# Podocyte-Specific Overexpression of Human Angiotensin-Converting Enzyme 2 Attenuates Diabetic Nephropathy in Mice

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**ABSTRACT**

Angiotensin-converting enzyme 2 (ACE2) is an important component of the renin-angiotensin system (RAS). ACE2 is thought to have a renoprotective effect in diabetic nephropathy because it is capable of degrading profibrotic angiotensin II to potentially protective angiotensin-(1-7). Podocyte death and detachment is a key component of diabetic nephropathy. ACE2 is localized in the podocyte and during a diabetic state, podocyte ACE2 expression is reduced.

The purpose of this study was to determine the effects of podocyte-specific ACE2 overexpression on the course of diabetic nephropathy. Diabetes was induced using streptozotocin in transgenic mice with podocyte-specific overexpression of human ACE2. The following parameters were assessed: systolic blood pressure, glomerular filtration rate, urinary albumin excretion, mesangial and glomerular area, and podocyte number.

Transgenic diabetic mice showed a significant transient attenuated increase in albuminuria, an attenuated increase in mesangial area, decreased glomerular area, and preserved podocyte number, compared to wildtype diabetic mice. This was independent of a change in blood pressure.

This study showed that the podocyte-specific overexpression of human ACE2 attenuates the development of diabetic nephropathy.

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**LIST OF ABBREVIATIONS**

ACE: angiotensin-converting enzyme

ACE2: angiotensin-converting enzyme 2

AGEs: advanced glycation end products

Ang: angiotensin

AT1: angiotensin II type 1 receptor

AT2: angiotensin II type 2 receptor

BP: blood pressure

CKD: chronic kidney disease

hrACE2: human recombinant angiotensin-converting enzyme 2

MAPK: mitogen-activated protein kinase

NADPH: nicotinamide adenine dinucleotide phosphate

PKC: protein kinase C

RAS: renin-angiotensin system

ROS: reactive oxygen species

TG: transgenic

TGF-β: transforming growth factor-β

TG-STZ: streptozotocin-induced diabetic transgenic mice

WT: wild-type

WT-STZ: streptozotocin-induced diabetic wild-type mice

## ACKNOWLEDGMENTS

Parts of this work have been published as a manuscript in Kidney International (Nadarajah et al. 2012) and were presented in abstract form and as a poster at the World Congress of Nephrology in April 2011 (Vancouver, BC, Canada).

All experiments were conducted by Renisha Bose unless stated otherwise. Transgenic mice were already established previous to this study as is described in methods. Mice were under the care of Renisha Bose from birth to study finish at which point they were euthanized by Mr. Anthony Carter under the supervision of Renisha Bose. Processing and storage of tissue was done by Renisha Bose except paraffin embedding, cutting, and slide mounting, which was completed by technical services of the Department of Pathology and Laboratory Medicine (University of Ottawa, Ottawa, ON, Canada). Immunohistochemical staining was done by Dr. Alex Gutsol.

I would like to thank Dr. Kevin Burns for all of his guidance and support throughout this project. He has gone above and beyond what is expected of a supervisor and it is incredibly appreciated. I would also like to thank the members of my lab, Mr. Joe Zimpelmann, Dr. Fengxia Xiao, and Danielle Zimmerman for all of their support during this project as well as the members of the Kidney Research Centre. I would like to thank my family and my husband Reetesh Bose, without whom, none of this would be possible, and my daughter Priana for all the hugs and kisses.

# INTRODUCTION

## *Diabetic Nephropathy*

The function of the kidneys is essential to life. The smallest functional unit of the kidney is the nephron and each human kidney contains approximately one million nephrons. Each nephron is composed of a glomerulus, proximal tubule, loop of Henle, distal tubule, and collecting duct. The glomerulus is composed of specialized capillaries that are surrounded by a basement membrane and epithelial cells known as podocytes. Glomerular podocytes are terminally differentiated highly specialized cells that are key components of the selective permeability of the glomerular basement membrane (Pavenstadt, 2003). The epithelial podocytes have primary, secondary, and tertiary foot processes that interdigitate to form intercellular slit diaphragms (Schnabel et al. 1990). Nephrin is an important podocyte-specific protein that is integral to the development of a functional slit diaphragm. People born with certain nephrin gene mutations develop congenital nephrotic syndrome, which is characterized by massive proteinuria and foot process developmental abnormalities (Kestila et al. 1998).

Diabetes mellitus is characterized by a chronic state of hyperglycemia that may lead to many complications including diabetic nephropathy. Diabetic nephropathy first appears clinically as glomerular hyperfiltration, which leads to microalbuminuria, which is characterized in humans as urinary albumin-excretion of 20-200 μg/min (Cooper 1998). This is accompanied by modest yearly increases in blood pressure (BP). Renal-biopsies reveal significant glomerular damage early on (Cooper 1998). After the microalbuminuria phase there is the clinical albuminuria phase, characterized by urinary

albumin excretion surpassing 200 μg/min. During this phase, there is a decline in glomerular filtration rate (GFR) and generally a progression of increased BP to hypertension. The Unites States Kidney Disease Outcomes Quality Initiative (KDOQI) uses five stages to describe the progression of chronic kidney disease (CKD) and this includes diabetic nephropathy (National Kidney Foundation 2002). The five stages are assigned based on reductions in GFR lasting for three months or more. Stage 1-2 refers to patients with kidney damage and a GFR between 60-90 mL/min/1.73m$^2$. Stage three is described as a moderate stage of CKD and is assigned when the GFR is between 30-59 mL/min/1.73m$^2$. Severe CKD occurs at a GFR between 15-29 mL/min/1.73m$^2$ and when GFR is below 15 mL/min/1.73m$^2$ this is referred to as kidney failure, at which point dialysis will usually be initiated unless a kidney is available for transplant (National Kidney Foundation 2002).

The clinical progression of diabetic nephropathy is well mapped out but the mechanism behind this progression remains unclear. Chronic hyperglycemia leads to changes in the kidney that manifest as thickening of the glomerular basement membrane, glomerular hypertrophy, glomerulosclerosis, mesangial cell expansion, podocyte loss, tubular atrophy, and tubulointerstitial fibrosis and inflammation (Mauer 1994, Fioretto et al. 2007, Balakumar et al. 2009). The mechanisms behind these changes are multi-faceted and remain to be fully understood, although many contributors have been implicated and studied. Aldose reductase is an enzyme that catalyzes the reduction of glucose to sorbitol. It has been implicated in both the progression of diabetic nephropathy because it provides an initial protection against hyperfiltration when inhibited (Pedersen et al. 1991), and the amelioration of diabetic nephropathy by decreasing oxidative stress-induced dicarbonyls

in a transgenic study (Dunlop 2000). The hexosamine pathway converts fructose-6-phosphate to glucosamine-6-phosphate by glutamine: fructose-6-phosphate-amidotransferase, which is the rate-limiting enzyme of the pathway. This enzyme has been implicated in the progression of diabetic nephropathy by inducing production of cytokines such as transforming growth factor-β (TGF-β) and basic fibroblast growth factor, which ultimately induce renal extracellular matrix expansion and fibrosis (Schleicher & Weigert 2000). Protein kinase C (PKC) is activated in a hyperglycemic environment leading to altered gene expression affecting cell proliferation, matrix expression, and apoptosis (Dempsey et al. 2000). This is thought to occur through accumulation of its co-factor, diacylglycerol or reactive oxygen species (ROS), or through advanced glycation end products (AGEs) (Meier et al. 2007). Mitogen-activated protein kinase (MAPK), particularly p38 MAPK is activated in podocytes in nephrotic syndromes and its inhibition prevents podocyte injury and proteinuria (Koshikawa et al. 2005). Thus p38 MAPK may be another contributor to the pathogenesis of diabetic nephropathy. TGF-β and other growth factors and cytokines such as vascular endothelial growth factor, platelet-derived growth factor, insulin-like growth factor, and tumour necrosis factor-α are also increased in a hyperglycemic state and contribute to the induction and progression of diabetic nephropathy (Rincon-Choles et al. 2002, Wolf 2003, Balakumar et al. 2009). Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase is upregulated in a hyperglycemic environment and in turn leads to production of superoxides (Forbes et al. 2008). This increases renal fibronectin and collagen-1 expression as well as mesangial expansion and albuminuria, all of which have been

shown to be attenuated when NADPH oxidase is inhibited by apomycin treatment in diabetic rats (Asaba et al. 2005).

It has been over three decades since Morgensen first showed that antihypertensive treatments could attenuate the rate of renal decline in diabetic patients (Morgensen 1976) and since then, targeting the decrease in vasoactive agents such as angiotensin (Ang) II has proven to be very effective in the treatment and management of diabetic nephropathy. Angiotensin-converting enzyme (ACE) inhibitors and Ang II type 1 (AT1) receptor antagonists are currently the best treatment option for diabetic nephropathy (Lewis et al. 1993, Brenner et al. 2001, Lewis et al. 2001). They are effective in significantly delaying the progression of diabetic nephropathy by blocking the generation and signaling of Ang II, and thus its effects of vasoconstriction, promotion of water and sodium retention, inflammation, ROS production, and pro-fibrotic mediation. However, this efficacy is limited and does not offer a cure for the disease. Managing blood glucose levels and the current symptoms is the best option available.

*Renin-Angiotensin System (RAS)*

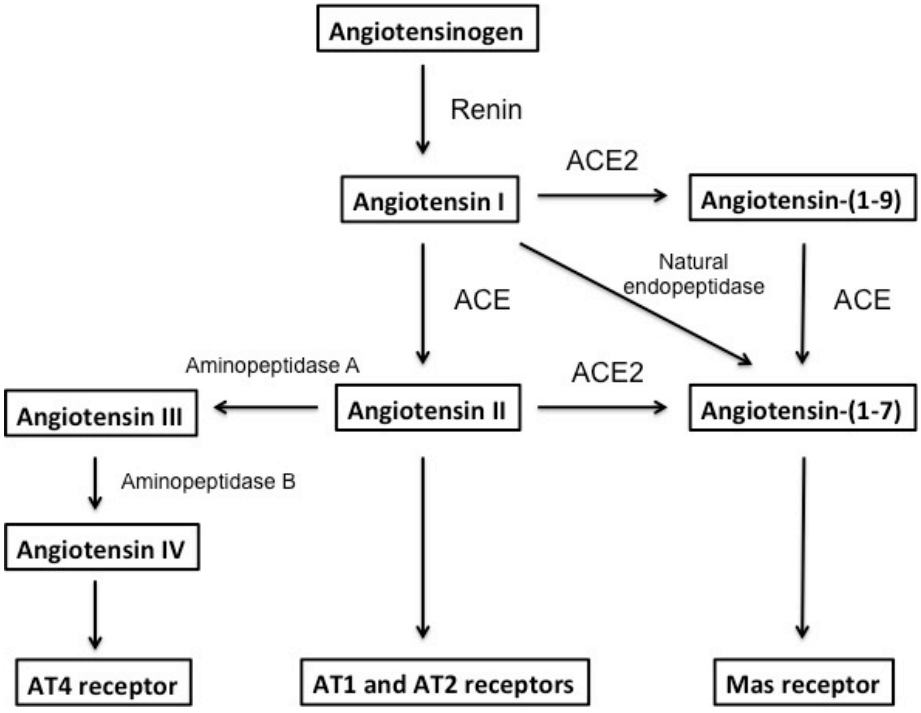


Diagram 1. Renin-Angiotensin Aldosterone System

The classic RAS has been studied for many years. Renin is stored in and released from the juxtaglomerular cells on the kidneys' afferent arterioles into the circulation (Hackenthal et al. 1990) where it cleaves circulating angiotensinogen, which has been formed and released from the liver, to form the decapeptide Ang I (Navar et al. 1997). Ang I is then activated to Ang II by ACE. There are two forms of ACE; somatic and testicular and they are both derived from alternative splicing of the same gene (Ehlers et al. 1989, Hubert et al. 1991). Somatic ACE is present in endothelial cells throughout the body, but is mainly found in the lung, intestine, and kidney. The main function of Ang II in a normal state is to maintain blood pressure in the body in order to deliver sufficient

oxygen to the brain and to other organs. Ang II acts directly on blood vessels as a potent vasoconstrictor and it stimulates the sympathetic nervous system. Ang II also stimulates tubular sodium and chloride reabsorption along with potassium excretion and water retention. It does this directly and by stimulating the adrenal gland to secrete aldosterone from its cortex, which effects the same changes to tubular permeability. Ang II also stimulates the release of anti-diuretic hormone from the posterior pituitary gland, which increases water permeability of the collecting ducts resulting in significant water reabsorption. These functions make Ang II very effective at increasing blood pressure, and in a normal state its function is essential to maintaining adequate oxygen distribution. However, in pathologic states, increased Ang II can be detrimental. Ang II effects its actions by activating its primary G-protein coupled AT1 receptor (Timmermans et al. 1993). Ang II also activates the Ang II type 2 (AT2) receptor; the expression of this receptor decreases dramatically after birth (Norwood et al. 2000). AT2 receptor activation may counteract the actions of AT1 receptor activation by stimulating the formation of bradykinin and nitric oxide (Siragy & Carey 1999). Until recently this has been the most studied pathway, but now we know that the system is more complicated with the discovery of new members such as Ang III, Ang IV, and Ang-(1-7), which are all biologically active (Haulica et al. 2005). Thus along with its classical actions, Ang II has secondary actions through these smaller metabolites and through local activation of its receptors, and so the RAS has been described as an endocrine, paracrine, autocrine, and intracrine system (Re 2003).

There are elements of the RAS in every organ, some organs are more established than others in this regard. For instance, the kidney has been found to express every

component of the RAS. This system is referred to as the intrarenal RAS. Thus substantial levels of Ang II are produced directly in the kidney and exert significant effects locally (Ingert et al. 2002). Renal Ang II is generated primarily from angiotensinogen originating in the liver. A significant proportion is filtered through the glomerulus and reabsorbed by proximal tubule cells in a megalin-dependent fashion (Matsusaka et al. 2012). Ang II is subsequently produced by renal ACE. It is well established that many kidney diseases are characterized by excessive production of Ang II and that targeting proteins responsible for its production and signaling can ameliorate the progression of these diseases. A potential target is another recently discovered member of the RAS; angiotensin-converting enzyme 2 (ACE2).

*Angiotensin Converting Enzyme 2*

ACE2 is a carboxypeptidase that is a homologue of ACE, a key player of the RAS. ACE2 is composed of 805 amino acids and has a single catalytic domain that shares 40% identity with the two catalytic domains found in ACE (Donoghue et al. 2000 & Tipnis et al. 2000). Unlike ACE, ACE2 is capable of cleaving the vasoconstrictive peptide Ang II to its vasodilative counterpart, Ang-(1-7) (Rice et al. 2004). ACE2 is also not inhibited by conventional ACE inhibitors and Ang II receptor blockers (Rice 2004). ACE2 protein is expressed in the endothelial cells of arterials and veins all over the body (Hamming et al. 2004). ACE2 mRNA is high in the kidney, lungs, gastrointestinal tract, heart, and testis (Tipnis 2000, Donoghue 2000, Harmer et al. 2002). In the kidney, ACE2 is located predominantly in the tubular epithelium, but is also found in the glomerular visceral and parietal epithelium as well as the podocytes (Donoghue 2000, Hamming

2004, Li et al. 2005, Ye et al. 2006). ACE2 knock out mice showed significantly higher levels of plasma Ang II and systolic BP (Gurley et al. 2006). ACE2 is also capable of converting Ang 1 to Ang-(1-9) which is subsequently converted to Ang-(1-7) by ACE (Rice 2004). Ang-(1-7) is suspected to have opposite and renoprotective effects compared to Ang II. For instance, treatment of hypertensive diabetic rats with Ang-(1-7) has been shown to decrease renal NADPH oxidase activity and albuminuria (Benter et al. 2008) and the genetic deletion of the Ang-(1-7) Mas receptor leads to glomerular hyperfiltration and proteinuria (Pinheiro et al. 2009).

*ACE2 and diabetes*

Just as ACE inhibitors and Ang II receptor blockers have been shown to be effective treatments against the progression of nephropathy, ACE2 has been thought to be potentially renoprotective (Batlle et al. 2012). To address this theory, ACE2 levels in the kidney in diabetic conditions have been examined in a number of studies. Mizuiri et al. found a decrease in ACE2 compared to ACE in the tubulointerstitium and glomeruli of diabetic patients with overt nephropathy (Mizuiri et al. 2008). Moon et al. found that in streptozotocin (STZ)-induced diabetic rats, ACE2 was decreased also in the glomeruli at early stages of diabetes but increased in tubules (Moon et al. 2008). Reich et al., in keeping with the Mizuiri study, also found a decrease in glomerular and tubular ACE2 protein expression levels in diabetic patients when compared to controls (Reich et al. 2008). Also in keeping with the Mizuiri group, Reich found an increase in ACE protein expression levels. This phenotype apparent in diabetic patients suggests that diabetic nephropathy progresses partly due to decreased ACE2 and an increase in ACE, which

results in increased Ang II levels and decreased Ang-(1-7) levels. In 2009, Wang et al. showed an increase in glomerular and tubulointerstitial mRNA expression levels of ACE and ACE2 in diabetic patients when compared to kidney donor patients. However, at the protein level glomerular ACE and ACE2 levels were again significantly higher but tubulointerstitial ACE remained unchanged, whereas there was a decrease in ACE2 in the diabetic patients (Wang et al. 2009). Although these studies are helpful in characterizing the phenotype of ACE2 under diabetic conditions, further studies are needed to understand if and how ACE2 affects the progression of diabetic nephropathy.

Studies have already begun to address this need. In 2007, Wong et al. used an ACE2 knockout mouse crossed with a type 1 diabetic model mouse to determine what effect the loss of ACE2 would have on the progression of diabetic nephropathy. They found increased albuminuria in the diabetic ACE2 knockout mice, compared to the diabetic mice with ACE2 present (Wong et al. 2007). They also found increased mesangial matrix and glomerular basement membrane thickness, along with increased fibrosis (Wong 2007). Soler et al. conducted a study whereby ACE2 activity was blocked by the administration of the specific ACE2 blocker, MLN-4760 to streptozotocin-induced diabetic mice (Soler et al. 2007). They found similar results in that proteinuria was increased along with the expansion of the glomerular matrix (Soler 2007). Both of these studies suggest that the progression of nephropathy in diabetes is partly dependent on the activity of ACE2, in a renoprotective way.

Oudit et al. administered human recombinant ACE2 (hrACE2) to male diabetic Akita mice daily for 4 weeks to assess the effects on the progression of diabetic nephropathy (Oudit et al. 2010). They found a decrease in BP for the hrACE2-treated

mice when compared to the non-treated diabetic mice, as well as a decrease in glomerular area, mesangial area, and glomerular basement membrane thickness (Oudit 2010). They concluded that hrACE2 treatment attenuated diabetic kidney injury in Akita mice.

Although the effects of administering circulating hrACE2 in diabetic mice have been addressed in the literature, the effect of overexpression of localized ACE2 in the kidney, independent of BP, has yet to be studied.

