NADPH Oxidase Nox5 Accelerates Renal Injury in Diabetic Nephropathy

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NADPH oxidase–derived excessive production of reactive oxygen species (ROS) in the kidney plays a key role in mediating renal injury in diabetes. Pathological changes in diabetes include mesangial expansion and accumulation of extracellular matrix (ECM) leading to glomerulosclerosis. There is a paucity of data about the role of the Nox5 isoform of NADPH oxidase in animal models of diabetic nephropathy since Nox5 is absent in the mouse genome. Thus, we examined the role of Nox5 in human diabetic nephropathy in human mesangial cells and in an inducible human Nox5 transgenic mouse exposed to streptozotocin-induced diabetes. In human kidney biopsies, Nox5 was identified to be expressed in glomeruli, which appeared to be increased in diabetes. Colocalization demonstrated Nox5 expression in mesangial cells. In vitro, silencing of Nox5 in human mesangial cells was associated with attenuation of the hyperglycemia and TGF-β1–induced enhanced ROS production, increased expression of profibrotic and proinflammatory mediators, and increased TRPC6, PKC-α, and PKC-β expression. In vivo, vascular smooth muscle cell/mesangial cell–specific overexpression of Nox5 in a mouse model of diabetic nephropathy showed enhanced glomerular ROS production, accelerated glomerulosclerosis, mesangial expansion, and ECM protein (collagen IV and fibronectin) accumulation as well as increased macrophage infiltration and expression of the proinflammatory chemokine MCP-1. Collectively, this study provides evidence of a role for Nox5 and its derived ROS in promoting progression of diabetic nephropathy.

Diabetic nephropathy (DN), a leading cause of end-stage renal disease, is characterized by the progressive expansion of the mesangium with accumulation of extracellular matrix (ECM) components in both the glomerular mesangium and basement membrane, ultimately leading to glomerulosclerosis (1). Multiple factors in diabetes contribute to the development of increased accumulation of mesangial matrix (2). The activation of profibrotic growth factors and cytokines by hyperglycemia leads to an increase in the synthesis of most collagens and fibronectin (3).

Several studies support the finding that renal reactive oxygen species (ROS) play a central role in mediating renal injury in diabetes (4–6). A range of enzymes are capable of generating ROS, but the prooxidant enzyme family of NADPH oxidases, Nox, are the only enzymes known to be solely dedicated to ROS generation in the kidney (7–10). Indeed, we previously showed that the Nox4 isoform contributes to renal injury in a mouse model of DN and that targeting of Nox4 using global or podocyte-specific genetic deletion or pharmacological inhibition resulted in attenuation

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of the diabetes-induced increase in albuminuria, glomerulosclerosis, and accumulation of ECM proteins via a reduction in ROS production in an experimental model of DN (7–10).

Recent studies have suggested a potential role for the more recently identified human Nox isoform, Nox5, in the pathogenesis and progression of diabetes-associated complications including nephropathy (11–13). However, there is a paucity of information about Nox5 in animal models of DN, since the Nox5 gene is absent in mice and rats. Recently, a study by Holterman et al. (11) showed increased expression of Nox5 in kidneys of patients with diabetes compared with control kidneys. Furthermore, in inducible human transgenic Nox5 mice with selective expression of Nox5 in podocytes, there was associated injury to podocytes and deleterious changes in renal function and structure in experimental diabetes (11). Given the important role of the mesangial cell with respect to renal fibrosis, in this study we examined the effect of inducible human Nox5 expression using Nox5 transgenic mice in the presence and absence of streptozotocin (STZ)-induced diabetes on renal injury with a particular focus on mesangial expansion and fibrosis.

RESEARCH DESIGN AND METHODS

Animals

The Nox5 transgenic mice were generated by our collaborators (Christopher Kennedy, University of Ottawa, Ottawa, Canada, and Rhian Touyz, University of Glasgow) (11). Briefly, the purified NOX5β gene-coding region was ligated with Tet-responsive promoter Pbi-1 (Clontech) to generate the NOX5β founder mouse on FVB/N background. The SM22-tTA-FVB/N mouse strain was generated by introducing the vascular smooth muscle cell (VSMC)-specific promoter SM22 to the tetracycline-regulated transcriptional activator (tTA-Off) gene. For subsequent generation of VSMC-Nox5–specific mice, the SM22-tTA-FVB/N strain was crossed with the Nox5β FVB/N strain to produce $SM22^{+}Nox5^{+}$ and $SM22^{+}Nox5^{-}$ mice. The $SM22^{+}Nox5^{+}$ transgenic mouse expresses Nox5 selectively in VSMCs, which include mesangial cells in the glomeruli. The inducible Nox5 transgenic mice express Nox5β under the control of the bidirectional tet0-responsive promoter. Gene expression of Nox5 is silenced by administration of doxycycline dose (1 mg/mL daily in drinking water). Doxycycline administration is maintained in lactating and pregnant mice and withdrawn from their offspring after weaning in advance of the induction of STZ diabetes. Control mice were littermates of SM22-tTA-FVB/N and Nox5β FVB/N mouse crosses. All animal studies were approved by the Alfred Medical Research and Education Precinct (AMREP) Animal Ethics Committee under guidelines laid down by the National Health and Medical Research Council (NHMRC) of Australia.

Diabetes was induced in 6-week-old male $SM22^{+}Nox5^{+}$ and $SM22^{+}Nox5^{-}$ mice by five daily i.p. injections of STZ (Sigma-Aldrich, St. Louis, MO), at a dose of 55 mg/kg/body wt in citrate buffer, with control mice receiving citrate buffer alone. Mice were placed individually into metabolic cages (Iffa Credo, L'Arbresle, France) for 24 h at 10 weeks after induction of diabetes. Urine and plasma samples were collected for subsequent analysis. Blood glucose, glycated hemoglobin, and systolic blood pressure were measured as previously described (8,14,15). Concentration of urinary albumin was measured by using a mouse albumin ELISA quantitation kit (Bethyl Laboratories, Montgomery, TX). Urinary and plasma creatinine were determined by a commercially available creatinine assay kit using the Cobas Integra 400 Plus computerized analyzer (Roche Diagnostics). Creatinine clearance and the urinary albumin-to-creatinine ratio (ACR) were determined as previously described (8,14).

A mouse cystatin C ELISA kit (BioVendor, Brno, Czech Republic) was used to determine serum cystatin C according to the manufacturer's instructions. After 10 weeks, animals were anesthetized by sodium pentobarbitone (100 mg/kg body wt i.p., Euthatal; Sigma-Aldrich, Castle Hill, Australia). The kidneys were rapidly dissected, weighed, and snap frozen or processed to paraffin for subsequent analysis.

