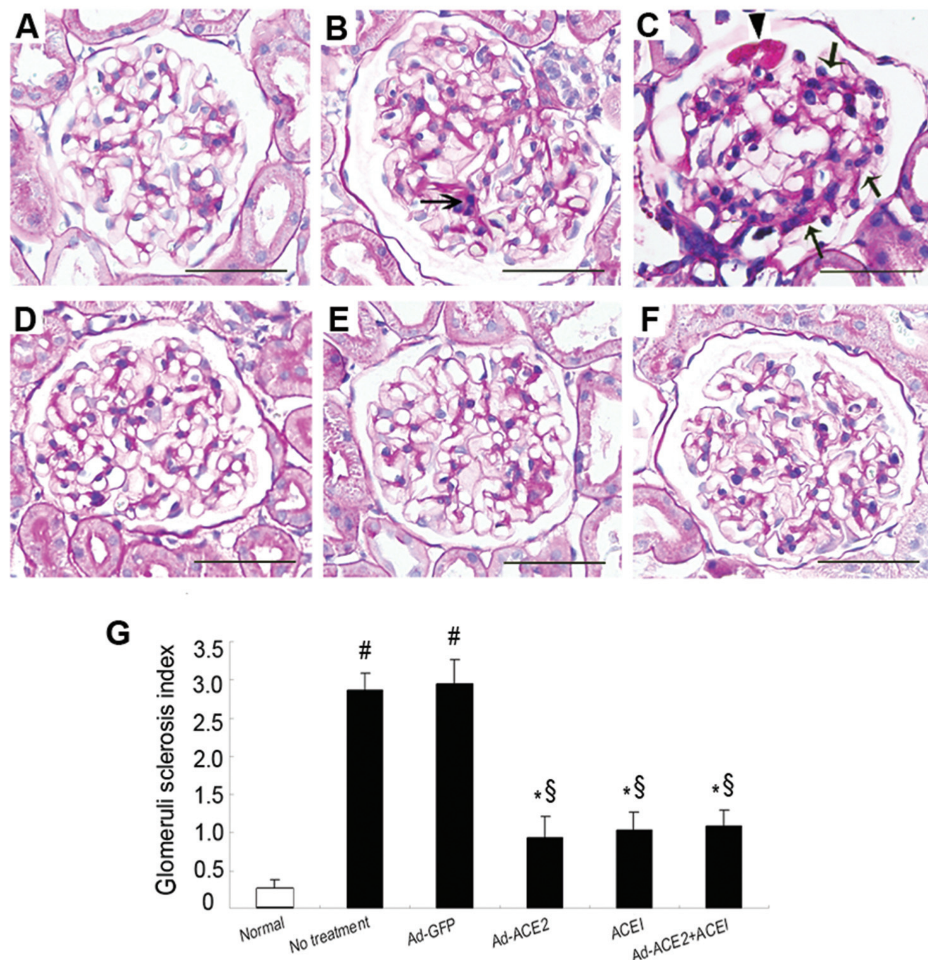

Figure 3. Representative images of periodic acid Schiff (PAS) staining from rats in the normal (A), no treatment (B), Ad-GFP (C), Ad-ACE2 (D), ACEI (E) and Ad-ACE2 + ACEI (F) groups, respectively. In the no treatment and Ad-GFP groups, large focal PAS-positive deposits were evident (arrows) in the mesangium, indicating mesangial expansion. Exudative lesions with fibrin-cap formation (arrowhead) in glomeruli were also observed in the no treatment and Ad-GFP groups. (G) Quantitative analysis of glomerulosclerosis. Bar = 50 μm. $^{*}P < 0.05$, $^{\#}P < 0.01$ versus normal group; $^{\S}P < 0.01$ versus no treatment group.

Histopathological Staining

Twelve wks after STZ injection, kidneys of the no treatment and Ad-GFP groups exhibited profound extracellular matrix deposition and frequent fibrin cap formation inside glomeruli (Figure 3B, C). These pathological abnormalities were remarkably improved in the Ad-ACE2, ACEI and Ad-ACE2 + ACEI groups (Figure 3D, E, F). The GSI was increased by nine-fold in the no treatment group compared with the normal group and was dramatically decreased in the Ad-ACE2, ACEI and Ad-ACE2 + ACEI groups (Figure 3G).