*The podocyte, the RAS, and diabetic nephropathy*

Podocytes are terminally differentiated cells that are essential to maintain the integrity of the glomerular filtation barrier (Pavenstadt, 2003). Podocyte precursors are polarized cuboidal epithelial cells that undergo differentiation and growth of octopus-like processes that ultimately surround the basement membrane and capillaries creating a selective filtration barrier in the glomerulus. There are three levels of branching of these actin-based processes. The tertiary or foot processes interdigitate to form the slit diaphragm and it is these processes which undergo morphological changes seen in pathological states involving spreading and shortening of these processes known as "foot process effacement" (Garg & Holzman 2012). It has been documented that podocyte death and detachment is directly correlated with the progression of diabetic nephropathy (Susztak et al. 2006, Toyoda, 2007). Urinary podocytes have been observed in diabetic patients with albuminuria, whereas this is not the case for healthy controls (Nakamura et al. 2000). Ang II negatively affects the podocyte. Ding and colleagues demonstrated that Ang II induces apoptosis in rat podocytes in vitro, an effect that is blocked when both AT1 and AT2 receptors are blocked by losartan and PD-123319 respectively. They also

showed that Ang II induced an increase in TGF-β (Ding et al. 2002). Durvasula et al. showed that mechanical strain on conditionally immortalized mouse podocytes induced an increase in Ang II, AT1 receptors, and TGF-β mRNA, which resulted in an Ang II dependent activation of podocyte apoptosis (Durvasula et al. 2004). ACE2 is localized in the podocyte and during a diabetic state, glomerular ACE2 expression is reduced (Ye 2006). These data suggest that enhancing podocyte ACE2 could have beneficial effects in the course of diabetic nephropathy. As such this thesis aims to determine the progression of diabetic nephropathy in mice that transgenically overexpress ACE2 specifically in the glomerular podocyte.

## HYPOTHESIS

The specific overexpression of ACE2 in the podocyte will have a protective effect against the progression of diabetic nephropathy.

## OBJECTIVES

The main objective of this study is to determine the effects of podocyte-specific human ACE2 overexpression on the progression of diabetic nephropathy in a diabetic mouse.

The effect of ACE2 overexpression on systolic BP, glomerular filtration rate (GFR), albuminuria, mesangial expansion, glomerular area, podocyte number, and urinary ACE2 activity will be assessed.

## MATERIALS AND METHODS

*Transgenic mouse*

The podocyte-specific ACE2 transgenic mice had already been established in our laboratory prior to this study. The cDNA encoding the open reading frame of the human ACE2 gene, which contained a double hemagglutinin (2XHA) epitope tag at the 5' end, was inserted immediately downstream from the murine nephrin promoter (provided by Dr. C. Kennedy, Ottawa Hospital Research Institute (OHRI), University of Ottawa, Ottawa, ON, Canada), and cloned into the *XhoI* restriction site of the expression vector pcDNA3 (Invitrogen, Carlsbad, CA, USA). The correct orientation of the insert was confirmed by DNA sequencing (StemCore Laboratories, OHRI, Ottawa, ON, Canada). This vector was then microinjected into FVB/n mouse embryos obtained from Charles River Laboratories, Inc. (Wilmington, MA) at a concentration of 2.0 ng/μL (Mouse embryo injections performed by Mr. Yves de Repentigny from the laboratory of Dr. R. Kothary, OHRI, Ottawa, ON, Canada). Founder mice were identified by DNA genotyping, using PCR with primers specific for the expression vector (forward primer; 5'-CAGGGAAGACAGCAACAAACAAG-3', reverse primer; 5'-GAGAAGGAGCCAGGAAGAGCTT-3'), which generated a band of 125 bp in transgenic mice. Three separate mouse founder lines were characterized for podocyte ACE2 expression, and one founder line was selected for this study.

Dr. Alex Gutsol did immunofluorescent staining to locate the ACE2 transgenic protein in the glomerulus. He used a rabbit polyclonal antibody to hemagglutinin (1:1000, Abcam, Cambridge, MA, USA) followed by a secondary antibody (1:2000/red), and a

rabbit polyclonal anti-synaptopodin antibody (1:1000, Santa Cruz Biotechnology Inc.) followed by another secondary antibody (1:2000/green).

*Diabetes model*

FVB/n mice were used as opposed to the traditional C57BL/6J mice due to evidence that the C57BL/6J mice are relatively resistant to STZ-induced diabetes (Qi et al. 2005). Male mice were used in this study because it has been shown that estrogen protects females from pancreatic beta-cell apoptosis (Le May et al. 2006). At 8 weeks of age male transgenic (TG) and wildtype (WT) FVB/n mice were injected intraperitoneally over 5 consecutive days with either STZ (50 μg/g per day; Sigma-Aldrich, St Louis, MO, USA) (WT-STZ and TG-STZ) or sodium-citrate vehicle (WT and TG) (Qi 2005). Hindlimb blood glucose levels were monitored by glucometer readings and spot urines were taken weekly. Urine was analyzed at 16 weeks for ketones using Ketostix (Bayer HealthCare LLC, Elkhart, IN, USA). All mice were housed and cared for in the Animal Care Facility at the University of Ottawa with free access to food and water. All protocols were approved by the University of Ottawa Animal Care Committee and conducted according to the guidelines of the Canadian Council on Animal Care.

*Tissue preparation*

At 8 and 16 weeks post vehicle- or STZ-injections mice were anesthetized using isoflurane and euthanized by cardiac puncture, followed by phosphate buffered saline (PBS) perfusion. The heart and kidneys were dissected and weighed. One kidney was snap frozen in liquid nitrogen and then stored at -80ºC to be used for ACE2 enzymatic

activity assays, and immunoblots. The second kidney was transferred to 4% paraformaldehyde to be paraffin-embedded for histological analysis.

*Plasma analysis and hematocrit measurements*

At the time of euthanasia, blood was collected from the mice with a heparinized needle and transferred to Eppendorf tubes kept on ice. Plasma was separated by centrifugation at 3,000 g for 10 min at 4ºC. Plasma electrolytes, ($Na^+$, $K^+$, $Cl^-$, $CO_2$, $Ca^{2+}$, $Mg^{2+}$, $PO_4^{-2}$) glucose, albumin, urea, creatinine, triglycerides, and cholesterol were assayed by Dr. Gabrielle Cherton-Horvat using the Synchron CX5 Delta (Beckman Coulter, Fullerton, CA, USA). Blood was also collected in heparinized microhematocrit capillary tubes (Baxter, Deerfield, IL, USA) to measure hematocrit. Tubes were spun in a micro-capillary centrifuge (IEC, Needham HTS, MA, USA) for 10 min at room temperature and hematocrit was determined using a micro-capillary reader (Model CR, IEC, Needham HTS).

*Systolic blood pressure (BP) measurements*

Systolic BP was measured using tail-cuff plethysmography (BP-2000; Visitech Systems, Apex, NC, USA). Mice were trained at 6 weeks of age for 5 consecutive days and a baseline measurement was taken at 7 weeks of age as the average of measurements taken over 3 consecutive days. Systolic BP was then measured weekly from 1 week after STZ or vehicle injection until 16 weeks later.

*Measurement of glomerular filtration rate*

The glomerular filtration rate (GFR) was measured in mice at 16 weeks of study using plasma fluorescein isothiocyanate (FITC)-inulin clearance kinetics, following the Animal Models of Diabetic Complications Consortium (AMDCC) protocol entitled Determination of Glomerular Filtration Rate in Conscious Mice using FITC-inulin exempting some differences (available at http://www.DiaComp.org). A 5% FITC-inulin solution is prepared in 2 mL of 0.9% NaCl and heated in boiling water to allow the solution to homogenize. This is then placed in a dialysis membrane and suspended in 1000 mL of 0.9% NaCl for 24 hrs to remove any unbound FITC. The solution is sterilized by filtration through a 0.22 μm filter. Unlike the AMDCC protocol, mice were restrained in a large tube with air-holes and injected with 5% FITC-inulin (3.74 μL/g body weight) by tail vein. Approximately 20 μL of blood was then collected from the saphenous vein located at the inner thigh at 3, 7, 10, 15, 35, 55, and 75 minutes. Samples were spun at 2000 g for 10 min to separate plasma.

Samples are then buffered to pH 7.4 by mixing 10 μL of plasma to 90 μL of 0.5 M HEPES buffer. 100 μL of sample were loaded onto a 96-well plate and fluorescence was determined at each time point using the FLUOstar Galaxy fluorometer (BMG Labtechnologies, Durham, NC, USA, software version 4.31-0) (excitation 485 nm, emission 538 nm). The plasma fluorescence decay was plotted on a two-phase exponential decay curve using nonlinear regression (GraphPad Prism, Software version 4.02, San Diego, CA, USA). GFR was calculated using the equation: $GFR=I(A/\alpha + B/\beta)$, where I is the amount of FITC-inulin delivered by tail vein, A (Span 1) and B (Span 2) are the y-intercepts of the rapid and slow decay rates respectively, and $\alpha$ and $\beta$ are the

decay constants for the distribution (rapid) and elimination (slow) phases respectively. GFR values were corrected for body weight and expressed as μLs of FITC-inulin cleared per minute per gm body weight (Dilauro et al. 2010).

*Glomerular isolation*

Mice were anesthetized using isoflurane and then perfused through the heart with $4x10^7$ Dynabeads (Invitrogen Dynal AS, Oslo, Norway) diluted in 20 ml of PBS. The kidney cortices were digested in collagenase Type 1 (1.0 mg/ml; Worthington Biochemical, Lakewood, NJ, USA) and DNase 1 (0.1 mg/ml; Roche Diagnostics, Indianapolis, IN, USA) at 37ºC for 30 min, and tissue was then passed through a 100 μm sieve. Glomeruli were isolated using a magnetic particle concentrator as previously described (Takemoto et al. 2002). Glomeruli purity was assessed qualitatively by light microscope. Glomeruli were assessed for ACE2 activity and endogenous and transgenic ACE2 protein expression.

*Immunoblotting*

Kidney cortical tissue was homogenized in lysis buffer (62.5 mM Tris-HCl (pH 6.8), 2% w/v sodium dodecyl sulphate, 10% glycerol, 50 mM dithiothreitol, 0.01% w/v bromophenol blue) then boiled for 5 min and centrifuged at 9,500 g for 10min to remove cellular debris. Glomeruli were isolated and sonicated in reaction buffer (25 mM HEPES buffer, 125 mM NaCl, 10 μL $ZnCl_2$, pH 7.4) and then centrifuged at 2,500 g for 5 min at 4ºC. The supernatant was then spun at 21,000 g for 15 min at 4ºC and the pellet was incubated with 0.5% Triton X-100 for 10 hrs at 4ºC. Samples were then centrifuged at

21,000 g for 6 min and the protein concentration of the supernatant was determined using the Bradford method (Bio-Rad Laboratories, Mississauga, ON, Canada). Samples with 7 μg of total protein each were run on 10% SDS-polyacrylamide gels and then transferred to nitrocellulose membranes (Bio-Rad). Membranes were blocked for one hr at room temperature with 5% skim milk in Tris-buffered saline (pH 7.6) containing 0.1% Tween 20 (TBS-T). Blocking agent was removed and membranes were washed with 0.1% TBS-T for 5 min 3 consecutive times. A goat polyclonal human ACE2 antibody (1:500) (R&D Systems Inc., Minneapolis, MN, USA) was then incubated with membranes for 16 hr at 4°C. Membranes were washed again for 3 consecutive 5 min intervals. Blots were then probed with horseradish peroxidase-conjugated secondary antibody (1:2000, Jackson Immuno-Research Laboratories, West Grove, PA). Blots were then washed for 4 consecutive 5 min intervals. Proteins were detected by enhanced chemiluminescence (ECL) (Amersham, GE Healthcare, Buckinghamshire, UK) on high performance chemiluminescence film (Amersham Hyperfilm, GE Healthcare).

*Urinary albumin excretion*

At 4 and 16 weeks after injections, urine was collected over a 24 hour period by housing mice in metabolic cages. Urinary albumin excretion was measured using a murine albumin ELISA (Bethyl Laboratories Inc., Montgomery, TX, USA).

*ACE2 enzymatic activity assay*.

Kidney cortical, glomerular, plasma, and urinary ACE2 enzymatic activity was determined following incubation with a synthetic ACE2 substrate (Mca-Ala-Pro-

Lys(Dnp)-OH (AnaSpec, San Jose, CA, USA). Kidney cortex and glomerular tissue were prepared for this assay as previously described (Joyner et al. 2007). 20 μg of cortical tissue, 1-2 μg of glomerular tissue, and 5 μL of plasma or urine was each incubated separately with assay buffer (50 mM MES, 300 mM NaCl, 10 μM $ZnCl_2$, pH 6.8) with protease inhibitors added (1 mM N-ethylmaleimide, 1 mM phenylmethylsulfonyl fluoride, plus 30 μM substrate, with and without $10^{-6}$ M MLN-4760 (Millenium Pharmaceuticals, Boston, MA (Soler 2007)), a specific ACE2 inhibitor, to a total of 100 μL per well. The reaction was allowed to proceed at room temperature and fluorescence was observed periodically using the FLUOstar Galaxy fluorometer (BMG Labtechnologies, Durham, NC, USA, software version 4.31-0) (excitation 320 nm, emission 405 nm) until a peak increase in fluorescence was observed. The activity with MLN-4760 was subtracted from the activity without MLN-4760 and that value was normalized to a standard curve created using recombinant human ACE2 (R&D Systems, Catalog number: 933-ZN, Minneapolis, MN, USA). Urine ACE2 activity was corrected for creatinine excretion, measured using a murine creatinine assay kit (Creatinine Companion, Exocell, Philadelphia, PA, USA).

*Kidney histology*

Dissected kidneys were incubated with 4% paraformaldehyde for 48 hrs at 4ºC. They were subsequently dehydrated, paraffin-embedded, and cut into sections 4 μm thick. Sections were stained with Periodic-acid Schiff (PAS) reagent. Assessment of the glomerular and mesangial cross-sectional areas was done for six mice in each group (10 glomeruli per mouse) in a blinded fashion, under 40X magnification (Zeiss AX10

microscope, Carl Zeiss Canada Ltd, Toronto, ON, Canada). High-resolution digital images were generated using AxioVision (AxioVs40 V4.6.1.0, Carl Zeiss Imaging Solutions). Mesangial and glomerular areas were assessed by pixel counts on each section, and results are reported in $\mu m^2$.

Glomerular podocyte number was determined by counting nuclei in glomerular cells that stained positively for the podocyte protein WT-1 (Yu et al. 2005). Staining was conducted by Dr. Alex Gutsol (Kidney Research Centre, OHRI, University of Ottawa, Ottawa, ON, Canada). Kidney tissue was immersion fixed in 10% neutral buffered formalin, embedded in paraffin and cut into 5 µm-thick sections. After deparaffinization and hydration, heat-induced epitope retrieval was performed in a buffer consisting of 10 mM sodium citrate and 0.05% Tween 20 (pH 6.0). Sections were blocked with 10% donkey serum in 1% BSA in PBS for 1 hr and then incubated with a primary rabbit polyclonal antibody to WT-1 (1:500, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Slides were incubated for 16 hr at 4ºC and endogenous peroxidase was blocked with 0.3% $H_2O_2$ in PBS for 30 min. A secondary biotinylated donkey anti-rabbit IgG (1:200) was applied for 1 hr at room temperature, followed by streptavidin-conjugated horseradish peroxidase (Vector Laboratories, Burlingame, CA, USA) for 30 min. Slides were counterstained with Mayer's hematoxylin for 40 sec, dehydrated, cleared in xylene and mounted. Immunopositive cells were counted in a blinded fashion under light microscopy.

*Statistics*

Data are presented as mean ±s.e. Data were analyzed using GraphPad Prism (Software version 4.02, San Diego, CA, USA). For multiple comparisons, analysis was by one-way analysis of variance followed by Bonferroni and Newman-Keuls post-comparison test. For comparisons involving two groups, Student's t-test was used. A P-value of $<0.05$ was considered statistically significant.

## RESULTS

*Characterization of ACE2 TG mice*

Prior to the start of this study, the ACE2 TG mice had already been established in our laboratory. Direct immunofluorescence revealed co-localization of the human ACE2 hemagglutinin epitope tag with the podocyte-specific protein synaptopodin, and RT-PCR demonstrated expression of human ACE2 in the glomeruli at levels ~50 fold higher than in kidney cortex and not significantly in heart, brain, liver, spleen, or lung (Nadarajah et al. 2012). It was also shown that endogenous mouse ACE2 levels were not significantly affected by the presence of the transgene (Nadarajah 2012).

In the current studies, glomeruli from TG mice were isolated and the human ACE2 protein was detected by immunoblot as a single band of ~120 kDa (Figure 1A). Only the endogenous murine ACE2 at ~100 kDa was detectable in WT mice. In TG mice, the human ACE2 protein was not readily detectable in kidney cortical homogenates by immunoblot analysis. As further evidence for glomerular ACE2 overexpression, ACE2 activity levels were significantly increased in glomeruli from TG mice versus WT mice (Figure 1B: 2.82-fold increase in TG vs WT mice, $p<0.02$, n=9-10).

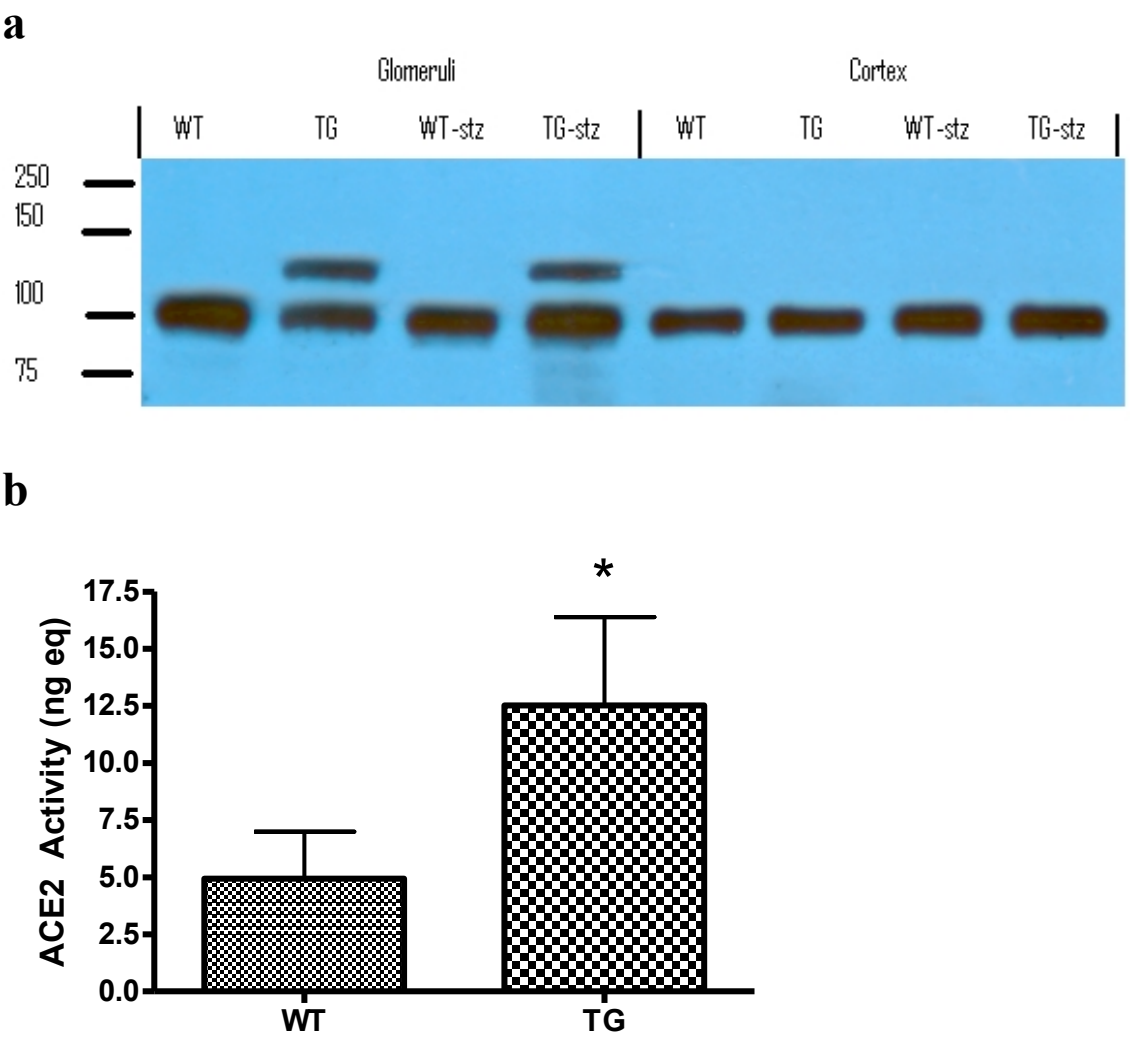


Figure 1. **Characterization of transgenic (TG) mice.** (**a**) Immunoblot analysis of ACE2 in glomeruli and cortex tissue. Shown is a representative immunoblot of ACE2 protein in glomeruli and kidney cortex from WT and TG mice, with or without STZ, at 16 weeks post injections (n=2). The human ACE2 transgenic protein is present as a single band at ~120 kDa in glomeruli from TG and TG-STZ mice. Endogenous mouse ACE2 protein is observed in all lanes as a single band at ~100 kDa. (**b**) Glomerular ACE2 activity. ACE2 activity was measured in isolated glomeruli from WT or TG mice, as described in Materials and Methods. *P<0.02, n=9 (WT), n=10 (TG).