Histochemistry on Renal Sections

Kidney sections were stained with periodic acid Schiff (PAS) for measurement of mesangial expansion and glomerulosclerotic injury as previously described (8,14,16). Immunostaining for Nox5, collagen IV, fibronectin, nitrotyrosine, F4/80, and PKC-α and dihydroethidium (DHE) staining for superoxide production were conducted as previously described (8,14) (Supplementary Data). All sections were examined using microscopy and digitized with a high-resolution camera. For the quantification of the proportional area of staining, 20 glomeruli (×400) were analyzed using Image-Pro plus 7.0 (Media Cybernetics, Bethesda, MD).

Quantitative RT-PCR

Total RNA from glomerular fraction and human mesangial cells was extracted with Trizol and analyzed and cDNA generated as previously described (8,9). Gene expression using probes and primers as described in Supplementary Table 1 was analyzed quantitatively and relative to the expression of the housekeeping gene 18S (18S ribosomal RNA Taqman Control Reagent kit) using the Taqman system (ABI Prism 7500; Perkin Elmer, Foster City, CA) (8,14). Results were expressed relative to nondiabetic $SM22^{+}Nox5^{-}$ mice, which were arbitrarily assigned a value of 1.

Western Blot

Glomerular protein extracts (5 μg) were electrophoresed on 7.5–10% acrylamide gels under nonreducing conditions (17). Western blot analysis was then performed after incubation with a primary antibody to Nox5 (goat polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA), SM22-α (goat polyclonal; Abcam, Cambridge, MA), MCP-1 (rabbit polyclonal; Abcam), and PKC-α (rabbit polyclonal; Santa Cruz Biotechnology) and assessed with rabbit anti-goat for Nox5 and SM22-α and goat anti-rabbit for MCP-1 and PKC-α (Dako Corp., Carpinteria, CA) secondary antibodies. Membranes were subsequently probed for α-tubulin and β-actin

(Sigma-Aldrich, St. Louis, MO) for determination of equal loading of samples. Blots were detected using the ECL detection kit (Sigma-Aldrich, St. Louis, MO), and densitometry was performed using Quantity One software (Bio-Rad Laboratories, Richmond, CA).

Nitrotyrosine: ELISA

The concentration of nitrotyrosine was identified in the protein extracts obtained from the glomerular fraction by using the manufacturer's instructions for the ELISA kit (Abcam). The ELISA results were expressed relative to protein concentration.

Lucigenin Assays

The glomerular fraction was harvested in 100 μL ice-cold phosphate buffer of 50 mmol/L KH_2PO_4, 1 mmol/L EGTA, and 150 mmol/L sucrose (pH 7.4) with protease inhibitors as previously described (9,11). Baseline activity was measured by adding 50 μL glomerular extract to 175 μL buffer and 2.5 μL of 1 mmol/L lucigenin (Sigma-Aldrich, St. Louis, MO). NADPH-dependent superoxide production was measured by the addition of 25 μL of 1 mmol/L NADPH (Sigma-Aldrich, St. Louis, MO). Baseline activity was subtracted and normalized to protein concentration.

In Vitro Experiments

Normal human mesangial cells were obtained from Lonza (Clonetics Mesangial Cell Systems, Lonza, Walkersville, MD). Cells were grown in mesangial cell medium (Lonza) with 10% FCS in a humidified incubator (5% CO_2 at 37°C). The knockdown of Nox5 was performed in human mesangial cells using MISSION short hairpin RNA (shRNA)-expressing lentivirus vectors (Sigma-Aldrich, St. Louis, MO) as previously described (18). The sequence targeting Nox5 knockdown corresponds to 5′-GCCACTTTGAGGTGTTCTATT-3′ (TRCN0000046101). The cells transduced with the MISSION Nontarget shRNA control vector particles (Sigma-Aldrich, St. Louis, MO) were used as controls. Cells were seeded at 1×10^6 cells/dish in a 100-mm dish and infected by the lentivirus particles in the presence of 8 μg/mL polybrene, followed by selection in puromycin (1 μg/mL; Sigma-Aldrich, St. Louis, MO) for 4 days. The knockdown efficiency in the cells was verified by RT-PCR. Nontarget and Nox5-shRNA infected cells were then cultured in mesangial cell medium with 5 mmol/L glucose or 25 mmol/L glucose or in high glucose plus TGF-β1 (5 ng/mL) and incubated for 48 h at 37°C for subsequent gene expression analysis. Results were expressed relative to the control, which was arbitrarily assigned a value of 1. Human Probe and primer sequences used for quantitative RT-PCR are shown in Supplementary Table 2.

Measurement of ROS In Vitro

Nontarget and Nox5-knockdown human mesangial cells were incubated in normal glucose (NG) (5 mmol/L) or in the presence of high glucose (25 mmol/L) or in high glucose plus TGF-β1 (5 ng/mL) for 4 h at 37°C. Thereafter, cells were washed with PBS, harvested, and assayed in duplicates in clear 96-well microplates (Perkin Elmer) and prewarmed Krebs-HEPES supplemented with L-012 (Wako Chemicals, Richmond, VA) at a concentration of 100 μmol/L added in each well in the dark and incubated at 37°C for 10 min. After incubation, plates were read in a luminometer (Micro lumat plus; Berthold Technologies, Pforzheim, Germany) and luminescence was measured with a single measuring time of 1 s and cycle time of 111 s for 20 min at 37°C. Buffer blank was subtracted from each reading. A Pierce BCA protein assay (Thermo Scientific, Scoresby, Australia) was performed (samples 1:10 in PBS) according to kit instructions, and results were expressed relative to the total protein concentration (milligrams).

Statistical Analysis

All parameters were analyzed by one-way ANOVA using GraphPad Prism 6 for multiple comparison of the means or analyzed by the two-tailed unpaired Mann-Whitney *U* test when required. A P value <0.05 was considered to be statistically significant. Results are expressed as mean ± SEM unless otherwise specified.

RESULTS

Expression of Nox5 in Human Mesangial Cells and Kidney Biopsies

We examined the gene expression of Nox isoforms in response to hyperglycemia in human mesangial cells. Both gene and protein expression of Nox5 in human mesangial cells were increased in the presence of high glucose compared with NG (Fig. 1*A* and Supplementary Fig. 3*A*–*C*), whereas gene expression of the Nox1, Nox2, and Nox4 isoforms remained unchanged (Fig. 1*A*).

In addition, we examined the protein expression of Nox5 in the kidney biopsies obtained from individuals with or without diabetes. Nox5 expression appeared to be increased in the glomeruli from individuals with diabetes versus those without (Fig. 1*B*). Furthermore, Nox5 expression was localized to mesangial cells, as evident from the colocalization of Nox5 and SM22-α (Fig. 1*C*). Nox5 was also increased in renal blood vessels in kidney biopsies of individuals with diabetes versus those without (Supplementary Fig. 2*A*).