Immunohistochemical Staining

Immunohistochemical analysis showed that the level of ACE protein expression in the glomeruli of the no treatment and Ad-GFP groups was increased by approximately sixfold compared with the normal group, but was substantially reduced in the Ad-ACE2, ACEI and Ad-ACE2 + ACEI groups. The intensity of TGF-β1, collagen IV and VEGF staining and the number of proliferating nuclear antigen (PCNA)-positive cells were markedly increased in the no treatment and Ad-GFP groups compared with the normal group, but were significantly decreased in the Ad-ACE2, ACEI and Ad-ACE2 + ACEI groups. On the contrary, the expression of nephrin was significantly decreased in the no treatment and Ad-GFP groups compared with the normal group, but was substantially increased in the Ad-ACE2, ACEI and Ad-ACE2 + ACEI groups (Figure 4A, B).

GMC Proliferation

After stimulation of HBZY-1 cells with Ang II, cell counting revealed that cell number was significantly increased in the Ad-GFP group but was substantially decreased in the Ad-ACE2 group (Figure 5A). Flow cytometrical analysis showed that cell number in the S and G2-M phases was remarkably decreased in Ad-ACE2 transfected cells compared

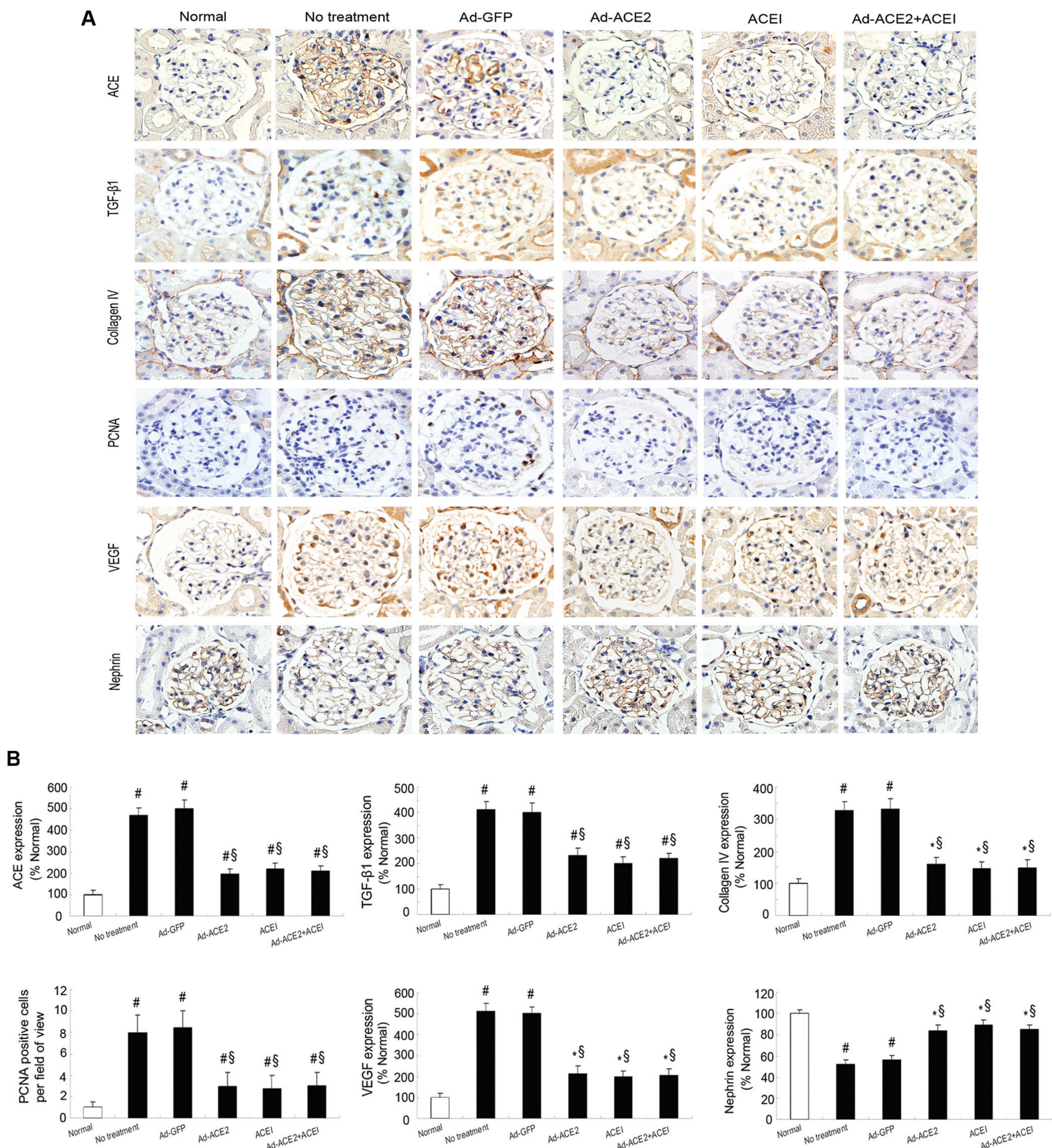

Figure 4. Immunohistochemical staining (A) and quantitative analysis (B) in six groups of rats. Brown color indicates the positive staining. Abundant expression of ACE, TGF-β1, VEGF and type IV collagen was detected within the glomeruli of kidneys from the no treatment and Ad-GFP groups compared with the normal group. In contrast, the Ad-ACE2, ACEI and Ad-ACE2 + ACEI groups showed markedly attenuated expression of ACE, TGF-β1, VEGF