*STZ diabetes: whole animal data*

Tables 1 and 2 depict whole animal data regarding body and organ weights, and plasma analysis, respectively. WT and TG mice treated with STZ (WT-STZ and TG-STZ, respectively) developed severe diabetes, with significantly increased plasma glucose compared to vehicle-treated mice, and with no significant difference between the two STZ groups. TG mice had significantly lower body weights when compared to WT mice at 16 weeks of the trial ($p<0.01$). Body weight was further decreased in STZ-treated mice ($p<0.001$) (Table 1). Kidney weight to body weight ratio was significantly higher for STZ-treated mice ($p<0.001$), indicating that these mice underwent significant kidney hypertrophy. There was no significant difference between all groups with respect to cardiac weight to body weight ratios. Kidney weight to body weight ratios were not significantly different between TG and WT mice, with or without STZ (Table 1). Despite the high levels of plasma glucose, diabetic mice did not develop ketosis, as determined by urine ketone testing at 16 weeks (not shown), and absence of metabolic acidosis (Table 2). Mortality rate was 12.2% (11/90) for all mice in the study, with no significant differences amongst the four groups.

As noted, glomerular ACE2 activity remained significantly high in TG-STZ mice compared to WT-STZ mice ($3.30 \pm 0.88$-fold increase, $P<0.01$, $n=5$). Glomerular ACE2 overexpression was also confirmed by immunofluorescent staining of kidney sections for ACE2. Results showed a marked increase in glomerular TG ACE2 expression compared to WT mice ($P<0.001$) which was not affected by diabetic conditions 16 weeks after STZ treatments (Figure 2). Glomerular expression of human ACE2 protein also remained robust by immunoblot after 16 weeks (Figure 1A).

Table 1. **Body weights, kidney and heart weight ratios, blood glucose**

| | WT | TG | WT-STZ | TG-STZ |
|---|---|---|---|---|
| BW (g) | 36.2±0.7 (6) | 31.1±1.1 (7)* | 27.2±0.5 (8)** | 25.9±0.5 (6)** |
| KW/BW (g/kg) | 11.4±0.4 (6) | 12.2±0.2 (7) | 20.8±0.7 (8)*** | 21.0±1.3 (6)*** |
| H/BW (g/kg) | 3.9±0.3 (6) | 4.3±0.1 (7) | 4.3±0.2 (8) | 4.2±0.2 (6) |
| Gluc (mM) | 19.1±1.0 (6) | 18.3±1.3 (6) | 55.5±8.0 (6)† | 72.2±17.1 (6)† |

Abbrevations: BW, body weight; KW, total kidney weights; H, heart weight; Gluc, plasma glucose.

Values are means ± s.e. (N).

*P<0.01 vs. WT.

**P<0.001 vs. WT and P<0.01 vs. TG.

***P<0.001 vs. WT and TG.

†P<0.05 vs. WT and TG.

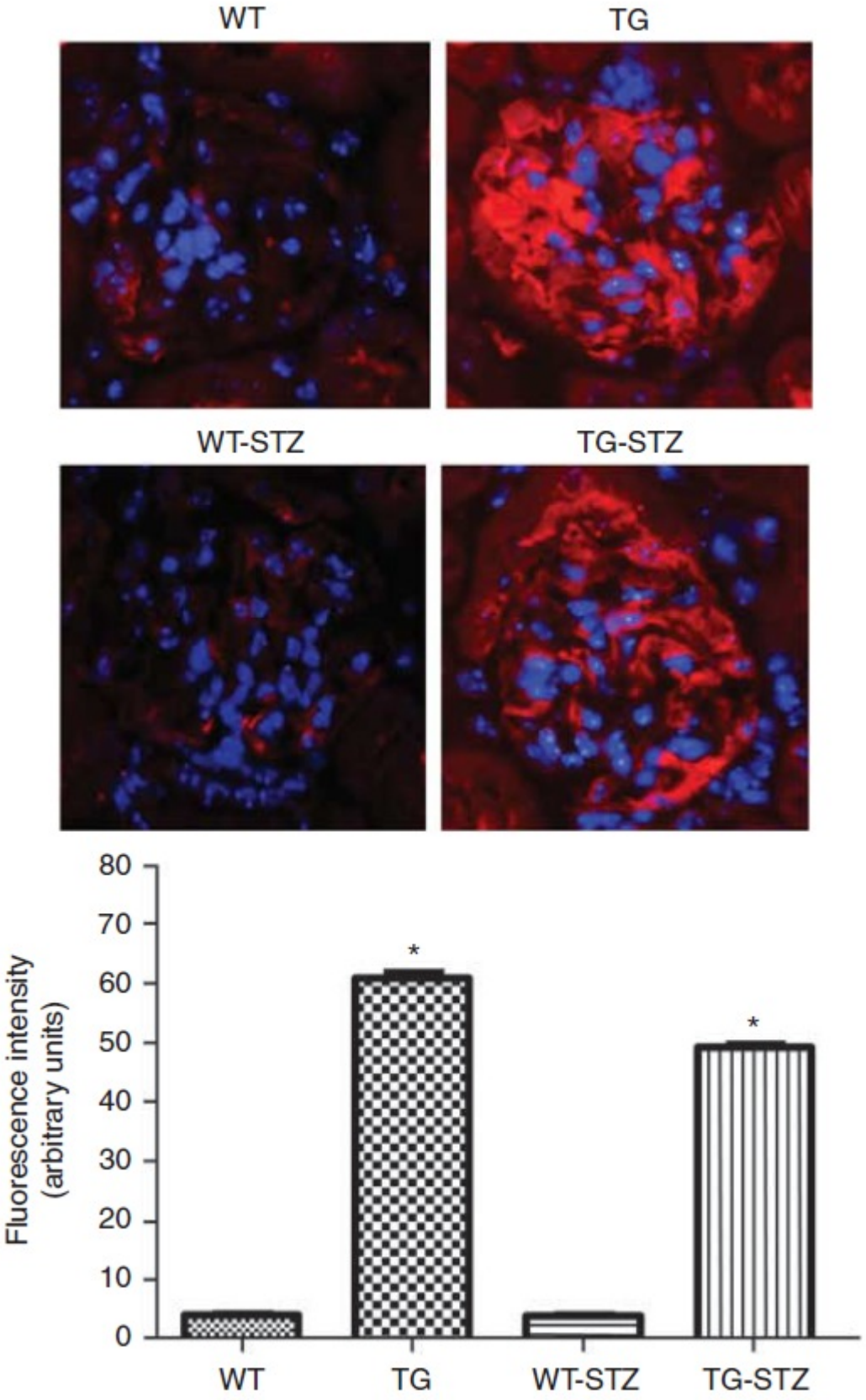
WT
TG
WT-STZ
TG-STZ
Fluorescence intensity (arbitrary units)
80
70
60
50
40
30
20
10
0
*
*
WT
TG
WT-STZ
TG-STZ

Figure 2. **Glomerular expression of ACE2 by immunofluorescence at 16 weeks.** Graph depicts semiquantitative analysis of ACE2 immunofluorescent staining in glomeruli at 16 weeks. Values are means ± s.e. *P<0.001 vs. WT and WT-STZ. Representative images depict ACE2 staining (red) in glomeruli along with Hoechst nuclear stain (blue). Original magnification X640. n=100 glomeruli per group (6-11 mice per group).

*Plasma components at 16 weeks*

16 weeks post-injections, mice were sacrificed and plasma was separated and analyzed. There were no significant differences between all groups with respect to plasma $Na^+$. $K^+$. $Cl^-$, $CO_2$, albumin, creatinine, total protein, phosphorus, blood urea nitrogen, and cholesterol (Table 2). However, at 16 weeks, TG-STZ mice had lower serum $Ca^{2+}$ levels compared to vehicle-treated wildtype mice ($p<0.05$). The concentration of blood $Mg^{2+}$ was significantly higher in STZ-treated versus vehicle-treated mice ($p<0.05$). Plasma triglycerides were significantly higher in WT-STZ mice compared to all other groups ($p<0.01$) (Table 2). Hematocrit was significantly higher in WT-STZ mice compared to vehicle-treated mice ($p<0.01$). The increase in hematocrit in TG-STZ mice, however, did not reach statistical significance compared to vehicle-treated mice (Table 2).

Table 2. **Plasma components at 16 weeks**

| | WT | TG | WT-STZ | TG-STZ |
|---|---|---|---|---|
| $Na^+$ (mM) | 163.0 ±4.4 | 156.5 ±2.2 | 150.1 ±1.9 | 146.4 ±4.2 |
| $K^+$ (mM) | 6.1 ±0.2 | 5.4 ±0.2 | 5.1 ±0.4 | 5.7 ±0.7 |
| $Cl^-$ (mM) | 118.7 ±5.5 | 123.1 ±1.4 | 114.7 ±1.1 | 113.6 ±3.0 |
| $CO_2$ (mM) | 19.0 ±0.8 | 16.7 ±0.9 | 16.4 ±1.6 | 14.0 ±1.6 |
| $Ca^{2+}$ (mM) | 2.4 ±0.1 | 2.3 ±0.05 | 1.9 ±0.1 | 1.8 ±0.1* |
| Albumin (g/L) | 12.5 ±0.3 | 12.7 ±0.4 | 12.4 ±0.4 | 11.8 ±0.8 |
| Creatinine (μM) | 24.8 ±1.1 | 25.7 ±0.9 | 35.1 ±1.8 | 51.3 ±15.8 |
| $Mg^{2+}$ (mM) | 0.9 ±0.05 | 0.9 ±0.1 | 1.1 ±0.1* | 1.1 ±0.1* |
| Tot Protein (g/L) | 50.7 ±1.5 | 49.3 ±1.2 | 47.0 ±1.4 | 48.8 ±3.8 |
| Phosphorus (mM) | 2.6 ±0.2 | 2.4 ±0.2 | 3.2 ±0.3 | 3.6 ±0.7 |
| BUN (mM) | 8.4 ±0.5 | 8.1 ±1.0 | 12.1 ±1.0 | 20.8 ±7.7 |
| Cholesterol (mM) | 3.8 ±0.2 | 3.3 ±0.1 | 3.5 ±0.1 | 3.1 ±0.5 |
| Triglycerides (mM) | 2.7 ±0.4 | 3.4 ±0.5 | 11.4 ±2.2** | 5.4 ±1.2 |
| Hematocrit (%) | 36.2±0.5 (9) | 36.0±0.9 (7) | 40.1±0.7 (11)** | 38.5±1.1 (8) |

Values are means ±SE (hematocrit N). N=5-8. *p<0.05, **p<0.01 vs. WT. BUN: blood urea nitrogen.

*Systolic BP is increased in STZ-treated mice*

Systolic BP was measured weekly throughout the study. By 15 weeks BP was significantly higher in both STZ-treated groups compared to vehicle-treated mice ($p<0.05$) and this difference persisted at 16 weeks ($p<0.01$) (Figure 3). However, systolic BP did not significantly differ between WT-STZ and TG-STZ mice at any point in the study, nor did it differ between WT and TG mice (Figure 3).

*GFR*

At 16 weeks post-injections, GFR was significantly higher in STZ-treated groups compared to WT and TG mice ($p<0.05$), but there was no significant difference between the two STZ groups (Figure 4).

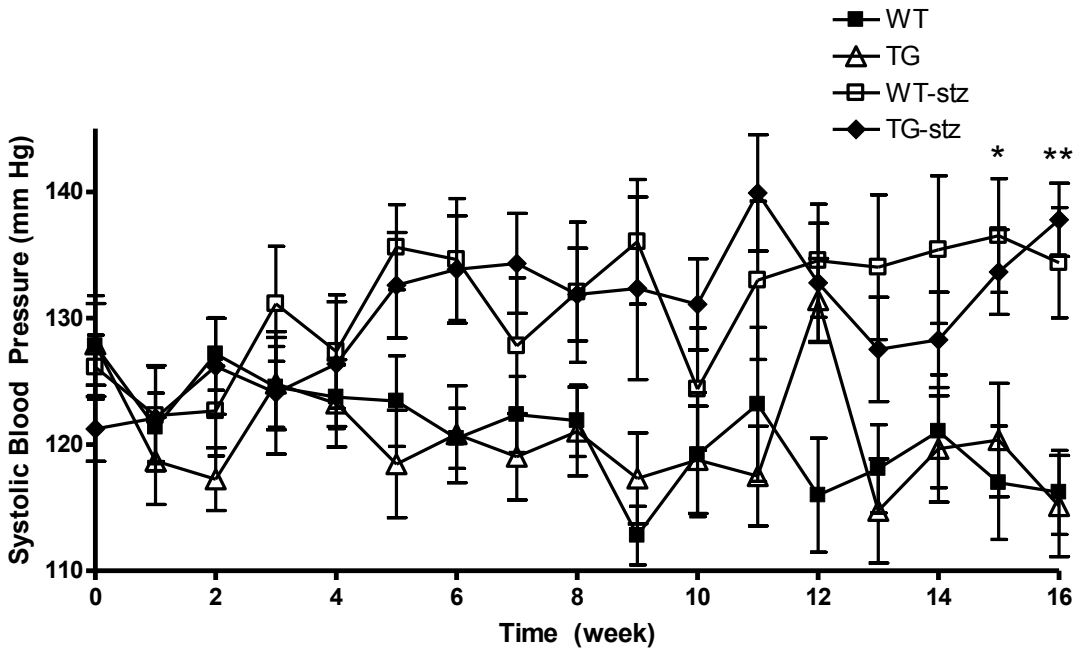


Figure 3. **Systolic Blood Pressure (BP)**. Systolic BP was measured weekly with tail-cuff plethysmography. Values are means ± s.e. **P<0.01 vehicle-treated vs. streptozotocin (STZ)-treated, *P<0.05 vehicle-treated vs. STZ-treated. There was no significant difference between WT-STZ and TG-STZ mice throughout study; n=14 mice per group.

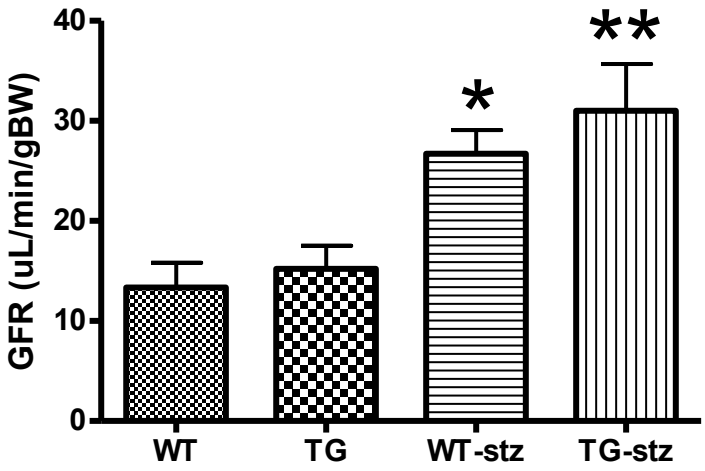


Figure 4. **Glomerular filtration rate (GFR).** Graph shows GFR measurements in mice 16 weeks after injections. Values are means ± s.e. *P<0.05 vs. WT and TG, **P<0.01 vs. WT and TG; n=4-6.

*Albuminuria*

At 4 weeks, WT-STZ mice had significantly increased levels of albuminuria as compared to vehicle-treated mice (Figure 5A). In contrast, TG-STZ mice demonstrated no significant increase in albuminuria at 4 weeks ($p<0.05$ vs WT-STZ, n=5). However, there was no significant difference in albuminuria between WT-STZ and TG-STZ mice at 8 weeks (WT-STZ: 1,312 ± 527 vs TG-STZ: 1,233 ± 380 μg per 24 h; p=NS, n=6). Similarly, at 16 weeks, both WT-STZ and TG-STZ mice demonstrated markedly increased levels of albuminuria, with no significant difference between the two groups (Figure 5B).

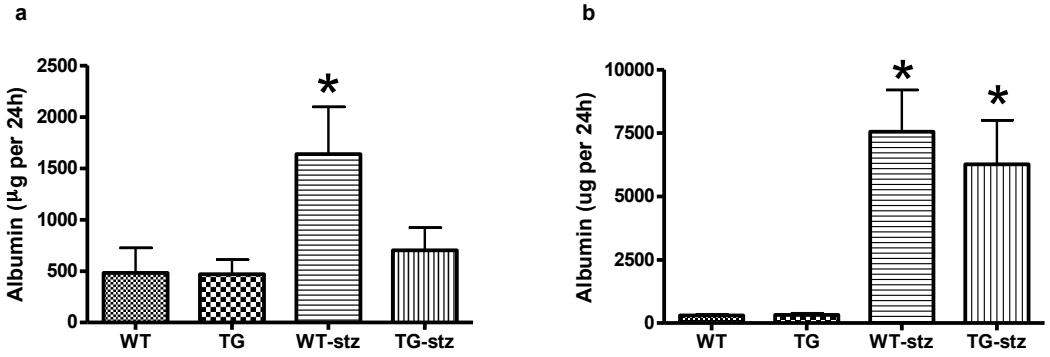


Figure 5. **Urinary albumin excretion.** (**a**) The 24h urinary albumin excretion in mice at 4 weeks post injections. *P<0.05 vs. all other groups (WT, TG, and TG-STZ mice); n=5. (**b**) The 24h urinary albumin excretion at 16 weeks. *P<0.01 vs. WT and TG. There was no significant difference between WT-STZ and TG-STZ mice; n=14.