Characterization of Nox5 Expression in Mesangial Cells of Nox5 Transgenic Mice

Nox5 expression in mesangial cells was confirmed by colocalization of Nox5 and SM22-α (a marker of smooth muscle cells) in the mesangial cells of $SM22^+Nox5^+$ transgenic mice (Fig. 1*D*). In addition, induction of diabetes showed increased protein expression of Nox5 in $SM22^+Nox5^+$ transgenic mice compared with control $SM22^+Nox5^+$ transgenic mice (Fig. 1*E* and *F*). With respect to other Nox isoforms, there were no changes in Nox1 expression, but expression of Nox4 and Nox2 was increased in $SM22^+Nox5^-$ diabetic mice compared with $SM22^+Nox5^-$ control mice (Supplementary Fig. 1*A*). Nox2 expression was increased in both control and diabetic $SM22^+Nox5^+$ transgenic mice at the gene and protein level (Supplementary Fig. 1*A*–*C*). Nox4 expression was not increased in diabetic $SM22^+Nox5^+$ transgenic mice (Supplementary Fig. 1*A*).

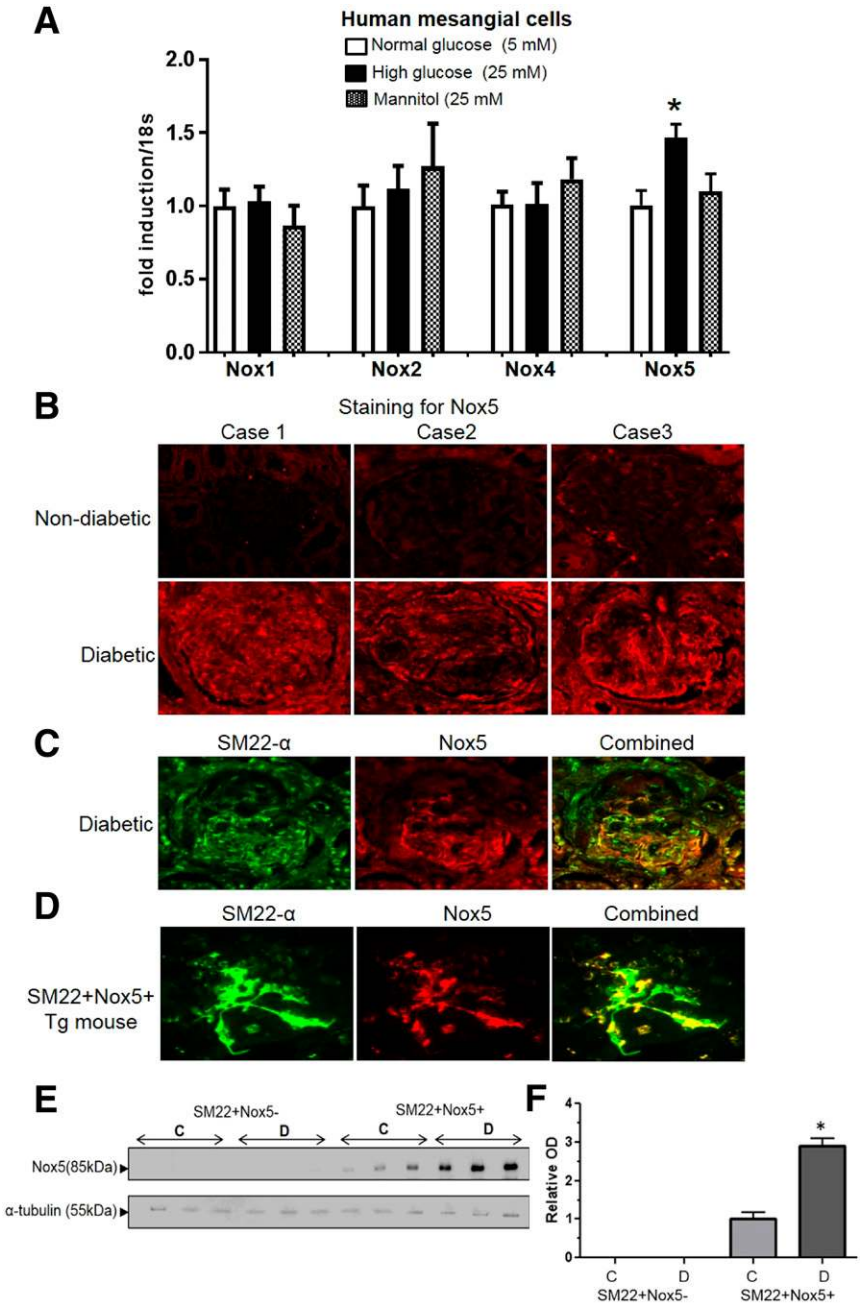

Figure 1—Expression of Nox5 in human kidney and mesangial cells and characterization of Nox5 transgenic mice ($SM22^{+}Nox5^{+}$) expressing Nox5β in VSMCs including mesangial cells in the glomeruli. *A*: Gene expression of Nox1, Nox2, Nox4, and Nox5 in response to NG (5 mmol/L), high glucose (25 mmol/L), and mannitol (25 mmol/L + 5 mmol/L NG) in human mesangial cells. *$P < 0.05$ vs. NG. *B*: Immunofluorescence for Nox5 (red staining) in kidney biopsies obtained from individuals without and with diabetes ($n = 3$/group). *C* and *D*: Colocalization of Nox5 (red staining) and the marker of smooth muscle cells, transgelin (SM22-α [green staining]), in kidney biopsies obtained from individuals with diabetes (*C*) as well as in frozen kidney sections of $SM22^{+}Nox5^{+}$ transgenic mice (*D*) (magnification ×20). *E*: Western blot for the expression of Nox5 in the glomerular fraction of the control (C) and diabetic (D) $SM22^{+}Nox5^{-}$ and $SM22^{+}Nox5^{+}$ mice after 10 weeks of diabetes and its quantitation (*F*) ($n = 3$/group). *$P < 0.05$ vs. control $SM22^{+}Nox5^{+}$ mice. Data are shown as mean ± SEM. OD, optical density.

Gene and protein expression of glomerular SM22-α in $SM22^{+}Nox5^{+}$ mice was not altered by diabetes per se (Supplementary Fig. 2*C–E*), but expression of Nox5 was increased in the glomeruli of diabetic mice (Fig. 1*E* and *F* and Supplementary Fig. 2*B*). There was no expression of Nox5 in podocytes in $SM22^{+}Nox5^{+}$ mice (Supplementary Fig. 2*F*).

Metabolic Parameters

We examined the effects of human Nox5 gene expression selectively in vascular smooth muscle/mesangial cells in a mouse model of STZ-induced DN on various metabolic and biochemical parameters after 10 weeks of diabetes. Diabetic $SM22^{+}Nox5^{-}$ mice had reduced body weight, elevated plasma glucose and glycated hemoglobin levels, increased

food and water intake, and polyuria compared with nondiabetic controls. Overexpression of Nox5 in mesangial cells had no effect on the diabetes-induced changes in these metabolic parameters (Table 1). In addition, systolic blood pressure was similar in all groups. The kidney weight–to–body weight ratio was increased in $SM22^{+}Nox5^{-}$ diabetic mice, and this parameter was further increased in $SM22^{+}Nox5^{+}$ diabetic mice (Table 1).