and type IV collagen but enhanced expression of nephrin compared with the Ad-GFP group. More proliferative cells (arrow) were seen in the no treatment and Ad-GFP groups than the normal group. The number of PCNA-positive cells was greatly diminished in the Ad-ACE2, ACEI and Ad-ACE2 + ACEI groups compared with the no treatment group. $^{*}P < 0.05$, $^{\#}P < 0.01$ versus normal group; $^{\S}P < 0.01$ versus no treatment group.

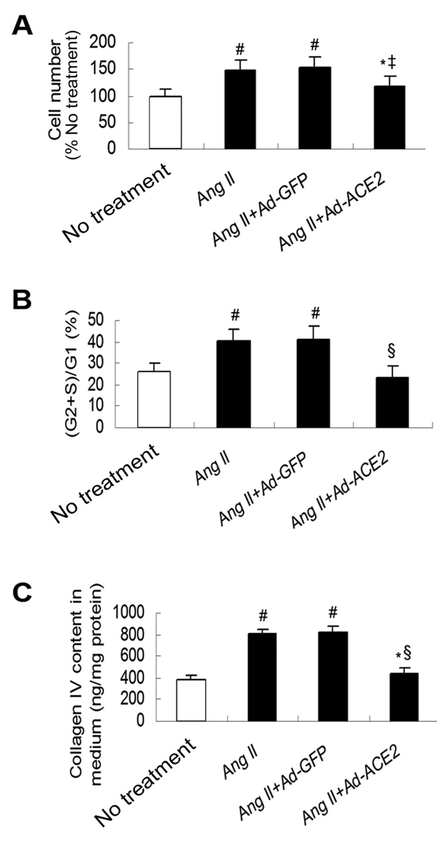

Figure 5. Effects of Ad-ACE2 on Ang II–induced cell proliferation (A, B) and type IV collagen production (C) in GMCs. Cell number was expressed as a percentage of the no treatment group. Type IV collagen contents were expressed as nanograms per milligram of cellular protein. *P < 0.05, #P < 0.01 versus no treatment group; ‡P < 0.05, §P < 0.01 versus Ad-GFP group. S, synthesis phase; G1, gap1; G2, gap2.

with Ad-GFP transfected cells (Figure 5B). Ad-ACE2 transfection significantly inhibited Ang II–induced collagen IV protein synthesis in HBZY-1 cells (Figure 5C).

Measurements of Oxidative Stress

In the renal cortex, SOD activity was significantly lower, whereas MDA content was higher in the no treatment and Ad-GFP groups than in the normal group. In contrast, *ACE2* gene transfer and ACEI treatment significantly increased renal SOD activity and decreased the renal MDA content compared with the no treatment and Ad-GFP group. There was no significant difference in renal SOD activity and MDA content among the Ad-ACE2, ACEI and Ad-ACE2 + ACEI groups (Figure 6A, B). In HBZY-1 cells, Ad-ACE2 transfection markedly reduced Ang II–induced O_2^- production, as revealed by DHE staining (Figure 6C, D).