*Histology*

Histologic analysis was performed in a blinded fashion on kidney sections at 8 and 16 weeks. At 8 weeks, WT-STZ mice and TG-STZ mice demonstrated increases in mesangial and glomerular areas compared to vehicle-treated mice (Figure 6A and B). However, the increase in mesangial area was significantly attenuated in TG-STZ mice. At 16 weeks, mesangial and glomerular areas remained significantly higher in WT-STZ and TG-STZ groups, compared to non-diabetic mice, although glomerular diameter was significantly diminished in TG-STZ mice, compared to WT-STZ mice (Figure 7A and B).

Podocyte numbers were assayed at 16 weeks by counting numbers of glomerular nuclei staining positively for WT-1. As shown in Figure 8, WT-STZ mice demonstrated a significant decrease in average podocyte number per glomerulus, compared to vehicle-treated mice ($p<0.05$). In contrast, TG-STZ mice did not exhibit a significant change in podocyte number compared to non-diabetic mice.

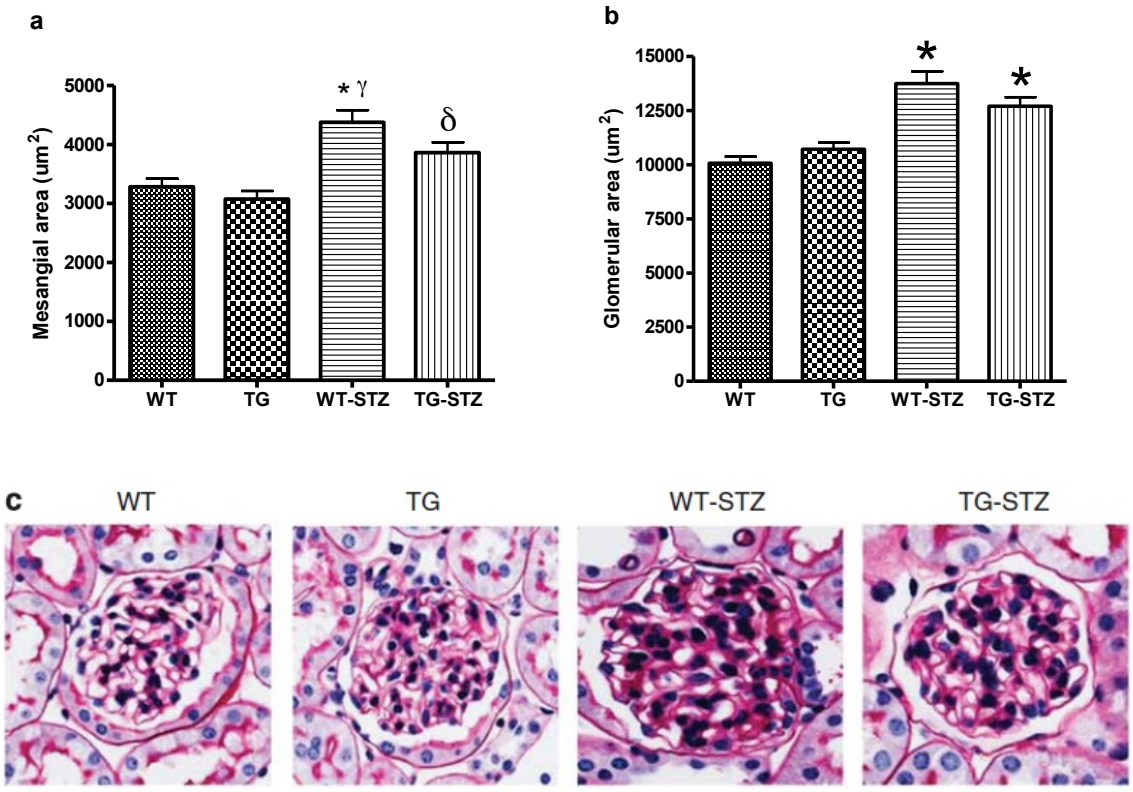


Figure 6. **Effect of podocyte angiotensin-converting enzyme (ACE2) overexpression on mesangial and glomerular area at 8 weeks.** Graphs depict (**a**) mesangial and (**b**) glomerular areas in mice at 8 weeks post injections. (**a**) Mesangial area at 8 weeks. Values are means ± s.e. (n=40 glomeruli per group of 4 mice). *P<0.001 vs. WT and TG, $^{\delta}$P<0.02 vs. WT and TG, $^{\gamma}$P<0.03 vs. TG-STZ. (**b**) Glomerular area at 8 weeks. Values are means ± s.e. (n=40 glomeruli per group of 4 mice). *P<0.001 vs. WT and TG. There was no significant difference between WT-STZ and TG-STZ mice. (**c**) Representative photomicrographs depicting periodic acid-Schiff (PAS)-stained glomeruli in WT, TG, WT-STZ, and TG-STZ mice at 8 weeks. Glomerular hypertrophy and increased mesangial staining are evident in STZ groups as compared to non-STZ mice. Original magnification 640X.

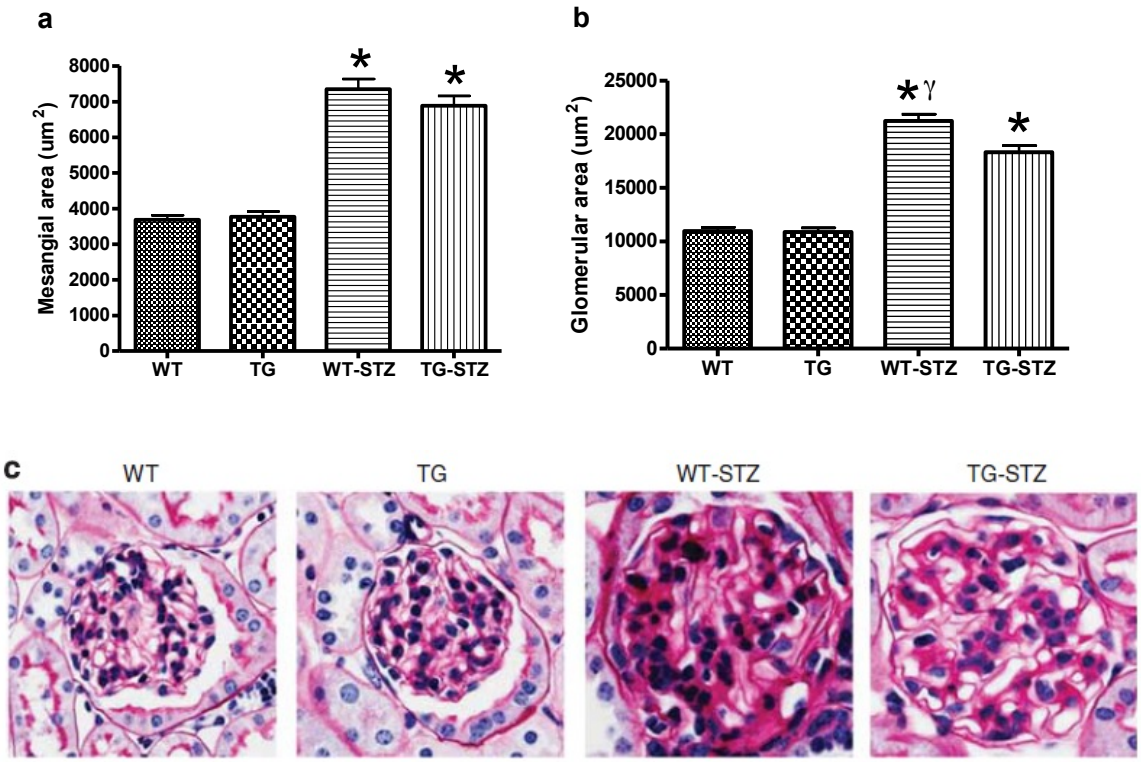


Figure 7. **Effect of podocyte angiotensin-converting enzyme 2 (ACE2) overexpression on mesangial and glomerular area at 16 weeks.** Graphs depict (**a**) mesangial and (**b**) glomerular area in mice at 16 weeks post injections. (**a**) Mesangial area at 16 weeks. Values are means ± s.e. (n=60 glomeruli per group of 6 mice). *P<0.001 vs. WT and TG. There was no significant difference between WT-STZ and TG-STZ mice. (**b**) Glomerular area at 16 weeks. Values are means ± s.e. (n=60 glomeruli per group of 6 mice). *P<0.001 vs. WT and TG, $^{\gamma}$P<0.001 vs. TG-STZ. (**c**) Representative photomicrographs depicting periodic acid-Schiff (PAS)-stained glomeruli in WT, TG, WT-STZ, and TG-STZ mice at 16 weeks. Glomerular hypertrophy and increased mesangial staining are evident in STZ groups as compared to non-STZ mice. Original magnification 640X.

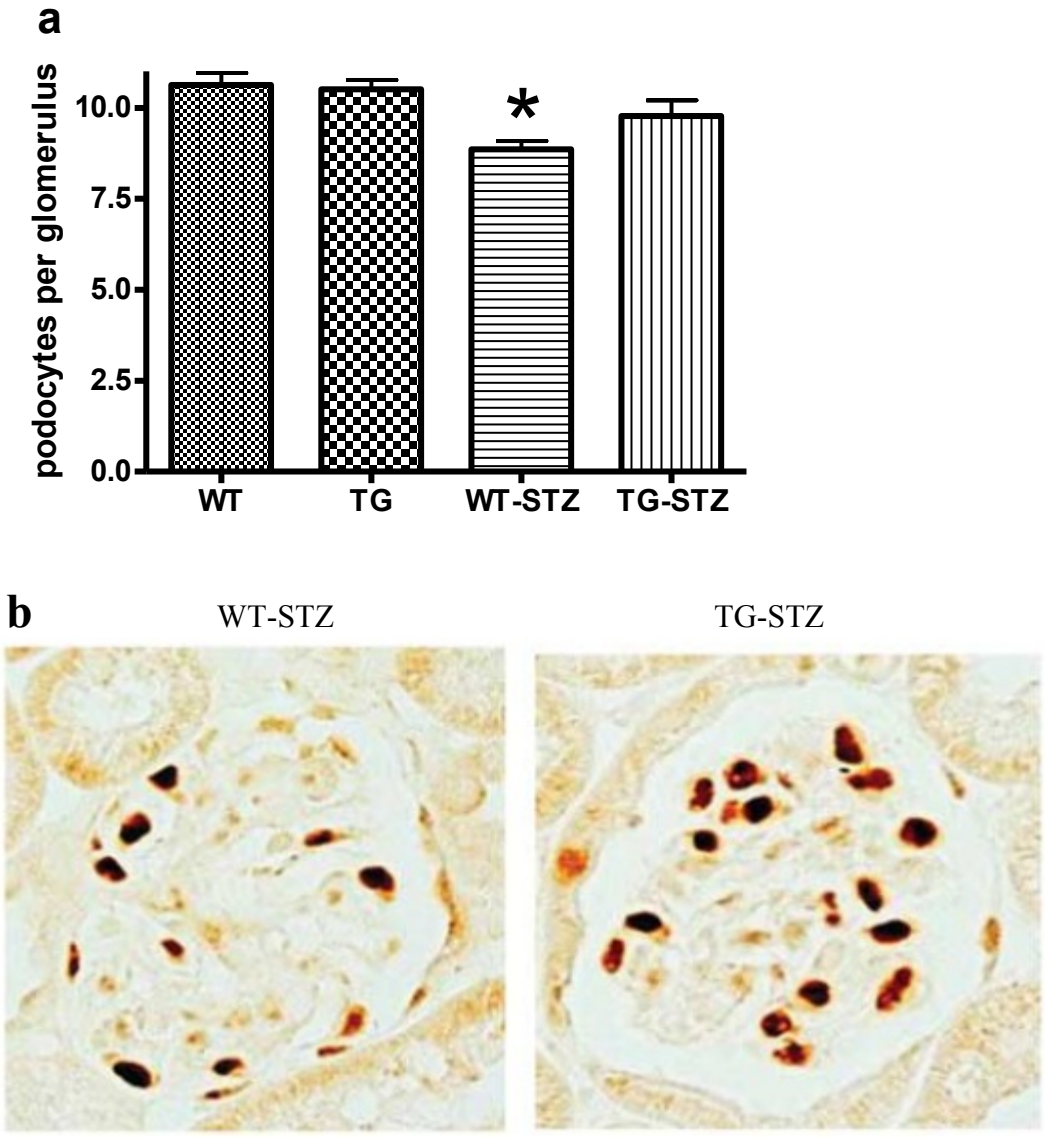


Figure 8. **Podocyte number at 16 weeks.** (**a**) Graph shows average number of podocytes per glomerulus in mice at 16 weeks, as determined by counting WT-1 positive nuclei in kidney sections. Values are means ± s.e. (n= 120 glomeruli per 6 mice in each group). *P<0.01 vs. WT and TG. There was no significant difference between TG-STZ and WT, TG, or WT-STZ mice. (**b**) Representative photomicrographs depicting glomerular WT-1 staining in glomeruli from WT-STZ vs. TG-STZ mice at 16 weeks. Original magnification 400X.

*Urinary and plasma ACE2 activity*

ACE2 enzymatic activity was measured on spot urines from all groups of mice at 1, 4, 8, 12, and 16 weeks. Vehicle-treated mice had undetectable levels of urinary ACE2 activity throughout the study. In contrast, there was a significant increase in urinary ACE2 activity in both WT-STZ and TG-STZ mice, starting at 4 weeks and persisting to 16 weeks ($p<0.0001$ vs WT and TG vehicle-treated mice at all time points) (Figure 9). At 4 weeks TG-STZ mice had a significantly attenuated increase in urinary ACE2 compared to WT-STZ mice ($p<0.05$), but the levels of urinary ACE2 activity did not differ between the two STZ groups at later time points.

At 16 weeks, plasma ACE2 activity was significantly higher in both WT-STZ and TG-STZ mice, compared to vehicle-treated groups ($p<0.001$). However, TG-STZ mice had significantly lower plasma ACE2 activity compared to WT-STZ mice ($p<0.05$) (Figure 10).

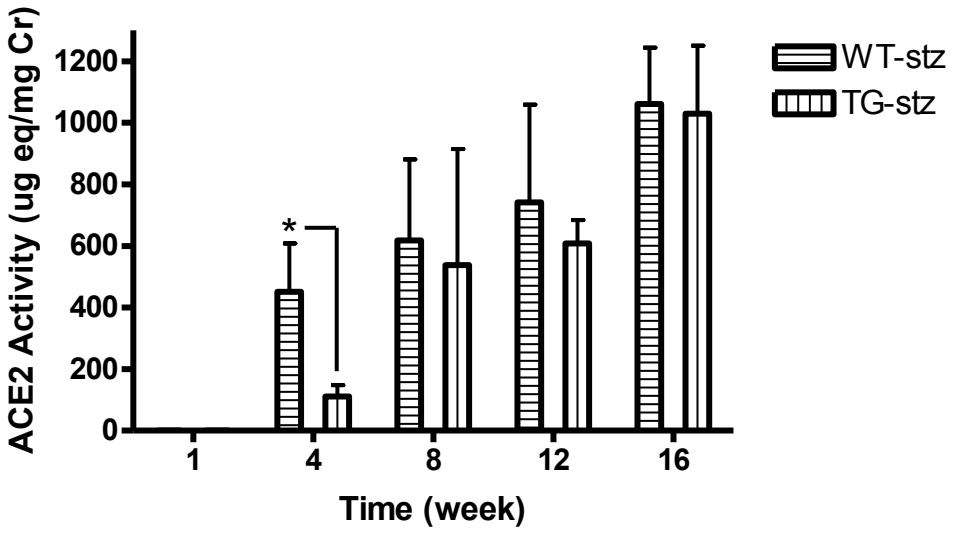


Figure 9. **Urinary angiotensin-converting enzyme 2 (ACE2) activity.** Results depict urinary ACE2 activity levels in WT-STZ and TG-STZ mice only, since urinary ACE2 activity was not detected in WT and TG mice. Values are means ± SE. (n=6 for weeks 1-12, n=15 for week 16). *$p<0.05$, WT-STZ vs TG-STZ.

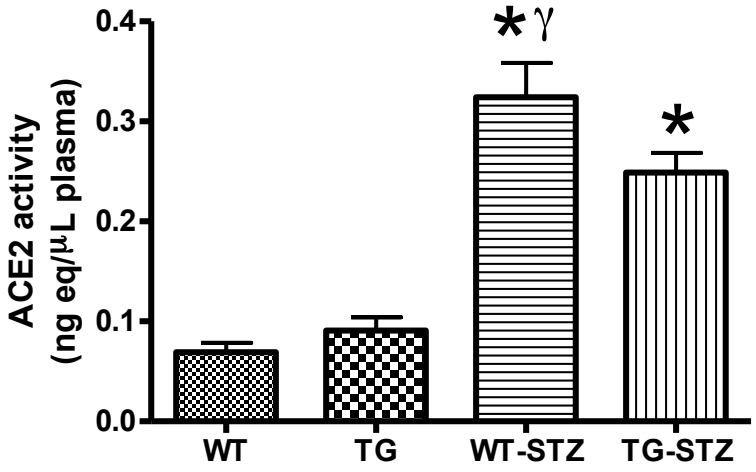


Figure 10. **Plasma angiotensin-converting enzyme 2 (ACE2) activity.** Graph depicts plasma ACE2 enzymatic activity at 16 weeks post vehicle or streptozotocin injections. Values are means ± s.e. *P<0.001 vs. WT and TG, $^{\gamma}$P<0.05 vs. TG-STZ; n=6.

*ACE2 activity in cultured podocytes*

Podocyte cells were studied in vitro throughout the course of this project to determine what effect high glucose had on ACE2 expression and activity. There were many difficulties that needed to be overcome but towards the end some preliminary data were obtained using human podocyte cells (HPODs) produced in Dr. Kennedy's laboratory (University of Ottawa, Ottawa, ON, Canada). HPODs were plated in normal media (5.5 mM D-glucose), high glucose media (25 mM D-Glucose) and control media for osmolarity (5.5 mM D-glucose + 19.5 mM L-glucose). Firstly ACE2 activity was detected in these cell extracts. Secondly, preliminary results showed a trend towards a decrease in ACE2 activity seen in the HPODs plated in high glucose media. This experiment was only conducted once in duplicates and further studies are required to confirm this finding.

## DISCUSSION

The major finding of this study is that overexpression of human ACE2 in the glomerular podocyte attenuates the development of nephropathy in mouse STZ-diabetes. This was shown by (1) a delay in the development of albuminuria, independent of any effect on systolic BP; (2) histological evidence of renal protection, namely an early reduction in mesangial expansion, and attenuation of glomerular hypertrophy at 16 weeks; and (3) prevention of podocyte loss by ACE2 overexpression. Significant amounts of urinary ACE2 activity in STZ-diabetic mice were also observed, which were transiently attenuated in diabetic mice with overexpressed podocyte ACE2. In addition to protection against diabetic nephropathy, this model of ACE2 overexpression should be useful to examine the pathology and progression of chronic kidney disease in other forms of glomerular injury.