Glomerular Superoxide and ROS Levels

Nox5 generates superoxide and then interacts with nitric oxide to form peroxynitrite, which binds to tyrosine residues to form nitrotyrosine (19). NADPH-dependent glomerular superoxide production (Fig. 2*E*), glomerular intensity of DHE fluorescence (Fig. 2*D*) and nitrotyrosine accumulation (Fig. 2*A* and *B*), and the level of glomerular nitrotyrosine (Fig. 2*C*) were all increased in $SM22^{+}Nox5^{-}$ mice after 10 weeks of diabetes compared with nondiabetic controls. Notably, a further increase in superoxide and nitrotyrosine levels was found in $SM22^{+}Nox5^{+}$ diabetic mice compared with $SM22^{+}Nox5^{-}$ diabetic mice (Fig. 2*A*–*E*).

Renal Functional Parameters

Induction of diabetes was associated with increased creatinine clearance and a decreased level of both plasma creatinine and cystatin C in both diabetic groups of mice compared with their respective controls, but there was no specific effect of the Nox5 transgene (Table 1). Nox5 expression in mesangial cells per se did not increase albuminuria in the absence of diabetes. The level of albuminuria was significantly increased in both groups of diabetic mice compared with their respective controls. A trend for increased albuminuria (P = 0.059) was observed in $SM22^{+}Nox5^{+}$ mice compared with $SM22^{+}Nox5^{-}$ after 10 weeks of diabetes. Similar effects were also observed when the data were expressed as ACR (P = 0.057)) after 10 weeks of diabetes (Table 1).

Renal Structural Assessment

PAS-positive mesangial area expansion (Fig. 3*A* and *B*) and glomerulosclerotic index (Fig. 3*C*) were not altered by Nox5 expression in the absence of diabetes. After 10 weeks of diabetes, glomerulosclerosis and mesangial area were significantly increased in both $SM22^{+}Nox5^{-}$ and $SM22^{+}Nox5^{+}$ mice compared with their respective controls. Mesangial area and glomerulosclerosis were significantly further increased in diabetic $SM22^{+}Nox5^{+}$ transgenic mice compared with diabetic $SM22^{+}Nox5^{-}$ mice (Fig. 3*A*–*C*).

Gene Expression of Profibrotic Markers

Consistent with the findings on glomerulosclerosis and mesangial expansion, glomerular gene expression of ECM proteins including fibronectin and collagens I and IV; expression of α-SMA, a marker of mesangial cell proliferation and fibrosis; and expression of p21, a marker of cell hypertrophy (Fig. 4*A*), were increased in both diabetic $SM22^{+}Nox5^{-}$ and $SM22^{+}Nox5^{+}$ mice compared with their respective control mice. A further increase in the expression of fibronectin, collagens I and IV, α-SMA, and p21 was observed in $SM22^{+}Nox5^{+}$ mice compared with $SM22^{+}Nox5^{-}$ mice after 10 weeks of diabetes (Fig. 4*A*).

Accumulation of ECM Proteins

Nox5 expression in the absence of diabetes did not affect fibronectin or collagen IV accumulation (Fig. 4*B*–*E*). However, in the presence of diabetes both collagen IV and fibronectin were increased in the glomeruli of diabetic $SM22^{+}Nox5^{-}$ and $SM22^{+}Nox5^{+}$ mice compared with their respective control mice. Importantly, the expression of the

Table 1—General and metabolic variables, albuminuria, creatinine clearance, plasma creatinine, and cystatin C in control and diabetic $SM22^{+}Nox5^{-}$ and $SM22^{+}Nox5^{+}$ mice (n = 10–20/group)

	$SM22^{+}Nox5^{-}$		$SM22^{+}Nox5^{+}$	
	Control	Diabetes	Control	Diabetes
Body weight (g)	32 ± 0.9	28 ± 0.6*	32 ± 0.7	26 ± 0.6^
Kidney weight/body weight (%)	0.64 ± 0.02	0.99 ± 0.03*	0.68 ± 0.02	1.12 ± 0.03^#
Plasma glucose (mmol/L)	11.9 ± 0.5	33.0 ± 2.4*	12.0 ± 0.8	34.2 ± 1.4^
Glycated hemoglobin (%)	4.3 ± 0.1	11.4 ± 0.5*	4.2 ± 0.1	11.2 ± 0.5^
Systolic BP (mmHg)	105 ± 0.9	109 ± 1.0	108 ± 0.9	110 ± 1.2
Food intake (g)	2.9 ± 0.2	5.8 ± 0.5*	3.1 ± 0.3	6.4 ± 0.3^
Water intake (mL)	2.85 ± 0.40	27.5 ± 2.0*	3.0 ± 0.4	26.9 ± 1.6^
Urine output (mL)	0.56 ± 0.08	22.7 ± 1.8*	0.57 ± 0.10	24.4 ± 1.61^
Creatinine clearance (mL/min/m^2)	6.8 ± 0.5	19.8 ± 2.6*	6.9 ± 1.1	13.9 ± 2.7^
Albuminuria (μg/24 h)	38 ± 4.5	651 ± 80*	27 ± 2.6	864 ± 70^†
ACR (μg/mg)	124 ± 21	1,130 ± 147*	97 ± 11	1,648 ± 203^††
Plasma creatinine (μmol/L)	22.7 ± 2.3	17.7 ± 2.5	29.6 ± 3.2	21.5 ± 4.7
Cystatin C (ng/mL)	346 ± 24	208 ± 32*	431 ± 28	134 ± 18*

Data are shown as mean ± SEM. BP, blood pressure. *$P < 0.05$ vs. control $SM22^{+}Nox5^{-}$ mice; ^$P < 0.05$ vs. control $SM22^{+}Nox5^{+}$ mice; †P = 0.059 and ††P = 0.057 vs. diabetic $SM22^{+}Nox5^{-}$ mice; #$P < 0.05$ vs. diabetic $SM22^{+}Nox5^{-}$ mice.