DISCUSSION

The major finding of the present study was that, in a rat model of diabetic nephropathy, *ACE2* gene transfection effectively reduced SBP, urinary albumin excretion, Ccr and GSI, and these beneficial effects were similar to ACEI treatment. The mechanisms underlying these effects involved decreased renal Ang II levels, increased renal Ang-(1–7) levels and inhibited oxidative stress, TGF-β1 and VEGF signaling pathways. To the best of our knowledge, this is the first study to demonstrate that the *ACE2* overexpression is as effective as standard ACEI treatment in improving nephrotic morphology and function, and thus ACE2 may provide an important therapeutic target in the treatment of diabetic nephropathy.

In the present study, an intraperitoneal injection of 60 mg/kg STZ was used to induce hyperglycemia, and this method has been widely applied to create rodent models of diabetes and diabetic nephrology similar to their human counterparts (27). Our results revealed that rats in the no treatment group developed severe hyperglycemia, albuminuria and renal pathological changes as well as enhanced Ccr, which are characteristic of early diabetic nephropathy, and demonstrated that an animal model of diabetic nephropathy was successfully established. In our previous study (8) and the current study, the high expression of GFP in the Ad-GFP group and the high expression and activity of ACE2 in the Ad-ACE2 group, as well as the lack of noticeable side effects in these groups, indicated a high efficiency and safety of *ACE2* gene transfer.

A wealth of evidence suggests that tissue RAS plays a pivotal role in the development of diabetic complications (19,28,29). In this study, local renal RAS was found to be highly activated, as manifested by the increased ACE expression and Ang II level and decreased ACE2 expression and Ang-(1–7) level in the cortex. As the key peptide of the RAS, Ang II exerts a variety of effects via its type 1 receptor, including vasoconstriction, sodium retention, cell proliferation and apoptosis, proinflammation and oxidative stress (30). Our study confirmed that Ang II promoted cell proliferation and enhanced reactive oxygen species production in cultured GMCs. These effects, acting either alone or synergistically, may lead to enhanced glomerular permeability and overt albuminuria in diabetic nephropathy.

Contrary to Ang II, Ang-(1–7) has vasodilation, antiinflammation and antiproliferation effects. The marked decrease in the renal MDA content and the number of the PCNA positive cells, and the substantial increase in the renal SOD activity in the Ad-ACE2, ACEI and Ad-ACE2 + ACEI groups confirmed the antiinflammation and antiproliferation effects of Ang-(1–7) *in vivo*. These beneficial effects were further confirmed by the results of GMC culture and *ACE2* gene transfer *in vitro*. Although the Ad-ACE2, ACEI and Ad-ACE2 + ACEI groups exhibited a similar decline in the renal Ang II concentration and a similar increase in the renal Ang-(1–7) concentration 4 weeks after treatment, the renal Ang-(1–7) concentration was the highest in the Ad-ACE2 group, leading to the lowest ratio of renal Ang II/Ang-(1–7) in this group. This result suggested a key role of ACE2 in producing Ang-(1–7) in the kidney. Alternatively, Ang-(1–7) can be converted from Ang I by neprilysin and hydrolyzed to Ang-(1–4) by aminopeptidase and neprilysin in the brush-border membrane of the renal cortex (31). Thus, the significant increase in the renal Ang-(1–7) concentration after ACEI treatment in our study probably reflects the secondary increase of renal ACE2 activity (32) or the increased neprilysin-dependent Ang-(1–7) produc-