Podocytes are highly specialized cells that form a critical component of the glomerular filtration barrier to proteins. Cultured podocytes express components of the RAS (Durvasula et al. 2008, Velez et al. 2007, Yoo et al. 2007) and ACE2 has been localized to the podocyte in glomeruli from diabetic mice by immunohistochemistry and immunogold staining (Ye 2006). Podocyte apoptosis is a key contributor to the pathogenesis of diabetic nephropathy (Susztak 2006, Toyoda 2007). Multiple signaling pathways may contribute to podocyte apoptosis in diabetes, including activation of the protein kinase mammalian target of rapamycin complex 1 (mTORC1) which also affects cellular growth and apoptosis (Inoki et al. 2011). Ang II, however, is recognized as a key contributor to podocyte apoptosis via interaction with AT1 receptors and production of

ROS, which influences apoptosis by numerous mechanisms including irreversible DNA damage, compromising mitochondrial membranes, and activation of proapoptotic proteins (Hsu et al. 2011, Jia et al. 2008, Toyokuni 1999, Fleury et al. 2002). Cultured podocytes increase Ang II production in high glucose media which may contribute to AT1 receptor–mediated apoptosis (Durvasula 2008). Podocyte ACE2 expression is decreased in the db/db mouse model of type 2 diabetes (Ye 2006), and renal biopsy specimens from humans with diabetic nephropathy similarly demonstrate decreased glomerular ACE2 mRNA and protein expression (Reich 2008). The reduced ACE2 expression promotes the accumulation of Ang II and the pathogenesis of nephropathy. As ACE2 degrades Ang II to Ang-(1–7), strategies to preserve or enhance podocyte ACE2 could prevent local increases in Ang II levels. Furthermore, ACE2 leads to enhanced production of Ang-(1–7), which has been associated with improvement of albuminuria in experimental diabetic models (Benter et al. 2007).

The glomerular ACE2-specific TG mice that are described in this study were characterized to confirm glomerular specificity and overexpression. Human ACE2 cDNA was linked to the mouse nephrin promoter, as nephrin gene expression is relatively specific to podocytes (Putaala et al. 2001). Several lines of evidence support the conclusion that human ACE2 continued to be selectively overexpressed in podocytes from TG mice. First, robust mRNA expression of the transgene occurred in glomeruli from TG mice, at a level ~50-fold higher than that in kidney cortex. Second, by immunoblot, the human ACE2 protein was detected in glomerular isolates, but not kidney cortex, as a protein of ~120 kDa. Third, the ACE2 protein colocalized with synaptopodin by immunofluorescence, indicating its presence in the podocyte (not shown). Fourth,

ACE2 activity was significantly enhanced in glomeruli from TG mice, and elevated activity (and glomerular expression by immunoblot and immunofluorescence) persisted after 16 weeks of STZ-induced diabetes. In this regard, nephrin expression decreases in diabetic nephropathy (Benigni et al. 2004, Doublier et al. 2003), and nephrin deficiency is associated with activation of nuclear factor-κB (NFκB) mediated pathways that enhance glomerular injury (Hussain et al. 2009). The data suggest that the nephrin promoter remained active and stimulated transcription of the human ACE2 gene in TG-STZ mice, despite the presence of severe diabetes. Finally, selective overexpression of the human ACE2 gene in podocytes was not associated with changes in systolic BP. Plasma ACE2 activity did not increase above the level of WT-STZ mice showing that the increase in ACE2 expression was specific to the podocyte.

TG mice did not exhibit major phenotypic differences compared with WT counterparts. For unclear reasons, TG mice had lower body weights compared with WT mice matched for age. However, both WT-STZ and TG-STZ mice developed similar elevations of plasma glucose, associated with loss of body weight, kidney hypertrophy (Table 1), glomerular hyperfiltration (Figure 4), and parallel increases in systolic BP. At no point during the study did systolic BP differ between TG and WT mice (Figure 3).

At 16 weeks of the study, mice were sacrificed and plasma was collected. There were no significant differences between all groups with respect to plasma $Na^+$. $K^+$. $Cl^-$, $CO_2$, albumin, creatinine, total protein, carbon dioxide, phosphorus, blood urea nitrogen, and cholesterol (Table 2). Serum $Ca^{2+}$ levels, however, were significantly lower in TG-STZ mice compared to vehicle-treated wildtype mice. It is well established that renal calcium excretion is increased in uncontrolled diabetes mellitus (Raskin et al. 1978), but

there is no basis to suspect that these findings relate in any way to the observations of this study. A decrease in serum $Ca^{2+}$ was not observed in the WT-STZ mice and thus further studies are required to investigate this issue. $Mg^{2+}$ is one of the most abundant cations in the body. It requires insulin to be transported intracellularly. When insulin is not present as in type 1 diabetes, $Mg^{2+}$ accumulates in the blood and is excreted in the urine at a higher rate, which ultimately leads to hypomagnesemia (Reed et al. 2008). In this study both WT-STZ and TG-STZ mice showed significantly higher plasma $Mg^{2+}$ levels compared to vehicle-treated mice. The reason for this is not clear, but this could be due to an accumulation of $Mg^{2+}$ in the blood due to the lack of insulin to transport $Mg^{2+}$ intracellularly. In addition to these changes, plasma triglycerides were found to be significantly increased in WT-STZ mice compared to all other groups, including TG-STZ mice. Circulating triglycerides are expected to increase in a diabetic state (Garg & Grundy 1990), however, the reason this increase was not seen in TG-STZ mice is not clear. Santos and colleagues observed dyslipidemia in Mas-knockout mice, which included increased muscle but normal liver triglyceride levels (Santos et al. 2008). There could be a connection between what was observed in that study and what was observed here but further studies are required to delineate the possibilities.

In WT-STZ mice, a significant increase in albuminuria occurred at 4 weeks as compared with nondiabetic mice. In contrast, urinary albumin excretion in TG-STZ mice at 4 weeks did not differ from levels in non-diabetic mice. Oudit and colleagues reported similar findings in 2010 when they administered hrACE2 to diabetic mice for 4 weeks. This observation, however, was accompanied by a decrease in BP (Oudit 2010). In this

study systolic BP remained constant between all groups at 4 weeks and even increased in diabetic groups by 16 weeks (Figure 3). In the study conducted by Oudit et al. it was not clear if the results observed happened independently of a change in BP or if they were a result of a change in BP. In this study it can be concluded that the decreased albuminuria seen in TG-STZ mice happened independently of any decreases in systolic BP.

The 24 h albuminuria levels in this diabetic mouse model were significantly higher than those reported by Qi et al. in FVB mice with STZ-diabetes (Qi 2005). There is considerable variability in urinary albumin measures in mouse models of diabetes, and assay procedures differ. Studies using the same assay procedure used in this study have reported high measures of urinary albumin in other mouse strains with diabetes (Gil et al. 2012, Zheng et al. 2011).

Despite the initial prevention of albuminuria in TG-STZ mice, albuminuria increased to the level of WT-STZ mice at later time points. In diabetes, increased Ang II is thought to promote podocyte dysfunction and albuminuria. Ang II reduces expression of the antioxidant protein peroxiredoxin 2 in podocytes in vitro and in vivo, associated with increased production of ROS, inhibition of the Akt pathway, and apoptosis (Hsu 2011).

As TG-STZ mice were only protected against albuminuria at 4 weeks, podocyte ACE2 activity may not have been sufficiently enhanced at later time points to reduce this Ang II–mediated adverse signaling, or other non-Ang II–dependent pathways affecting podocyte function may have dominated and contributed to albuminuria. Nonetheless, the early reduction in albuminuria in TG-STZ mice was associated with improvement in features of diabetic nephropathy. First, a significant decrease in mesangial area occurred

at 8 weeks, with decreased glomerular hypertrophy at 16 weeks. In diabetic nephropathy there is increased mesangial expansion and glomerular hypertrophy with an upregulation of extracellular matrix binding, fibronectin binding and laminin binding. Hyperglycemia also upregulates thrombospondin expression in mesangial cells (Brunskill & Potter 2012). Thrombospondin is important for activating TGF-β from its inactive procytokine form. TGF-β causes glomerular basement membrane thickening, matrix expansion, hypertrophy, and podocyte loss, thus a decrease in mesangial expansion and glomerular hypertrophy could indicate decreased TGF-β production. Second, partial protection against loss of podocyte number was evident at 16 weeks. Podocytes are terminally differentiated cells that are not thought to regenerate, thus podocyte loss can be correlated with glomerular scarring and damage (Garg & Holzman 2012). This partial protection may be because of enhanced ACE2-dependent degradation of Ang II in the diabetic glomerulus. In our laboratory further studies were conducted to investigate these findings. Mr. Joe Zimpelmann performed immunoblots on kidney cortex from all groups from this study for TGF-β and nephrin expression. He found that there was a significant increase in TGF-β in WT-STZ mice compared to all over groups at 8 weeks, and this increase was not seen in TG-STZ mice. At 16 weeks, however, WT-STZ again showed significant TGF-β levels, however by this time point TG-STZ also showed significant TGF-β levels. By immunoblot analysis, nephrin expression was significantly lower in WT-STZ mice compared to WT mice at 8 and 16 weeks of the study. However, glomerular nephrin expression did not significantly decrease in TG-STZ mice. Immunofluorescence analysis conducted by Dr. Alex Gutsol (University of Ottawa, Ottawa, ON, Canada) confirmed these observations at 16 weeks. At 8 weeks, however,

all groups showed a decrease in nephrin expression compared to WT mice. In the diabetic kidney, glomerular Ang II initiates a reduction in nephrin, synaptopodin, and stimulates TGF-β1, which increases podocyte loss (diabetic podocytopathy) leading to proteinuria and subsequent tubulointerstitial inflammation and fibrosis. This in turn is associated with increased TGF-β1 (Ziyadeh et al. 2008). Consistent with this view, in rats with STZ-diabetes, intravenous adenoviral gene transfer of ACE2 reduces kidney cortical levels of Ang II, and increases Ang-(1–7), associated with protection from glomerular injury. In rat mesangial cells, transfection of ACE2 inhibits Ang II–stimulated cell proliferation, oxidative stress, and collagen IV synthesis (Liu et al. 2011).

Although the beneficial effects of ACE2 may have derived largely from a reduction in Ang II levels in this study, the role of potentially enhanced podocyte generation of Ang-(1–7) in TG mice remains unclear. Although Ang-(1–7) inhibits Ang II–mediated proinflammatory signaling in proximal tubular cells and vasculature (Freeman et al. 1996, Sampaio et al. 2007, Su et al. 2006), its effects on podocyte function are unknown. In mesangial cells, Ang-(1–7) has been reported to either stimulate profibrotic pathways (Zimpelmann & Burns 2009) or inhibit Ang II signaling (Moon et al. 2011). Thus, although overexpression of ACE2 in the podocyte has a renoprotective effect, the relative contribution of diminished levels of Ang II versus increased Ang-(1–7) requires further study.

High urinary ACE2 activity levels were found in the STZ-induced diabetic groups and these levels steadily increased with the progression of the disease. Throughout the study urinary ACE2 levels were parallel between WT-STZ and TG-STZ mice, except for

early in the study at 4 weeks where urinary ACE2 levels were significantly attenuated in TG-STZ mice compared to WT-STZ. These observations mirrored the albuminuria results of this study. These events may not be connected, but the results suggest that urinary ACE2 is linked to albuminuria and perhaps urinary ACE2 may be used as a marker of kidney damage. At the time these experiments were conducted, these were novel findings. Recently, significant levels of urinary ACE2 have been discovered in patients with diabetes and CKD (Xiao et al. 2012, Mizuiri et al. 2011). Previous studies suggest that the urinary ACE2 is shed from the membranes of proximal tubular cells by tumour necrosis factor-α convertase (ADAM-17) (Xiao 2012, Mizuiri 2011). ADAM-17 has been found to be induced by Ang II treatment (Lautrette et al. 2005). This observation may provide one potential explanation for the attenuated urinary ACE2 at 4 weeks in our study. Perhaps overexpression of ACE2 at the podocyte results in less Ang II reaching the proximal tubule, and thus less ADAM-17 is induced to cleave ACE2. Another potential explanation for this protection may be the decreased albuminuria that corresponds to the decreased urinary ACE2 seen at 4 weeks. Proteinuria stimulates an inflammatory response in proximal tubular cells that leads to injury, fibrosis, and death (Eddy 2004). It is possible that the decreased proteinuria at 4 weeks translates to a direct protective effect on the proximal tubule cells. Further studies are necessary to delineate these theories.

Finally, this study focused on early diabetic nephropathy, and the long-term effects of overexpression of ACE2 on disease progression remain unclear. In summary, after STZ-induced diabetes, TG mice with podocyte-specific overexpression of the

human ACE2 protein are protected against the early development of albuminuria and show partial preservation of podocyte proteins and podocyte number, reduced glomerular histological injury, and decreased urinary ACE2 activity (at 4 weeks). ACE2 may represent a therapeutic target in the prevention and treatment of diabetic nephropathy.

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# APPENDICES

Open

# Podocyte-specific overexpression of human angiotensin-converting enzyme 2 attenuates diabetic nephropathy in mice

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**Angiotensin-converting enzyme 2 (ACE2) degrades angiotensin II to angiotensin-(1–7) and is expressed in podocytes. Here we overexpressed ACE2 in podocytes in experimental diabetic nephropathy using transgenic methods where a nephrin promoter drove the expression of human ACE2. Glomeruli from these mice had significantly increased mRNA, protein, and activity of ACE2 compared to wild-type mice. Male mice were treated with streptozotocin to induce diabetes. After 16 weeks, there was no significant difference in plasma glucose levels between wild-type and transgenic diabetic mice. Urinary albumin was significantly increased in wild-type diabetic mice at 4 weeks, whereas albuminuria in transgenic diabetic mice did not differ from wild-type nondiabetic mice. However, this effect was transient and by 16 weeks both transgenic and nontransgenic diabetic mice had similar rates of proteinuria. Compared to wild-type diabetic mice, transgenic diabetic mice had an attenuated increase in mesangial area, decreased glomerular area, and a blunted decrease in nephrin expression. Podocyte numbers decreased in wild-type diabetic mice at 16 weeks, but were unaffected in transgenic diabetic mice. At 8 weeks, kidney cortical expression of transforming growth factor-β1 was significantly inhibited in transgenic diabetic mice as compared to wild-type diabetic mice. Thus, the podocyte-specific overexpression of human ACE2 transiently attenuates the development of diabetic nephropathy.**

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KEYWORDS: ACE2; albuminuria; angiotensin; apoptosis; diabetes; podocyte

Angiotensin-converting enzyme 2 (ACE2), a recently discovered member of the renin–angiotensin system, has a single catalytic domain and shares 40% homology with ACE.[1,2] Despite this similarity, ACE2 activity is not affected by ACE inhibitors, nor does it generate angiotensin (Ang) II. On the contrary, ACE2 degrades Ang II to Ang-(1–7), a peptide that antagonizes Ang II signaling.[3–5] ACE2 also degrades Ang I to Ang-(1–9) that is subsequently converted to Ang-(1–7) by ACE.[6] Because of these properties, ACE2 has been postulated to be an endogenous renoprotective enzyme.[7]

ACE2 may be involved in the pathogenesis of diabetic nephropathy. Pharmacological inhibition of ACE2 in streptozotocin (STZ)-induced diabetic mice causes increased albuminuria and expansion of the glomerular matrix.[8] In Akita mice, a model of type 1 diabetes, deletion of the *ACE2* gene has been reported to exacerbate albuminuria, associated with increased mesangial matrix deposition, glomerular basement membrane thickening, and glomerulosclerosis, without significant changes in blood pressure (BP).[9] Recently, administration of human recombinant ACE2 to diabetic Akita mice significantly reduced albuminuria, associated with decreased BP.[10] Similarly, intravenous administration of recombinant adenovirus carrying the mouse *ACE2* gene to rats with STZ-diabetes was associated with diminished albuminuria and glomerulosclerosis, although systolic BP was also reduced.[11] Accordingly, there is increasing interest in the potential therapeutic action of ACE2 amplification to treat diabetic nephropathy, as recently reviewed.[12]

Glomerular podocytes are terminally differentiated epithelial cells that are key components of the selective permeability barrier of the glomerular basement membrane.[13] Podocyte apoptosis and detachment are directly correlated with the progression of diabetic nephropathy.[14] ACE2 has been localized to the podocyte, and podocyte ACE2 expression decreases in experimental diabetes.[15] However, there is no direct evidence regarding ACE2 overexpression in the podocyte as a protector against the development of diabetic nephropathy. In the current study, transgenic (TG) mice were generated that selectively overexpress human ACE2 in the

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podocyte to address this question. Specifically, we wished to examine the hypothesis that a podocyte-specific ACE2 transgene could protect against glomerular injury in the STZ model of diabetes, independent of changes in BP.

## RESULTS

### TG mouse characterization

TG mice with podocyte-specific overexpression of human ACE2 were characterized by reverse transcriptase–PCR, immunoblot analysis of isolated glomeruli, direct immunofluorescence, and measurement of glomerular ACE2 activity. By real-time reverse transcriptase–PCR, human ACE2 mRNA was readily detected in glomeruli from TG mice, at a level ~50-fold higher than in kidney cortex (Figure 1a). In contrast, the levels of endogenous mouse ACE2 mRNA in TG mice were significantly lower in glomeruli than in kidney cortex (Figure 1b). In wild-type (WT) mice, levels of endogenous mouse ACE2 mRNA were also significantly lower in glomeruli than kidney cortex ($n = 4$, not shown). In TG mice, expression of endogenous mouse ACE2 in kidney cortex was not significantly affected by the presence of the human ACE2 transgene (relative levels of mouse ACE2 mRNA, TG vs. WT mice: 1.8 ± 1.1-fold, $P =$ not significant, $n = 3$). Similarly, glomerular expression of endogenous mouse ACE2 was not affected by the human ACE2 transgene (WT: 1.37 ± 0.19 vs. TG: 1.65 ± 0.33 arbitrary units, $P =$ not significant, $n = 2$–4). Expression of the human ACE2 transgene was also not detected at significant levels in the heart, brain, liver, spleen, or lung (Figure 1a).

In glomerular isolates from TG mice, the human ACE2 protein was detected by immunoblot as a single protein band of ~120 kDa, compared with the endogenous mouse ACE2 at ~100 kDa (Figure 1c). In TG mice, the human ACE2 protein was not readily detected in kidney cortical homogenates (Figure 1c). By immunofluorescence of kidney cortical tissue, TG mice demonstrated positive glomerular staining for the hemagglutinin epitope tag, which colocalized with the podocyte protein synaptopodin (Figure 1d). In freshly isolated glomeruli, ACE2 enzymatic activity was significantly increased in TG mice as compared with WT mice (Figure 1e: 2.82-fold increase in TG vs. WT mice, $P < 0.02$, $n = 9$–10).