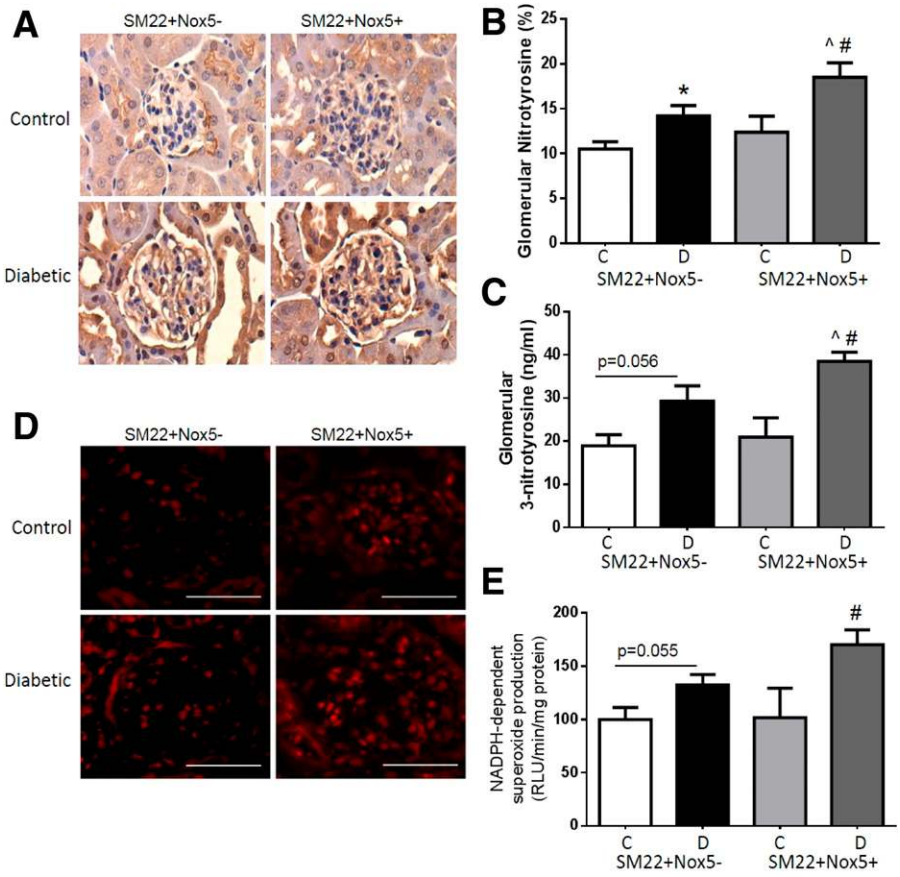

Figure 2—Overexpression of Nox5 in mesangial cells enhances glomerular ROS production in diabetic mice. Immunostaining of glomerular nitrotyrosine (*A*) and its quantitation (*B*) (n = 6–8/group) (magnification ×40). ELISA for nitrotyrosine in glomerular fraction (*C*). Immunofluorescence staining for DHE (*D*) (scale bars = 50 μm) and NADPH-dependent lucigenin assay (*E*) (n = 5–6/group) for superoxide production in the glomeruli of control (C) and diabetic (D) $SM22^{+}Nox5^{-}$ and $SM22^{+}Nox5^{+}$ transgenic mice after 10 weeks of diabetes. Data are shown as mean ± SEM. *P < 0.05 vs. control $SM22^{+}Nox5^{-}$ mice; ^P < 0.05 vs. control $SM22^{+}Nox5^{+}$ mice; #P < 0.05 vs. diabetic $SM22^{+}Nox5^{-}$ mice. RLU, relative light units.

ECM proteins collagen IV and fibronectin were further increased in $SM22^{+}Nox5^{+}$ diabetic mice compared with $SM22^{+}Nox5^{-}$ diabetic mice (Fig. 4*B*–*E*).

Glomerular Macrophage Infiltration and Inflammation

The macrophage marker F4/80 (Fig. 5*A* and *B*) and the gene (Fig. 5*C*) and protein (Fig. 5*D* and *E*) expression of MCP-1 were increased in the glomeruli of these mice after 10 weeks of diabetes compared with the respective control. Glomerular MCP-1 and F4/80 expression were significantly further increased in diabetic $SM22^{+}Nox5^{+}$ mice compared with diabetic $SM22^{+}Nox5^{-}$ mice (Fig. 5*A*–*E*). In addition, both glomerular F4/80 and MCP-1 expression were increased in control $SM22^{+}Nox5^{+}$ mice compared with control $SM22^{+}Nox5^{-}$ mice (Fig. 5*A*–*E*).

Glomerular Gene and Protein Expression of PKC-α

We also examined the expression of glomerular PKC-α at both the gene (Fig. 5*F*) and protein (Fig. 5*G*–*J*) levels. The diabetes-induced increase in PKC-α expression was further enhanced in $SM22^{+}Nox5^{+}$ transgenic mice (Fig. 5*F*–*J*). Gene expression of PKC-α was also found to be increased in control $SM22^{+}Nox5^{+}$ transgenic mice compared with control $SM22^{+}Nox5^{-}$ mice (Fig. 5*F*).

Human Mesangial Cells: In Vitro Studies

An upregulation of Nox5 (Fig. 6*A* and Supplementary Fig. 3*B* and *C*) and enhanced ROS and superoxide formation (Fig. 6*E* and *F*) were observed in human mesangial cells in response to both high glucose and TGF-β1. In addition, there was a significant increase in the expression of profibrotic genes including fibronectin, CTGF, collagen IV, and α-SMA (Fig. 6*C* and *D*). For examination of the role of Nox5-mediated ROS generation per se, human mesangial cells were infected with Nox5-specific shRNA–expressing lentivirus constructs. This resulted in downregulation of Nox5 gene expression by ~70% (Fig. 6*A*) and reduced protein expression of Nox5 (Supplementary Fig. 3*A*). Knockdown of Nox5 in human mesangial cells resulted in attenuation of high glucose and TGF-β1–induced increases in the gene expression of fibronectin, CTGF, collagen IV (Fig. 6*D*), and α- SMA (Fig. 6*C*) in association with reduced ROS formation (Fig. 6*E* and *F*).