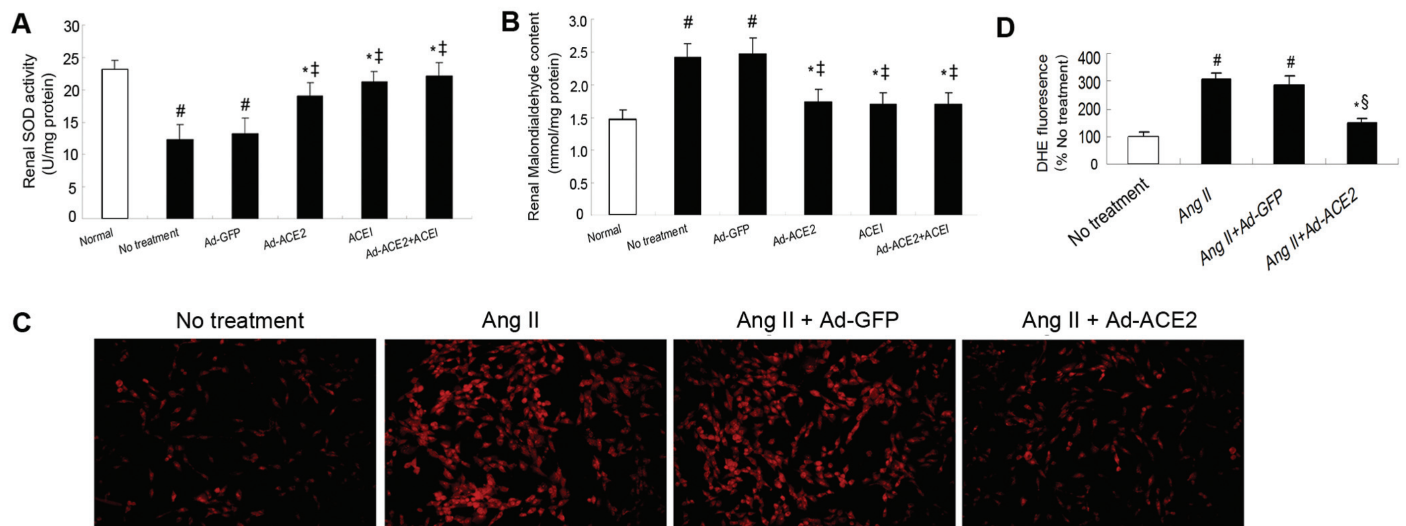

Figure 6. Measurements of oxidative stress. Renal cortical SOD activity (A) and MDA content (B) in six groups of rats. $^*P < 0.05$, $^\#P < 0.01$ versus normal group; $‡P < 0.05$, $^§P < 0.01$ versus no treatment group. C and D represent images of GMC DHE staining and quantitative analysis of DHE staining, respectively. $^*P < 0.05$, $^\#P < 0.01$ versus no treatment group; $^‡P < 0.05$, $^§P < 0.01$ versus Ang II group.

tion or the decreased Ang-(1–7) hydrolysis in brush-border membrane.

Current evidence indicates that hyperglycemia-induced oxidative stress is a key mechanism of diabetic nephropathy. The oxidative stress processes increase advanced glycated end products and activate protein kinase C and hexosamine pathways. These alternations in turn stimulate release of TGF-β1 and VEGF and other cytokines in the kidney, resulting in accumulation of extracellular matrix and aggravation of apoptosis, inflammation and proteinuria (33). Alternatively, hyperglycemia-induced Ang II directly stimulates TGF-β1 expression and suppresses nephrin expression in the kidney (34), with resultant release of TGF-β1 and VEGF and other cytokines in the kidney (35). In addition, podocyte-derived VEGF, a permeability and angiogenesis factor, acts in an autocrine mode to induce the podocytopathy of diabetes and albuminuria (36). The decreased podocytes and nephrin proteins in the slit diaphragm with podocyte foot process effacement underlie the principal feature of diabetic podocytopathy that clinically manifests as albuminuria and proteinuria. Thus, inhibition of TGF-β and VEGF signaling pathways is essential in the treatment of diabetic nephropathy, and blockade of RAS has proven promising to achieve this goal (33). The significant reduction in albuminuria, Ccr, GSI and oxidative stress, together with the downregulation in TGF-β1, VEGF and collagen IV expression and the upregulation in nephrin expression in the glomeruli of the Ad-ACE2, ACEI and Ad-ACE2 + ACEI groups demonstrated that *ACE2* overexpression and ACE inhibition offer effective approaches to the treatment of diabetic nephropathy. These salutary effects are likely mediated by inhibited oxidative stress, TGF-β1 and VEGF pathways. As expected, *ACE2* overexpression, ACEI or both in combination exhibited a similar degree in inhibiting oxidative stress, TGF-β1 and VEGF pathway.