### STZ-diabetes: whole animal data

Table 1 depicts whole animal data regarding body and organ weights, and plasma glucose. WT and TG mice treated with STZ (WT-STZ and TG-STZ, respectively) developed severe diabetes, with significantly increased plasma glucose compared with vehicle-treated mice, and with no significant difference between the two STZ groups. TG mice had lower body weights than WT mice matched for age. However, both WT-STZ and TG-STZ mice experienced weight loss, and renal hypertrophy, inferred from increased kidney weight to body weight ratio ($P < 0.001$ vs. non-STZ mice). In contrast, there was no difference in cardiac weight to body weight ratios among the four groups. Despite the high levels of plasma glucose, diabetic mice did not develop ketosis, as determined by urine testing at 16 weeks, and absence of metabolic acidosis (not shown). Mortality rate was 12.2% (11/90) for all mice in the study, with no significant differences among the four groups. In TG-STZ mice, glomerular ACE2 activity remained significantly increased after 16 weeks as compared with WT-STZ (3.30 ± 0.88-fold increase, $P < 0.01$, $n = 5$). By immunofluorescent staining, glomerular ACE2 expression was significantly increased in TG mice when compared with WT mice ($P < 0.001$), and this increase persisted 16 weeks after STZ

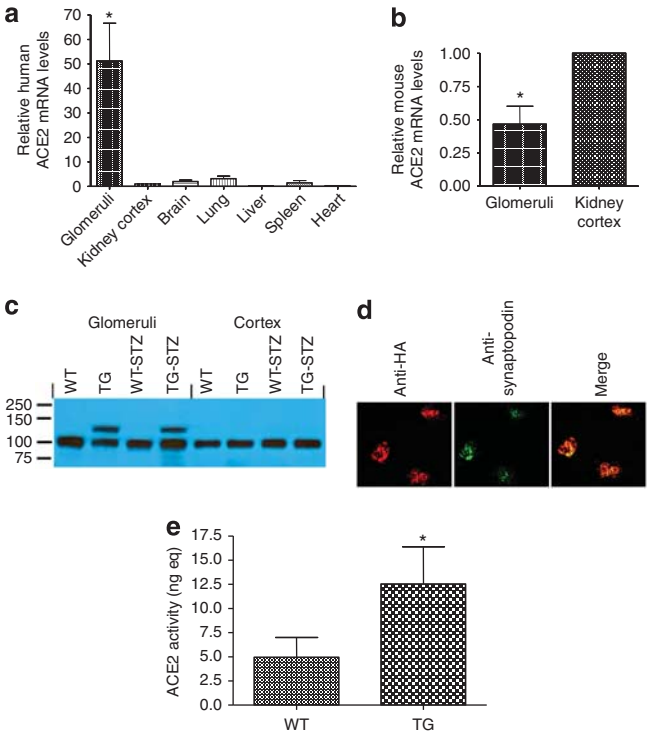


**Figure 1 | Characterization of transgenic (TG) mice.** (**a**) Expression of mRNA for the human angiotensin-converting enzyme 2 (ACE2) transgene in tissues from TG mice. Graph shows relative levels of human ACE2 mRNA in various mouse tissues, determined by real-time reverse transcriptase–PCR (RT–PCR). *$P < 0.001$, glomeruli vs. all other tissues; $n = 3$ (glomeruli) and $n = 6$–7 for other tissues. (**b**) Expression of endogenous mouse ACE2 mRNA in glomeruli and kidney cortex from TG mice. Graph depicts relative expression of mouse ACE2 mRNA in glomeruli and kidney cortex from TG mice, determined by real-time RT–PCR. *$P < 0.02$; $n = 3$. (**c**) Immunoblot analysis of ACE2 in glomeruli and cortex tissue. Shown is representative immunoblot of ACE2 protein in glomeruli and kidney cortex from wild-type (WT) and TG mice, with or without treatment with STZ, at 16 weeks ($n = 2$). Human ACE2 transgenic protein is present as a single band at ~120 kDa in glomeruli from TG or TG-STZ mice. Endogenous mouse ACE2 protein is observed in all lanes as a single band at ~100 kDa. (**d**) Colocalization of human ACE2 transgene with podocyte protein synaptopodin. Dual-color immunofluorescence was performed on kidney sections from TG mice. Sections were stained with rabbit polyclonal antibody to hemagglutinin (HA; present in the transgene as a double epitope tag; left panel), and with rabbit polyclonal anti-synaptopodin antibody (middle panel). Merged view is shown in the panel on the right. Original magnification × 400. (**e**) Glomerular ACE2 activity. ACE2 activity was measured in isolated glomeruli from WT or TG mice, as described in Materials and Methods. *$P < 0.02$, $n = 9$ (WT), $n = 10$ (TG).

**Table 1 | Whole animal data**

| | WT | TG | WT-STZ | TG-STZ |
|---|---|---|---|---|
| BW (g) | 36.2 ± 0.7 (6) | 31.1 ± 1.1 (7)** | 27.2 ± 0.5 (8)* | 25.9 ± 0.5 (6)* |
| KW/BW (g/kg) | 11.4 ± 0.4 (6) | 12.2 ± 0.2 (7) | 20.8 ± 0.7 (8)*** | 21.0 ± 1.3 (6)*** |
| HW/BW (g/kg) | 3.9 ± 0.3 (6) | 4.3 ± 0.1 (7) | 4.3 ± 0.2 (8) | 4.2 ± 0.2 (6) |
| Plasma glucose (mmol/l) | 19.1 ± 1.0 (11) | 18.3 ± 1.3 (11) | 55.8 ± 8.0 (11)† | 72.2 ± 17.1 (11)† |

Abbreviations: BW, body weight; HW, heart weight; KW, kidney weight; TG, transgenic; TG-STZ, transgenic streptozotocin-treated; WT, wild type; WT-STZ, wild-type streptozotocin-treated.
Values are means ± s.e. (*n*).
*$P<0.001$ vs. WT and *$P<0.01$ vs. TG.
**$P<0.01$ vs. WT.
***$P<0.001$ vs. WT and TG.
†$P<0.05$ vs. WT and TG.

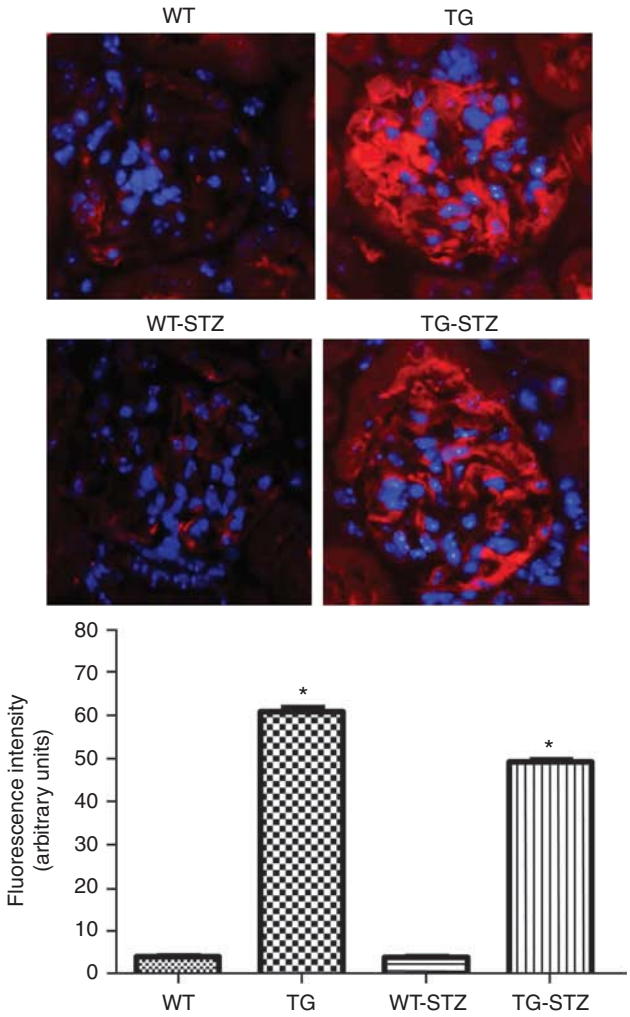


**Figure 2 | Glomerular expression of angiotensin-converting enzyme 2 (ACE2) by immunofluorescence at 16 weeks.** Graph depicting semiquantitative analysis of ACE2 immunofluorescent staining in mouse glomeruli at 16 weeks. TG, podocyte-specific ACE2 transgenic; TG-STZ, transgenic streptozotocin-treated; WT, wild type; WT-STZ, wild-type streptozotocin-treated. Values are means ± s.e. *$P<0.001$ vs. WT and vs. WT-STZ; $n=100$ glomeruli per group, from 6 to 11 mice per group. Representative images depicting ACE2 staining in glomeruli (red) at 16 weeks are shown above graph. Blue staining represents Hoechst nuclear stain. Original magnification × 640.

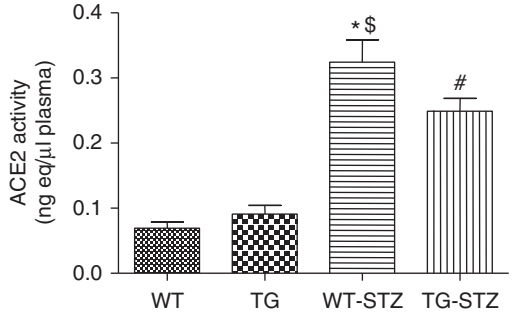


**Figure 3 | Plasma angiotensin-converting enzyme 2 (ACE2) activity.** Graph depicts plasma ACE2 enzymatic activity from wild-type (WT), transgenic (TG), wild-type streptozotocin-treated (WT-STZ), and transgenic streptozotocin-treated (TG-STZ) mice at 16 weeks. Values are means ± s.e. *$P<0.001$ vs. WT and TG, #$P<0.001$ vs. WT and TG, $$P<0.05$ vs. TG-STZ; $n=6$.

injection (Figure 2). Glomerular human ACE2 protein expression by immunoblot also remained robust at 16 weeks (Figure 1c). At this time point, in contrast, plasma ACE2 activity was actually decreased in TG-STZ mice compared with WT-STZ mice (TG-STZ: 0.25 ± 0.03 vs. WT-STZ: 0.32 ± 0.04 ng-eq/µl plasma, $P<0.05$, $n=6$; Figure 3). However, both diabetic groups had higher plasma ACE2 activity compared with non-STZ groups ($P<0.001$).

At 16 weeks, there were no significant differences in plasma electrolytes, total protein, or total cholesterol between WT-STZ and TG-STZ mice (not shown). WT-STZ mice had significant elevations in serum triglycerides compared with all other groups ($P<0.01$).

### Systolic BP

Systolic BP was measured weekly throughout the study and tended to be higher in both groups of STZ mice when compared with non-STZ mice, but the difference was not significant until the 15-week time point (Figure 4). Systolic BP did not significantly differ between WT-STZ and TG-STZ mice at any point in the study, nor did it differ between WT and TG mice (Figure 4).

### Glomerular filtration rate

At 16 weeks, glomerular filtration rate was significantly higher in WT-STZ and TG-STZ mice when compared with WT and TG mice, but there was no difference between the two STZ groups (Figure 5).

### Albuminuria

At 4 weeks, WT-STZ mice had significantly increased levels of albuminuria as compared with vehicle-treated mice (Figure 6a). In contrast, TG-STZ mice had no increase in albuminuria at 4 weeks ($P<0.05$ vs. WT-STZ, $n=5$). However, there was no significant difference in albuminuria between WT-STZ and TG-STZ mice at 8 weeks (WT-STZ: 1312 ± 527 vs. TG-STZ: 1233 ± 380 µg per 24 h; $P=$ not significant, $n=6$). Similarly, at 16 weeks, both WT-STZ and TG-STZ mice had markedly increased levels of albuminuria, with no significant difference between the two groups

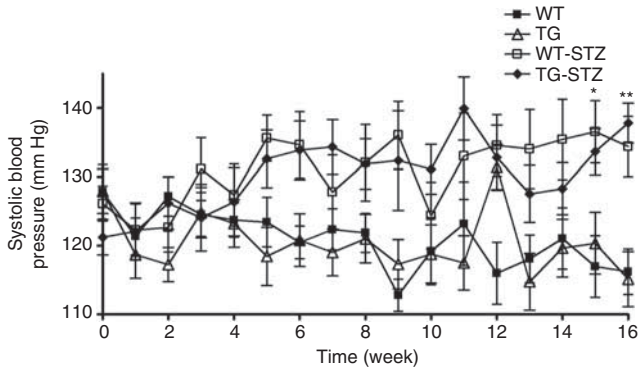


**Figure 4 | Systolic blood pressure (BP).** Systolic BP was measured weekly by tail-cuff plethysmography. TG, transgenic; TG-STZ, transgenic streptozotocin-treated; WT, wild type; WT-STZ, wild-type streptozotocin-treated. Values are means ± s.e. $^{*}P<0.01$ vs. WT and TG, $^{**}P<0.05$ vs. WT and TG. There was no significant difference between WT-STZ and TG-STZ mice; $n=14$ mice per group.

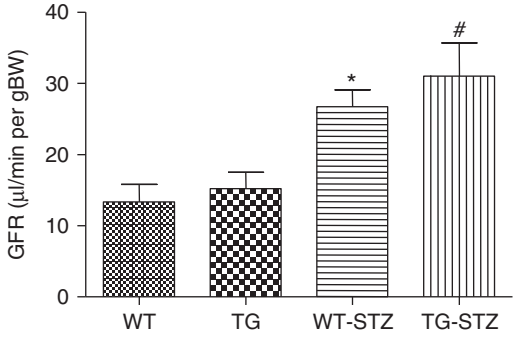


**Figure 5 | Glomerular filtration rate (GFR).** Graph shows GFR measurements in wild-type (WT), transgenic (TG), wild-type streptozotocin-treated (WT-STZ), and transgenic streptozotocin-treated (TG-STZ) mice after 16 weeks. Values are means ± s.e. $^{*}P<0.05$ vs. WT and TG, $^{\#}P<0.01$ vs. WT and TG; $n=4$–6.

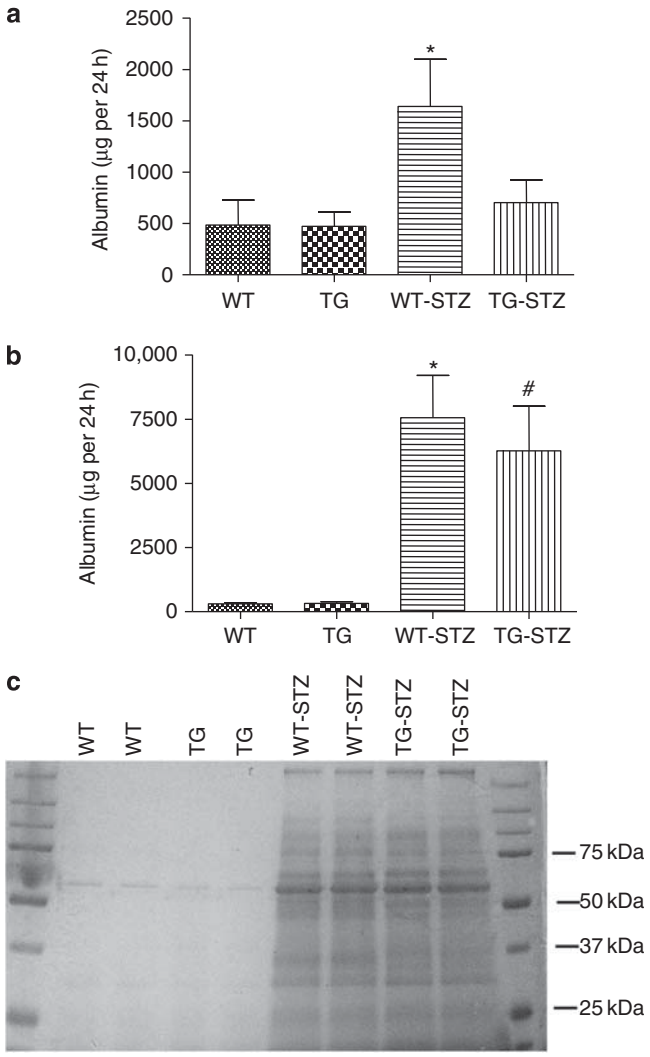


**Figure 6 | Urinary protein excretion.** (**a**) The 24 h urinary albumin excretion in wild-type (WT), transgenic (TG), wild-type streptozotocin-treated (WT-STZ), and transgenic streptozotocin-treated (TG-STZ) mice at 4 weeks. $^{*}P<0.05$ vs. all other groups (WT, TG, and TG-STZ mice); $n=5$. (**b**) The 24 h urinary albumin excretion at 16 weeks. $^{*}P<0.01$ vs. WT and TG, $^{\#}P<0.01$ vs. WT and TG. There was no significant difference between WT-STZ and TG-STZ mice; $n=14$. (**c**) Representative Coomassie blue-stained sodium dodecyl sulfate (SDS) gel of urine samples from WT, TG, WT-STZ, and TG-STZ mice at 8 weeks. Multiple protein bands are evident in urine samples from WT-STZ and TG-STZ mice. Molecular weight marker is depicted in the first and last lanes.

(Figure 6b). Coomassie blue staining of sodium dodecyl sulfate gels revealed generalized proteinuria in STZ-treated mice compared with nondiabetic mice (Figure 6c).

### Histological analysis and expression of podocyte proteins

Histological analysis was performed on kidney sections at 8 and 16 weeks. At 8 weeks, WT-STZ mice and TG-STZ mice demonstrated increases in mesangial and glomerular areas compared with vehicle-treated mice (Figure 7). The increase in mesangial area, however, was significantly attenuated in TG-STZ mice. At 16 weeks, mesangial and glomerular areas were significantly increased in WT-STZ and TG-STZ groups as compared with nondiabetic mice (Figure 8). Glomerular diameter was significantly diminished in TG-STZ mice when compared with WT-STZ mice (Figure 8).

Expression of the podocyte protein nephrin was examined by immunoblots and immunofluorescence. In kidney cortical lysates from WT-STZ mice, nephrin expression by immunoblot was significantly decreased at 8 weeks as compared with WT mice (Figure 9a). TG-STZ mice, in contrast, exhibited no significant difference in nephrin expression compared with their TG counterparts, although baseline levels were lower. Similarly, at 16 weeks, nephrin expression was significantly reduced in WT-STZ mice compared with WT mice, whereas nephrin levels did not significantly differ between TG and TG-STZ mice (Figure 9b). Semiquantitative immunofluorescence analysis at 8 and 16 weeks revealed a similar pattern of glomerular nephrin expression as the immunoblots (Figure 10). In particular, whereas nephrin expression was significantly reduced in WT-STZ mice, there was no significant difference in nephrin expression between TG

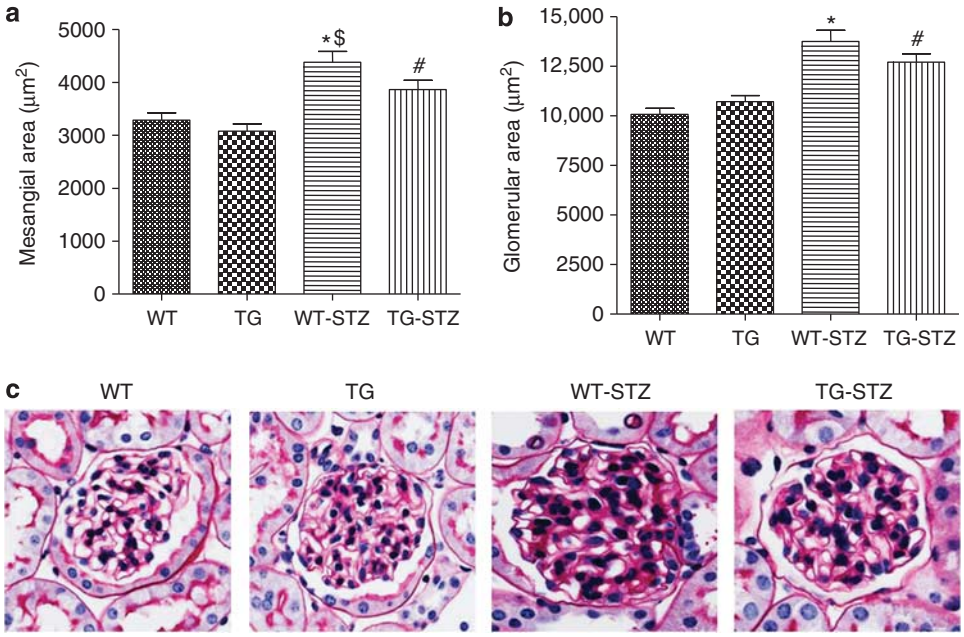


**Figure 7 | Effect of podocyte angiotensin-converting enzyme 2 (ACE2) overexpression on mesangial and glomerular area at 8 weeks.** Graphs depict (**a**) mesangial and (**b**) glomerular areas at 8 weeks in wild-type (WT), transgenic (TG), wild-type streptozotocin-treated (WT-STZ), and transgenic streptozotocin-treated (TG-STZ) mice. (**a**) Mesangial area at 8 weeks. Values are means ± s.e. ($n = 40$ glomeruli per group of 4 mice). *$P < 0.001$ vs. WT and TG, $^{\#}P < 0.02$ vs. WT and TG, $^{\$}P < 0.03$ vs. TG-STZ. (**b**) Glomerular area at 8 weeks. Values are means ± s.e. ($n = 40$ glomeruli per group of 4 mice). *$P < 0.001$ vs. WT and TG, $^{\#}P < 0.001$ vs. WT and TG. There was no significant difference between WT-STZ and TG-STZ mice. (**c**) Representative photomicrographs depicting periodic acid–Schiff (PAS)-stained glomeruli in WT, TG, WT-STZ, and TG-STZ mice at 8 weeks. Glomerular hypertrophy and increased mesangial staining are evident in STZ groups as compared with non-STZ mice. Original magnification × 640.