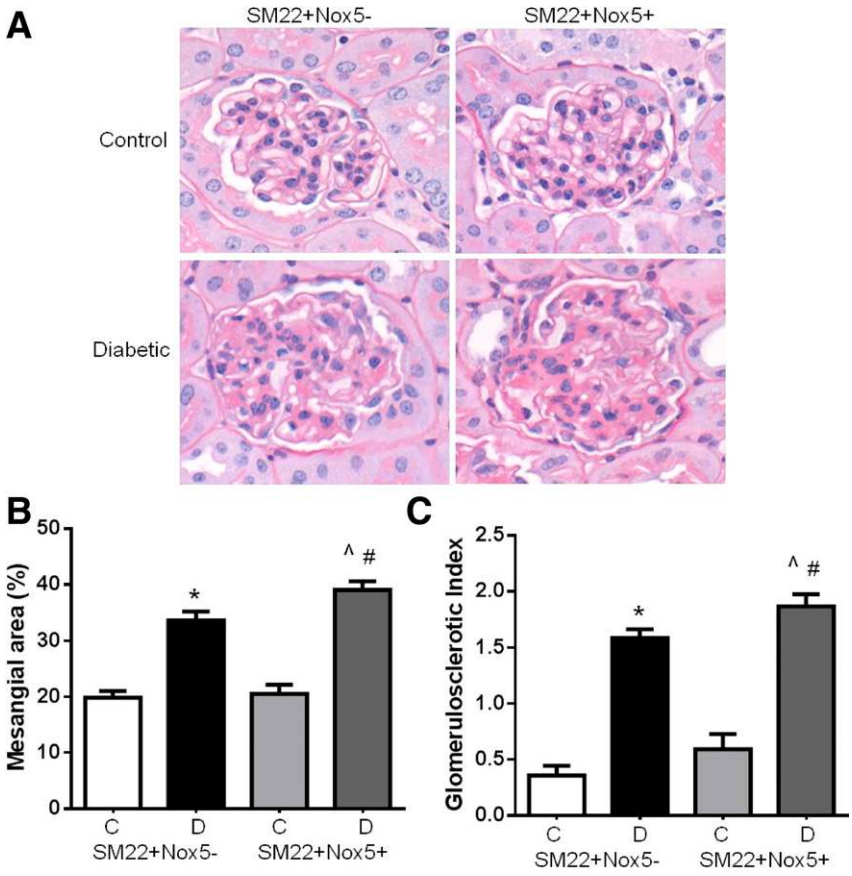

Figure 3—Overexpression of Nox5 in mesangial cells exacerbates glomerular injury in diabetic mice. PAS staining (*A*) (magnification ×40), mesangial area expansion (*B*), and glomerulosclerosis index (*C*) in control (C) and diabetic (D) $SM22^{+}Nox5^{-}$ and $SM22^{+}Nox5^{+}$ transgenic mice (n = 6–9/group) after 10 weeks of diabetes. Data are shown as mean ± SEM. *P < 0.05 vs. control $SM22^{+}Nox5^{-}$ mice; ^P < 0.05 vs. control $SM22^{+}Nox5^{+}$ mice; #P < 0.05 vs. diabetic $SM22^{+}Nox5^{-}$ mice.

MCP-1, NFκB, TRPC6, PKC-α, and PKC-β

We further investigated the effect of Nox5 silencing on key genes involved in inflammation and in the regulation and activation of Nox5 in the presence or absence of the diabetic milieu. High glucose alone or in combination with TGF-β1 increased the gene expression of MCP-1, TRPC6 (Fig. 6*C*), PKC-α, and PKC-β in human mesangial cells (Fig. 6*B*). Silencing of Nox5 in human mesangial cells attenuated expression of MCP-1, TRPC6 (Fig. 6*C*), PKC-α, and PKC-β (Fig. 6*B*). In addition, silencing of Nox5 resulted in decreased protein expression of PKC-α, α-SMA, TRPC6, and NFκB in human mesangial cells in response to high glucose (Supplementary Fig. 3*D–I*).

DISCUSSION

The current study has provided key findings emphasizing the potential role of Nox5-derived ROS in the pathogenesis and progression of DN. It appears that Nox5 expression in mesangial cells aggravates the diabetes-induced increase in albuminuria, renal hypertrophy, glomerulosclerosis, mesangial expansion, ECM accumulation, and renal inflammation via enhanced ROS production. As major cellular sources of ROS, NADPH oxidases have been a central focus in the field of ROS-mediated diabetes complications including nephropathy (20–22). While Nox4 and to a lesser degree Nox2 have been shown to be mediators of diabetes-induced excessive ROS production in the kidney and have been linked to renal injury (8,9,22,23), little is known about the role of Nox5 in diabetic renal disease.

Expression of Nox5 has been shown to be increased in kidneys from patients with diabetes (11). Here, we show increased expression of Nox5 in glomeruli of patients with DN compared with kidney biopsies from healthy individuals. Furthermore, we confirm the localization of Nox5 in mesangial cells with increased expression in mesangial cells in kidneys from individuals with diabetes.

In human mesangial cells we found upregulation of Nox5 in response to hyperglycemia, whereas there were no changes in the expression of other Nox isoforms including Nox1, Nox2, and Nox4. These findings are consistent with a potential role for Nox5 in mesangial cells in diabetes. A recent study reported that mice expressing inducible human Nox5 selectively in podocytes exhibited albuminuria, podocyte effacement, and interstitial fibrosis even in the absence of diabetes. These renal changes were further accelerated in the presence of diabetes (11).