In this study, the therapeutic effects of *ACE2* overexpression and ACE inhibition on experimental diabetic nephropathy were first compared, and the results showed that both interventions had similar effects on SBP and renal functional and histopathological parameters. As a pair of enzymes, ACE and ACE2 act to mutually regulate their protein expression (37) by using each other's product as its own substrate and producing a product with opposing functions, thus forming a forward negative feedback loop in RAS and coordinating functional performance in physical and pathophysiological conditions. Our results showed that *ACE2* overexpression and ACE inhibition had similar effects on reducing renal Ang II levels, increasing renal Ang-(1–7) levels and decreasing the number of proliferative cells and the protein expression levels of TGF-β1, VEGF, type IV collagen and nephrin in glomeruli after 4 weeks of treatment. An interesting finding of the present study was that *ACE2* overexpression and ACE inhibition had no impact on elevated serum glucose levels in the diabetic rats, which was consistent with the results of Wong *et al.*, who showed that glycemic control was not worsen by the loss of *ACE2* in the Akita diabetic mode (20). These results indicated that the renal protective effects of ACE2 were not attributable to glycemic control. In contrast, *ACE2* overexpression and ACE inhibition did have blood pressure–lowering effects in diabetic rats, which may contribute to the renal protective effects of these two interventions. However, previous studies have shown that the renal protective effects of ACEI and angiotensin receptor

blockers are well beyond what is expected from blood pressure lowering alone (38). Thus, the beneficial effects of ACE2 observed in this study are most likely attributable to the net effect of decreased local Ang II level and increased local Ang-(1–7) level. Recent studies found that long-term *ACE2* overexpression or Ang-(1–7) injection accelerated myocardial fibrosis or diabetic nephropathy (7,39,40). In this study, the outcome of *ACE2* overexpression was assessed 4 weeks after gene transfer, and no detrimental effects were found, suggesting that our gene therapy offers a safe approach. Noticeably, our results indicated that *ACE2* overexpression is not superior to ACE inhibition in the treatment of diabetic nephropathy and vice versa.

A previous study suggested that increased ACE2 and decreased ACE protein in kidney from diabetic mice may offer a renoprotective combination (41). However, the present study found no significant difference in all physical, biochemical, renal functional and histopathological, and molecular biochemical parameters measured in the Ad-ACE2, ACEI and Ad-ACE2 + ACEI groups. A possible explanation for the lack of additive or synergistic effects of *ACE2* overexpression and ACE inhibition is that although ACE inhibition reduced the level of renal Ang II, it also cut down the substrate for ACE2 and the production of renal Ang-(1–7) by ACE2. Therefore, combined use of Ad-ACE2 and ACEI led to only insignificant changes in renal Ang II and Ang-(1–7) levels compared with solo treatment with Ad-ACE2 or ACEI. Consequently, combined use of Ad-ACE2 and ACEI had no impact on the net production of renal Ang-(1–7). These results indicate that the combination of *ACE2* overexpression and ACE inhibition is not advantageous over either of the two therapies alone in the treatment of diabetic nephrology. Our results lend support to previous study in which combined therapy of ACE inhibitor and Ang II receptor blocker had no additive benefits on the progression of glomerulopathy (42).

Our study contains several limitations. First, adenoviral gene transfer can only provide a short-term treatment, and the long-term effects of *ACE2* overexpression on diabetic nephropathy are unknown. Further studies using lentiviral vector or adeno-associated viral vector are warranted. Second, the diabetic rats used in the present study represented the early stage of diabetic nephropathy. Whether *ACE2* overexpression is able to improve advanced diabetic nephropathy needs further investigation. Third, further studies using electron microscopic stereology are required to determine whether *ACE2* gene transfer can improve diabetic podocytopathy and prevent glomerular permeability defects.

In conclusion, in a rat model of diabetic nephropathy, *ACE2* gene transfer effectively reduces SBP, urinary albumin excretion, Ccr and GSI. *ACE2* overexpression or ACEI has similar efficacies in ameliorating glomerular injury. Combined use of *ACE2* gene transfer and ACE inhibition offer no additional benefits. The net effect of decreased local Ang II level and increased local Ang-(1–7) level, as well as inhibited renal oxidative stress, are the possible mechanisms involved.

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DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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