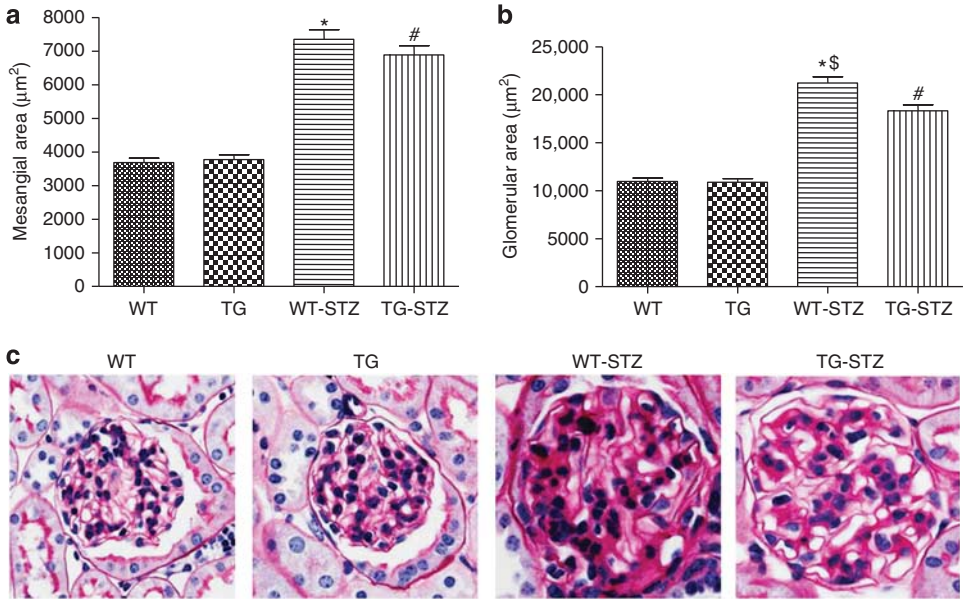


**Figure 8 | Effect of podocyte angiotensin-converting enzyme 2 (ACE2) overexpression on mesangial and glomerular area at 16 weeks.** Graphs depict (**a**) mesangial and (**b**) glomerular areas at 16 weeks in wild-type (WT), transgenic (TG), wild-type streptozotocin-treated (WT-STZ), and transgenic streptozotocin-treated (TG-STZ) mice. (**a**) Mesangial area at 16 weeks. Values are means ± s.e. ($n = 60$ glomeruli per group of 6 mice). *$P < 0.001$ vs. WT and TG, $^{\#}P < 0.001$ vs. WT and TG. There was no significant difference between WT-STZ and TG-STZ mice. (**b**) Glomerular area at 16 weeks. Values are means ± s.e. ($n = 60$ glomeruli per group of 6 mice). *$P < 0.001$ vs. WT and TG, $^{\#}P < 0.001$ vs. WT and TG, $^{\$}P < 0.001$ vs. TG-STZ. (**c**) Representative photomicrographs depicting periodic acid–Schiff (PAS)-stained glomeruli in WT, TG, WT-STZ, and TG-STZ mice at 16 weeks. Glomerular hypertrophy and increased mesangial staining is evident in STZ groups as compared with non-STZ mice. Original magnification × 640.

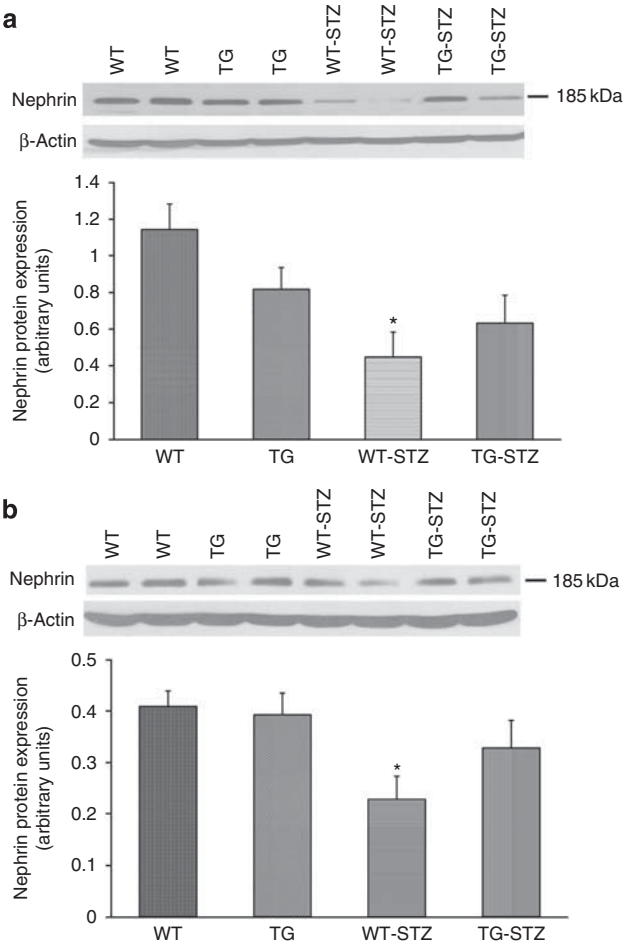


**Figure 9 | Nephrin expression at 8 and 16 weeks by immunoblot.** (**a**) Graph depicting nephrin expression by immunoblot analysis of cortical lysates in wild-type (WT), transgenic (TG), wild-type streptozotocin-treated (WT-STZ), and transgenic streptozotocin-treated (TG-STZ) mice at 8 weeks. Representative immunoblot is shown above graph, with nephrin at ~185 kDa, and β-actin signal below, as control for protein loading. Values are mean ± s.e., $n = 6$ per group. *$P < 0.02$ vs. WT. There was no significant difference between TG and TG-STZ mice or between WT-STZ and TG-STZ mice. (**b**) Graph depicting nephrin expression by immunoblot analysis of cortical lysates in WT, TG, WT-STZ, and TG-STZ mice at 16 weeks. Representative immunoblot is shown above graph, with nephrin at ~185 kDa, and β-actin signal below, as control for protein loading. Values are mean ± s.e., $n = 6$ per group. *$P < 0.05$ vs. WT. There was no significant difference between TG and TG-STZ mice.

and TG-STZ mice, although TG mice tended to have lower expression than WT mice.

By immunofluorescence, glomerular synaptopodin expression was not significantly affected at 8 weeks, although there was a tendency for reduced expression in WT-STZ and TG-STZ mice (Figure 11). However, at 16 weeks, expression of synaptopodin was significantly reduced in WT-STZ mice when compared with WT, TG, or TG-STZ mice (Figure 11).

Podocyte numbers were assayed at 8 and 16 weeks by counting glomerular nuclei staining positively for WT-1. At 8 weeks, both WT-STZ and TG-STZ mice had significantly decreased podocyte number per glomerulus compared with non-STZ mice (Figure 12a). In TG mice, podocyte number was lower than in WT mice, and the decrease with STZ was less pronounced compared with WT-STZ mice. At 16 weeks, WT-STZ mice showed a significant decrease in average podocyte number compared with vehicle-treated mice. In contrast, TG-STZ mice did not exhibit a significant decrease in podocytes as compared with nondiabetic mice (Figure 12b).

### Kidney cortical expression of TGF-β1

As shown in Figure 13, protein expression of the profibrotic cytokine transforming growth factor-β1 (TGF-β1) was significantly increased in kidney cortices from WT-STZ mice at 8 weeks when compared with nondiabetic mice. In contrast, TG-STZ mice exhibited no increase in cortical TGF-β1 at 8 weeks. At 16 weeks, expression of TGF-β1 was increased in both WT-STZ and TG-STZ mice, with no significant difference between the two groups.

## DISCUSSION

The major finding of this study is that overexpression of human ACE2 in the glomerular podocyte attenuates the development of nephropathy in mouse STZ-diabetes. This was shown by (1) a delay in the development of albuminuria, independent of any effect on systolic BP; (2) histological evidence of renal protection, namely an early reduction in mesangial expansion, and attenuation of glomerular hypertrophy at 16 weeks; (3) partial preservation of expression of the podocyte proteins nephrin and synaptopodin; (4) prevention of podocyte loss by ACE2 overexpression; and (5) reduction in cortical TGF-β1 expression at 8 weeks. In addition to protection against diabetic nephropathy, this model of ACE2 overexpression should be useful to examine questions related to pathology and progression of CKD in other forms of glomerular injury.

Podocytes are highly specialized cells that form a critical component of the glomerular filtration barrier to proteins. Cultured podocytes express components of the renin–angiotensin system,[16–18] and ACE2 has been localized to the podocyte in glomeruli from diabetic mice by immunohistochemistry and immunogold staining.[15] Loss of podocytes via apoptosis contributes importantly to the pathogenesis of diabetic nephropathy. Multiple signaling pathways may mediate podocyte apoptosis in diabetes, including involvement of mammalian target of rapamycin signaling.[19] However, Ang II is recognized as a key contributor to podocyte apoptosis, via interaction with $AT_1$ (angiotensin II receptor, type 1) receptors, and production of reactive oxygen species.[20,21] In cultured podocytes exposed to high glucose, production of Ang II is enhanced, which may contribute to $AT_1$ receptor–mediated apoptosis.[16] In the db/db mouse model of type 2 diabetes, podocyte ACE2 expression is decreased,[15] and renal biopsy specimens from humans with diabetic nephropathy similarly demonstrate decreased glomerular ACE2 mRNA and protein expression.[22] As ACE2

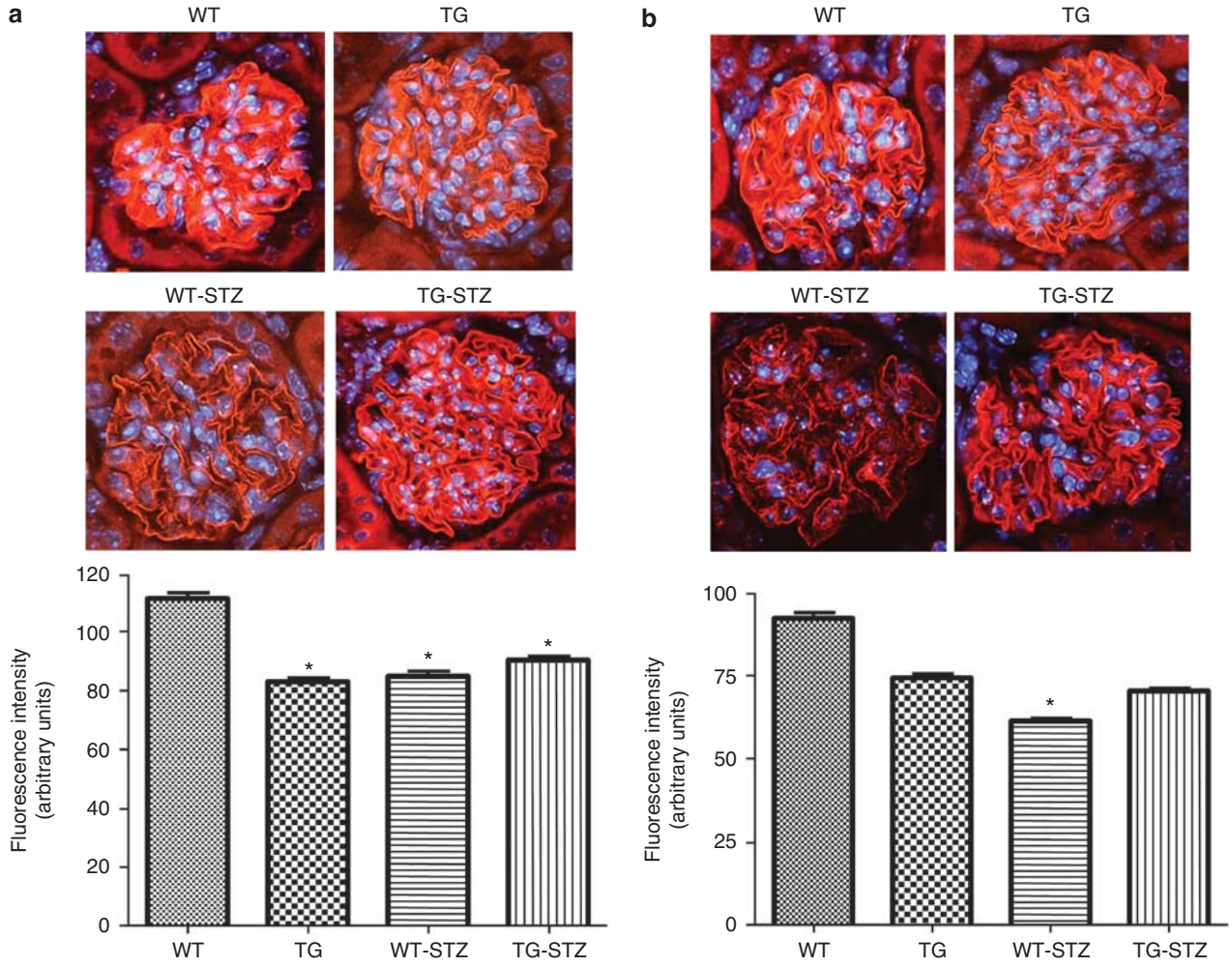


**Figure 10 | Nephrin expression at 8 and 16 weeks by immunofluorescence.** (**a**) Graph depicting semiquantitative analysis of nephrin expression by immunofluorescence in wild-type (WT), transgenic (TG), wild-type streptozotocin-treated (WT-STZ), and transgenic streptozotocin-treated (TG-STZ) mice at 8 weeks. Values are means ± s.e., $n = 100$ glomeruli per group, from 7 to 8 mice per group. *$P < 0.001$ vs. WT. There was no significant difference between TG and TG-STZ mice. Representative images depicting glomerular nephrin expression (red) at 8 weeks are shown above graph. Nuclear Hoechst staining (blue) is also shown. Original magnification × 640. (**b**) Graph depicting nephrin expression by immunofluorescence at 16 weeks. Values are means ± s.e., $n = 100$ glomeruli per group, from 6 to 11 mice per group. *$P < 0.001$ vs. WT. Representative images depicting glomerular nephrin expression (red) at 16 weeks are shown above graph. Nuclear Hoechst staining (blue) is also shown. Original magnification × 640.

degrades Ang II to Ang-(1–7), strategies to preserve or enhance podocyte ACE2 could prevent local increases in Ang II levels. Furthermore, ACE2 leads to enhanced production of Ang-(1–7), which has been associated with improvement of albuminuria in experimental diabetes.[23]

The glomerular ACE2-specific TG mice that we describe in this study did not exhibit major phenotypic differences compared with WT counterparts. For unclear reasons, TG mice had lower body weights compared with WT mice matched for age. However, both WT-STZ and TG-STZ mice developed similar elevations of plasma glucose, associated with loss of body weight, kidney hypertrophy, glomerular hyperfiltration, and parallel increases in systolic BP. The human ACE2 cDNA was linked to the mouse nephrin promoter in these studies, as nephrin gene expression is relatively specific to podocytes.[24] Several lines of evidence support the conclusion that human ACE2 was selectively overexpressed in podocytes from TG mice. First, robust mRNA expression of the transgene occurred in glomeruli from TG mice, at a level ~50-fold higher than that in kidney cortex, whereas endogenous mouse ACE2 expression was lower in glomeruli than cortex. Other tissues from TG mice, including brain (which expresses nephrin[24]) did not significantly express human ACE2 mRNA. Second, by immunoblot, the human ACE2 protein was detected in glomerular isolates, but not kidney cortex, as a protein of ~120 kDa. Third, the ACE2 protein colocalized with synaptopodin by immunofluorescence, indicating its presence in the podocyte. Fourth, ACE2 activity was significantly enhanced in glomeruli from TG mice, and elevated activity (and glomerular expression by immunoblot and immunofluorescence) persisted after 16 weeks of STZ-diabetes. In this