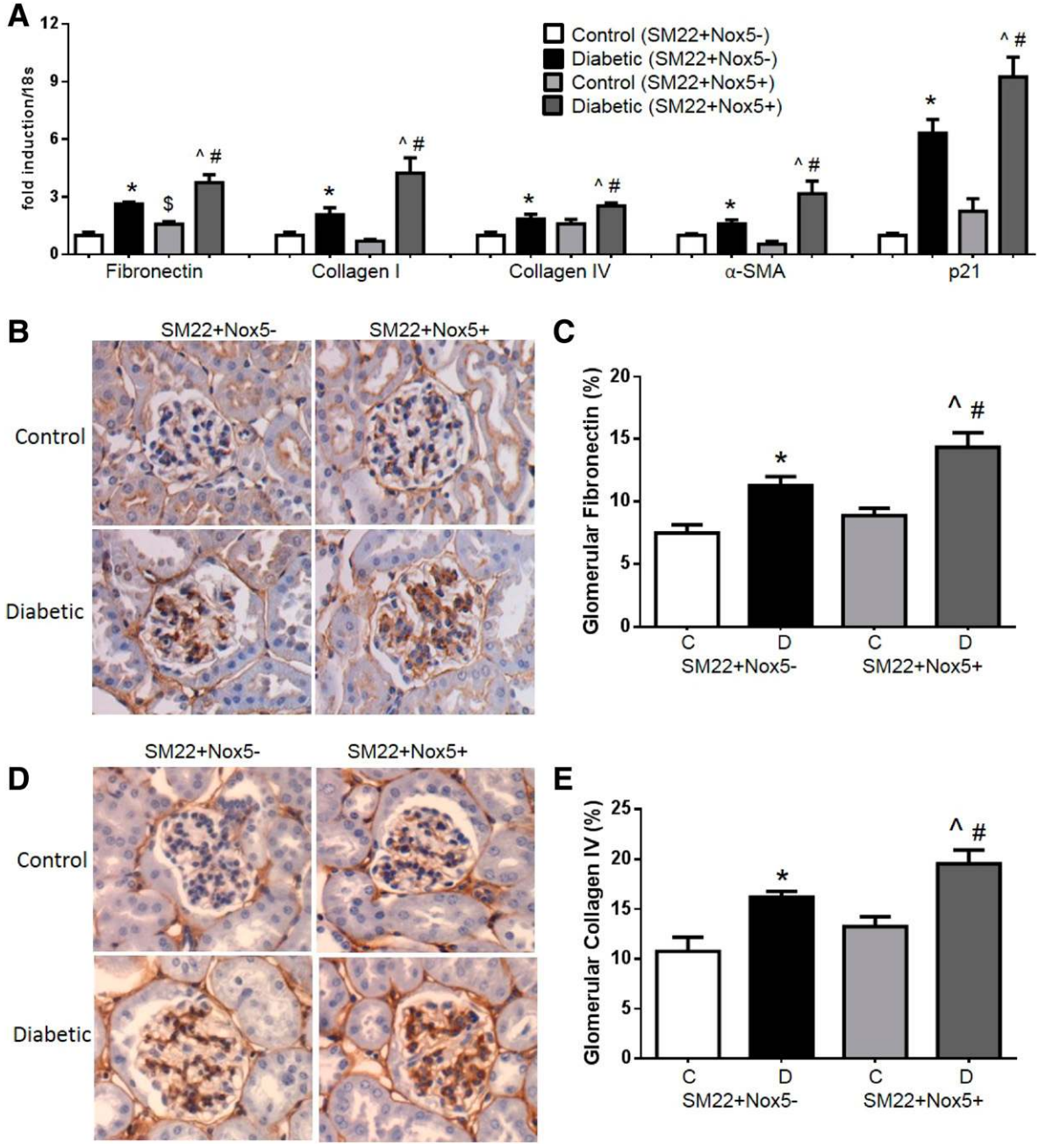

Figure 4— Overexpression of Nox5 in mesangial cells exacerbates glomerular fibrosis in diabetic mice. Glomerular gene expression of fibronectin, collagen I, collagen IV, α-SMA, and cyclin-dependent kinase inhibitor 1A (p21) (*A*). Immunostaining of fibronectin (*B*) and its quantitation (*C*). Immunostaining of collagen IV (*D*) and its quantitation (*E*) in the glomeruli of control (C) and diabetic (D) SM22^{+}Nox5^{-} and SM22^{+}Nox5^{+} transgenic mice (n = 5–8/group) after 10 weeks of diabetes (magnification ×40). Data are shown as mean ± SEM. *P < 0.05 vs. control SM22^{+}Nox5^{-} mice; ^P < 0.05 vs. control SM22^{+}Nox5^{+} mice; \$$P$ < 0.05 vs. control SM22^{+}Nox5^{-} mice; #P < 0.05 vs. diabetic SM22^{+}Nox5^{-} mice.

The mesangium provides the central support for the glomerular capillaries and is composed of mesangial cells, which contribute to the secretion of ECM proteins including collagen IV and fibronectin. The glomerular mesangium is the site of prominent structural lesions in DN including excess deposition of ECM proteins and mesangial expansion (24,25). Since mesangial cells are key components of glomeruli, we examined the effect of Nox5 on ROS formation and subsequent renal injury using an inducible human Nox5 transgenic mouse model expressing Nox5 selectively in VSMCs/mesangial cells in the absence and presence of diabetes.

Consistent with previous studies, diabetic mice showed increased nitrotyrosine levels in the glomeruli compared with their respective control counterparts. The presence of Nox5 in mesangial cells in the diabetic mice further enhanced nitrotyrosine levels in the glomeruli. In addition, DHE staining, a marker of superoxide, demonstrated enhanced levels of

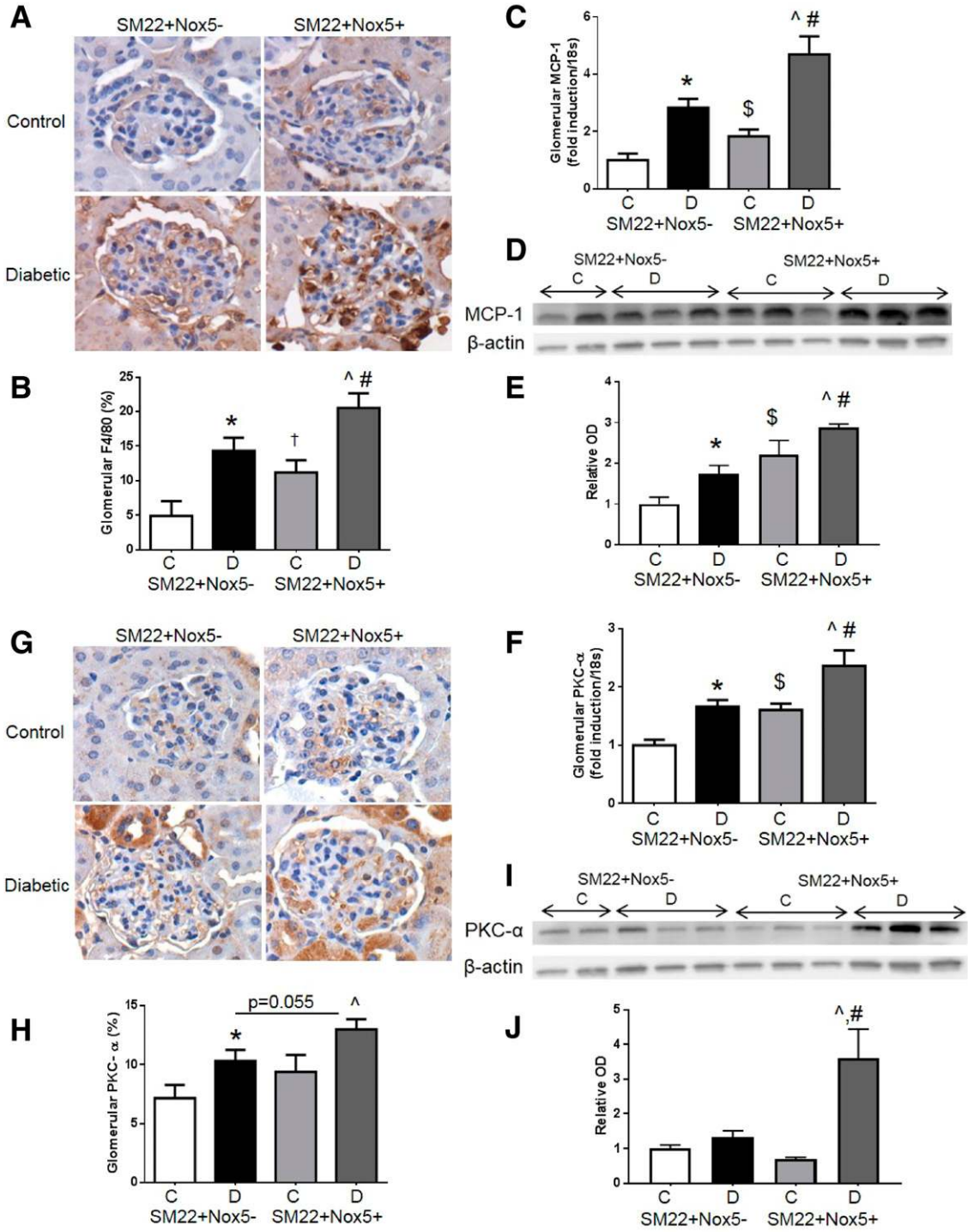

Figure 5—Overexpression of Nox5 in mesangial cells increases glomerular macrophage infiltration and PKC-α expression in diabetic mice. Immunostaining for macrophage infiltration marker F4/80 and its quantitation (*A* and *B*) (magnification ×40). Glomerular gene expression of MCP-1 (*C*) and Western blot for MCP-1 (25kDa) and its quantitation (*D* and *E*) (n = 2–3/group). Glomerular gene expression of PKC-α (*F*), immunostaining and its quantitation (*G* and *H*) as well as Western blot for PKC-α and its quantitation (*I* and *J*) in the glomeruli of control (C) and diabetic (D) SM22^{+}Nox5^{-} and SM22^{+}Nox5^{+} transgenic mice (n = 5–8/group) after 10 weeks of diabetes (magnification ×40). Data are shown as mean ± SEM. *P < 0.05 vs. control SM22^{+}Nox5^{-} mice; ^P < 0.05 vs. control SM22^{+}Nox5^{+} mice; \$$P$ < 0.05 vs. control SM22^{+}Nox5^{-} mice; †P = 0.05 vs. control SM22^{+}Nox5^{-} mice; #P < 0.05 vs. diabetic SM22^{+}Nox5^{-} mice. OD, optical density.