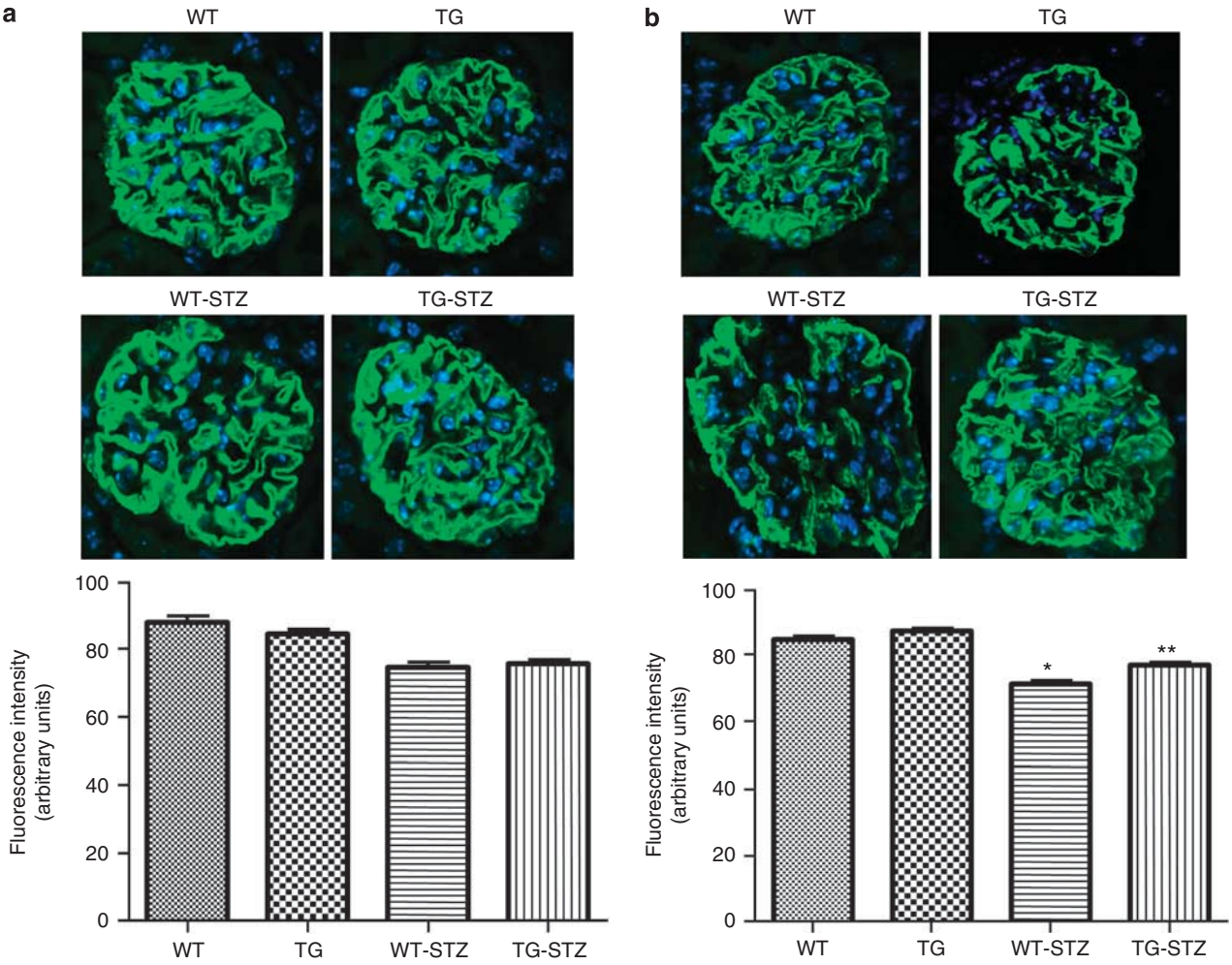


**Figure 11 | Synaptopodin expression at 8 and 16 weeks by immunofluorescence.** (**a**) Graph depicting semiquantitative analysis of synaptopodin expression by immunofluorescence in wild-type (WT), transgenic (TG), wild-type streptozotocin-treated (WT-STZ), and transgenic streptozotocin-treated (TG-STZ) mice at 8 weeks. Values are means ± s.e., $n = 100$ glomeruli per group, from 7 to 8 mice per group. There was no significant difference among the groups. Representative images depicting glomerular synaptopodin expression (green) at 8 weeks are shown above graph. Nuclear Hoechst staining (blue) is also shown. Original magnification × 640. (**b**) Graph depicting synaptopodin expression at 16 weeks. Values are means ± s.e., $n = 100$ glomeruli per group, from 6 to 11 mice per group. *$P < 0.001$ vs. WT and TG, **$P < 0.001$ vs. WT-STZ. There was no significant difference between TG and TG-STZ mice. Representative images depicting glomerular synaptopodin expression (green) at 16 weeks are shown above graph. Nuclear Hoechst staining (blue) is also shown. Original magnification × 640.

regard, nephrin expression decreases in diabetic nephropathy,[25,26] and nephrin deficiency is associated with activation of nuclear factor-κB–mediated pathways that enhance glomerular injury.[27] Our data suggest that the nephrin promoter remained active and stimulated transcription of the human ACE2 gene in TG-STZ mice, despite the presence of severe diabetes. Finally, selective overexpression of the human *ACE2* gene in podocytes was not associated with changes in systolic BP. Plasma ACE2 activity did not increase above the level of WT-STZ mice, in keeping with the localized increase in ACE2 at the podocyte level.

In WT-STZ mice, a significant increase in albuminuria occurred at 4 weeks as compared with nondiabetic mice. In contrast, urinary albumin excretion in TG-STZ mice at 4 weeks did not differ from levels in non-diabetic mice. The 24 h albuminuria levels in our diabetic mouse model were significantly higher than those reported by Qi *et al.*[28] in FVB mice with STZ-diabetes. There is considerable variability in urinary albumin measures in mouse models of diabetes, and assay procedures differ. Studies using the same assay procedure we used in this study have reported high measures of urinary albumin in other mouse strains with diabetes.[29,30] Despite the initial prevention of albuminuria in TG-STZ mice, albuminuria increased to the level of WT-STZ mice at later time points. In diabetes, increased Ang II is thought to promote podocyte dysfunction and albuminuria. Ang II reduces expression of the antioxidant protein peroxiredoxin 2 in podocytes *in vitro* and *in vivo*, associated with increased production of reactive oxygen species, inhibition of the Akt pathway, and apoptosis.[20] As TG-STZ mice were only

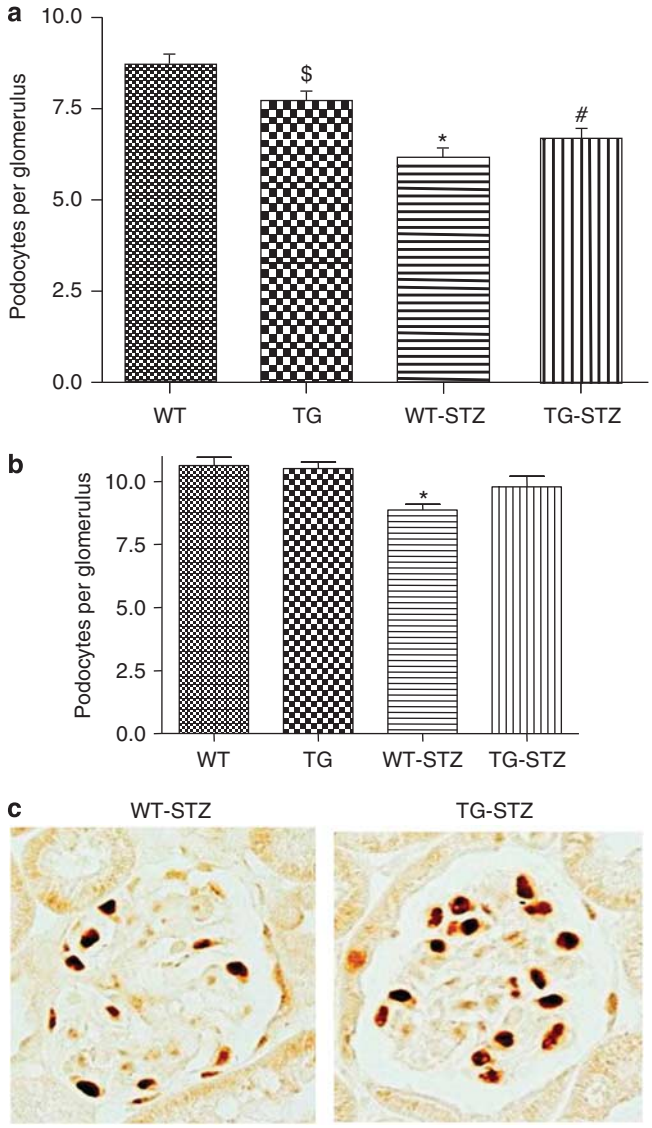


**Figure 12 | Podocyte number at 8 and 16 weeks.** (**a**) Graph shows average number of podocytes per glomerulus in wild-type (WT), transgenic (TG), wild-type streptozotocin-treated (WT-STZ), and transgenic streptozotocin-treated (TG-STZ) mice at 8 weeks, as determined by counting of WT-1-positive nuclei in kidney sections. Values are means ± s.e., $n = 78$–106 glomeruli per 7 to 8 mice in each group. $^{*}P < 0.001$ vs. WT and TG, $^{\#}P < 0.01$ vs. TG, $^{\$}P < 0.01$ vs. WT. (**b**) Podocyte numbers at 16 weeks. Values are means ± s.e., $n = 120$ glomeruli per 6 mice in each group. $^{*}P < 0.01$ vs. WT and TG. There was no significant difference between TG-STZ and WT, TG, or WT-STZ mice. (**c**) Representative photomicrograph depicting glomerular WT-1 staining in glomeruli from WT-STZ vs. TG-STZ mice at 16 weeks. Original magnification × 400.

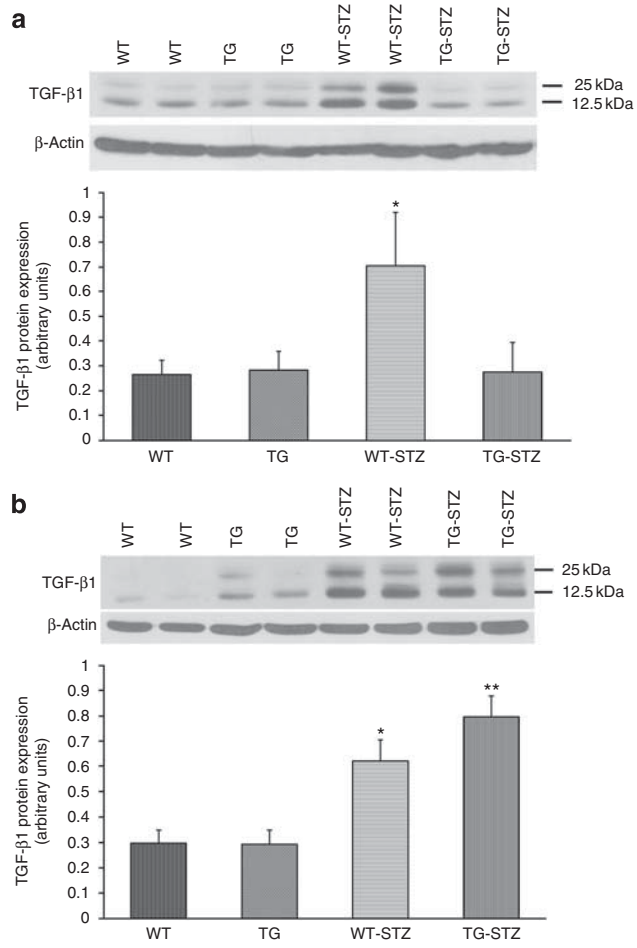


**Figure 13 | Expression of transforming growth factor-β1 (TGF-β1) at 8 and 16 weeks.** (**a**) Graph shows expression of TGF-β1 in kidney cortical lysates from wild-type (WT), transgenic (TG), wild-type streptozotocin-treated (WT-STZ), and transgenic streptozotocin-treated (TG-STZ) mice at 8 weeks, by immunoblot. Values are means ± s.e., $n = 6$ per group. $^{*}P < 0.02$ vs. all other groups (WT, TG, and TG-STZ). Above graph is a representative immunoblot for TGF-β1, depicting bands at 12.5 and 25 kDa, the latter representing the TGF-β1 dimer. Blot for β-actin, as loading control, is depicted below. (**b**) Graph shows expression of TGF-β1 from cortical lysates at 16 weeks, with representative immunoblot above graph. Values are means ± s.e., $n = 6$ per group. $^{*}P < 0.05$ vs. WT and TG. $^{**}P < 0.01$ vs. WT and TG. There was no significant difference between WT-STZ and TG-STZ mice.

protected against albuminuria at 4 weeks, podocyte ACE2 activity may not have been sufficiently enhanced at later time points to reduce this Ang II–mediated adverse signaling, or other non-Ang II–dependent pathways affecting podocyte function may have dominated and contributed to albuminuria.

Nonetheless, the early reduction in albuminuria in TG-STZ mice was associated with improvement in features of diabetic nephropathy. First, a significant decrease in mesangial area occurred at 8 weeks, with decreased glomerular hypertrophy at 16 weeks. Second, expression of the podocyte proteins nephrin and synaptopodin, reported to be decreased in diabetic models,[31,32] was partly preserved in TG-STZ mice. In TG mice, baseline nephrin expression was decreased compared with WT mice, and treatment with STZ did not induce a further decrease. As our model used the mouse nephrin promoter to induce podocyte-specific ACE2 overexpression, the decrease in basal nephrin expression in TG mice could be because of competition for transcription factors between the nephrin gene and the transgene. Importantly, partial protection against loss of podocyte

number was evident at 16 weeks, but not at 8 weeks. Finally, the expression of TGF-β1 in kidney cortex was significantly attenuated in TG-STZ mice at 8 weeks. We postulate that this partial protection is because of enhanced ACE2-dependent degradation of Ang II in the diabetic glomerulus. Our data support the hypothesis that in the diabetic kidney, glomerular Ang II initiates a reduction in nephrin and synaptopodin expression, and stimulation of TGF-β1, which induces podocyte loss (diabetic podocytopathy), leading to proteinuria and subsequent tubulointerstitial inflammation and fibrosis, which in turn is associated with increased elaboration of TGF-β1.[33] Consistent with this view, in rats with STZ-diabetes, intravenous adenoviral gene transfer of ACE2 reduces kidney cortical levels of Ang II, and increases Ang-(1–7), associated with protection from glomerular injury.[11] In rat mesangial cells, transfection of ACE2 inhibits Ang II–stimulated cell proliferation, oxidative stress, and collagen IV synthesis.[11]

Although the beneficial effects of ACE2 may have derived largely from a reduction in Ang II levels in our study, the role of potentially enhanced podocyte generation of Ang-(1–7) in TG mice remains unclear. Although Ang-(1–7) inhibits Ang II–mediated proinflammatory signaling in proximal tubular cells and the vasculature,[3–5] its effects on podocyte function are unknown. In mesangial cells, Ang-(1–7) has been reported to either stimulate profibrotic pathways[34] or inhibit Ang II signaling.[35] Thus, although overexpression of ACE2 in the podocyte has a renoprotective effect, the relative contribution of diminished levels of Ang II versus increased Ang-(1–7) requires further study. Finally, this study focused on early diabetic nephropathy, and the long-term effects of overexpression of ACE2 on disease progression remain unclear.

In summary, after STZ-induced diabetes, TG mice with podocyte-specific overexpression of the human ACE2 protein are protected against the early development of albuminuria and show partial preservation of podocyte proteins and podocyte number, reduced glomerular histological injury, and decreased kidney cortical TGF-β1 expression. ACE2 may represent a therapeutic target in the prevention and treatment of diabetic nephropathy.

## MATERIALS AND METHODS

### TG mice

The cDNA encoding the open reading frame of the human *ACE2* gene, which contained a double hemagglutinin epitope tag at the 5′ end, was inserted immediately downstream of the murine nephrin promoter (provided by Dr C Kennedy, University of Ottawa, Ottawa, ON, Canada), and cloned into the *Xho*I restriction site of the expression vector pcDNA3 (Invitrogen, Carlsbad, CA). The correct orientation of the insert was confirmed by DNA sequencing (StemCore Laboratories, Ottawa Hospital Research Institute, Ottawa, ON, Canada). Transfection of the expression plasmid containing the cytomegalovirus promoter but lacking the nephrin promoter into cultured COS-7 cells induced robust expression of the human ACE2 protein, as determined by western blot analysis (not shown). The linearized expression plasmid containing the nephrin promoter was microinjected into FVB mouse embryos (Charles River Laboratories, Wilmington, MA) and founder mice were identified by DNA genotyping. Three mouse founder lines were characterized for podocyte ACE2 expression, and one line was selected for further studies.

### Diabetes model

All mice used in these studies were on a congenic FVB/n background. At 8 weeks of age, male WT and TG mice were injected over 5 consecutive days with either STZ (50 μg/g per day intraperitoneally; Sigma-Aldrich, St Louis, MO) or sodium-citrate vehicle. Hindlimb blood glucose levels were monitored weekly by glucometer readings. Urine was analyzed for ketones using Ketostix (Bayer HealthCare LLC, Elkhart, IN). All mice were housed and cared for in the Animal Care Facility at the University of Ottawa with free access to food and water. All protocols were approved by the University of Ottawa Animal Care Committee and conducted according to the guidelines of the Canadian Council on Animal Care.

### Systolic BP

Systolic BP was measured using tail-cuff plethysmography (BP-2000; Visitech Systems, Apex, NC). Mice were trained at 6 weeks of age for 5 consecutive days and a baseline measurement was taken at 7 weeks of age. Systolic BP was measured weekly from 1 week after STZ or vehicle injection until 16 weeks.

### Plasma analysis

At 16 weeks after injections, mice were anesthetized using isoflurane and killed by cardiac puncture. Blood was collected with a heparinized needle and plasma was separated by centrifugation at 3000 *g* for 10 min at 4 °C. Plasma electrolytes, glucose, albumin, carbon dioxide, triglycerides, and cholesterol were assayed using the Synchron CX5 Delta analyzer (Beckman Coulter, Fullerton, CA).

### Glomerular isolation

Mice were anesthetized and cardiac perfusion was initiated with $4 \times 10^7$ Dynabeads (Invitrogen Dynal AS, Oslo, Norway), diluted in 20 ml of phosphate-buffered saline. Kidney cortices were digested in collagenase Type 1 (1.0 mg/ml; Worthington Biochemical, Lakewood, NJ) and DNase 1 (0.1 mg/ml; Roche Diagnostics, Indianapolis, IN) at 37 °C for 30 min, and tissue was passed through a 100 μm sieve. Glomeruli were isolated using a magnetic particle concentrator.[36]

### Reverse transcriptase–PCR for human and mouse ACE2

RNA was isolated from glomeruli and other tissues for real-time PCR for ACE2. Details of the protocol can be found in Supplementary Information online.

### Immunoblotting

Kidney cortical tissue and glomeruli were prepared for immunoblot analysis with antibodies to ACE2, nephrin, and TGF-β1. Details of the protocol can be found in Supplementary Information online.

### Measurement of glomerular filtration rate

The glomerular filtration rate was measured in mice at 16 weeks of study, using plasma fluorescein isothiocyanate–inulin clearance kinetics, as we have described.[37]

### Urinary albumin excretion

At 4, 8, and 16 weeks after injections, urine was collected over 24 h in metabolic cages. Urinary albumin excretion was measured using a murine albumin ELISA (Bethyl Laboratories, Montgomery, TX).

### Kidney histology

Dissected kidneys were incubated with 4% paraformaldehyde for 48 h at 4 °C, and were subsequently dehydrated, paraffin-embedded, and cut into 4 μm sections. Sections were stained with periodic acid–Schiff for measurement of mesangial and glomerular cross-sectional areas. Podocyte counts were determined by staining for WT-1,[38] and immunofluorescent staining was performed for ACE2, hemagglutinin, nephrin, and synaptopodin using standard procedures. All analyses were performed in a blinded fashion. Details on the histological protocols can be found in Supplementary Information online.

### ACE2 enzymatic activity assay

ACE2 activity in glomeruli and plasma was determined using a fluorogenic ACE2 synthetic substrate, essentially as described.[37,39] Details of the assay can be found in Supplementary Information online.

### Statistics

Data are presented as mean ± s.e. Data were analyzed using GraphPad Prism (Software version 4.02, San Diego, CA). For multiple comparisons, analysis was by one-way analysis of variance followed by Bonferroni or Newman–Keuls post-comparison test. For comparisons involving two groups, Student's *t*-test was used. A *P*-value of $<0.05$ was considered statistically significant.

### DISCLOSURE

All the authors declared no competing interests.

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### SUPPLEMENTARY MATERIAL

**Supplementary Information.** RT-PCR for human and mouse ACE2. Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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