superoxide in Nox5 transgenic diabetic mice, which was also supported by our data showing increased NADPH-dependent enhanced superoxide production in diabetic mice expressing Nox5 in mesangial cells. Importantly, Nox5 transgenic mice did not show a significant increase in the various ROS markers and hence did not show a pathological

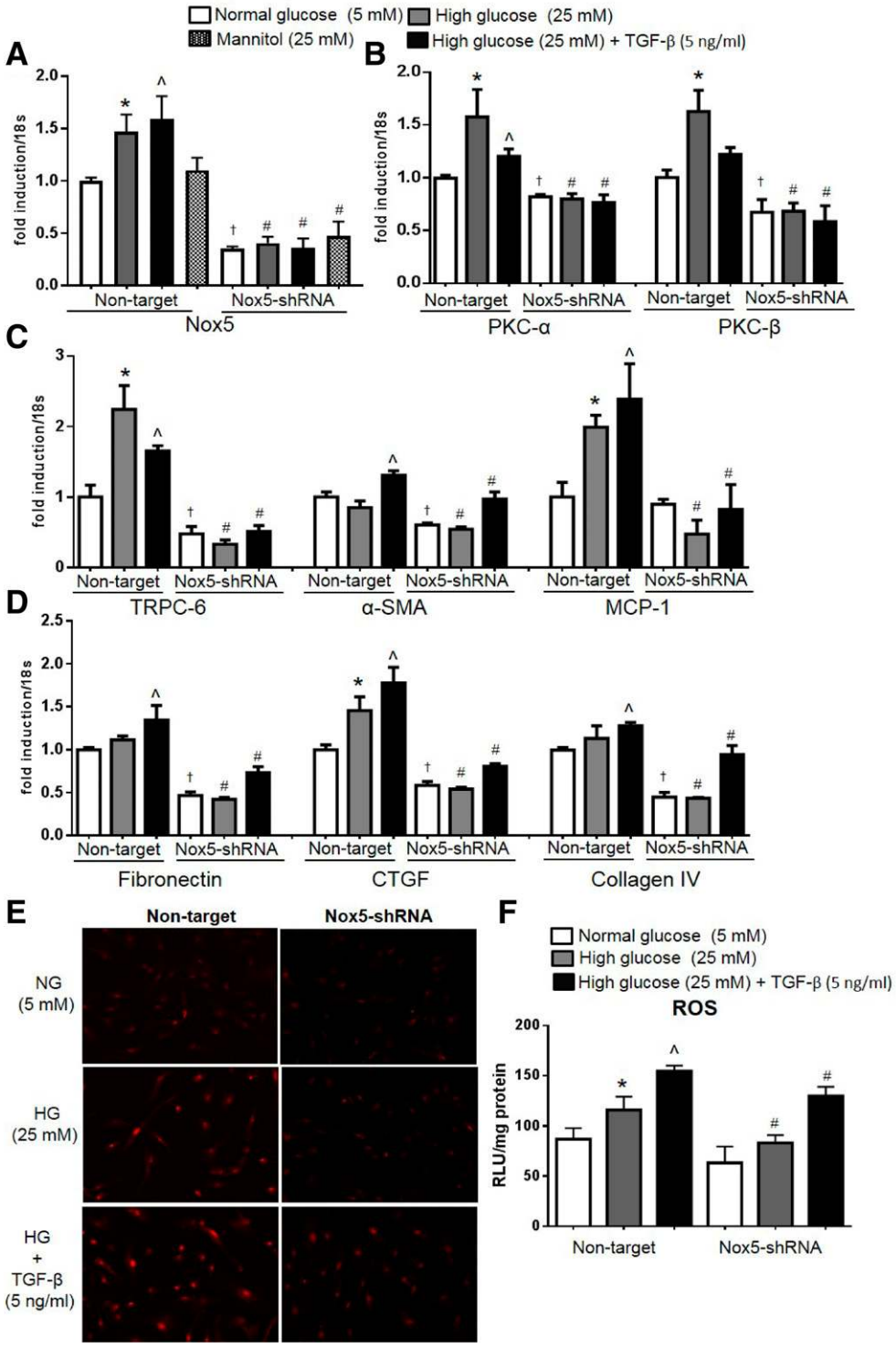

Figure 6—Silencing of Nox5 using shRNA attenuates high glucose and TGF-β1–mediated ROS formation and gene expression of markers of fibrosis, inflammation, and putative pathways in human mesangial cells. Human mesangial cells infected with shRNA specific for Nox5 are grown in NG (5 mmol/L) or in high glucose (HG group) (25 mmol/L) or in high glucose plus TGF-β1 (5 ng/mL) or in mannitol (25 mmol/L + 5 mmol/L NG, as osmotic control) for 2 days for the gene expression analysis and 4 h for the ROS measurement. Gene expression of Nox5 (*A*), putative pathways PKC-α and PKC-β (*B*) and TRPC6 (*C*), markers of inflammation MCP-1 and markers of fibrosis α-SMA (*C*), and fibronectin, CTGF, and collagen type IV (*D*) in human mesangial cells transfected with shRNA specific for Nox5. Analysis of ROS production in human mesangial cells transfected with shRNA specific for Nox5 by DHE immunofluorescence (*E*) and by chemiluminescence (L-012) (*F*). Results were expressed relative to nontarget plus NG. Data are mean ± SEM (n = 4–6/group). *P < 0.05 vs. nontarget plus NG; ^P < 0.05 vs. nontarget plus NG; †P < 0.05 vs. nontarget plus NG; #P < 0.05 vs. nontarget plus HG or nontarget plus high glucose and TGF-β1. RLU, relative light units.

phenotype in the absence of diabetes. There was also no increase in blood pressure in the Nox5-positive mice. However, in the context of diabetes there was enhanced Nox5 activity and ROS production leading to diabetic kidney disease.

After 10 weeks of diabetes, mice showed increased renal hypertrophy in association with increased mesangial expansion and glomerulosclerosis, and these structural parameters were further accelerated in diabetic mice expressing Nox5 in mesangial cells. In addition, these findings were observed in parallel with increased accumulation of ECM proteins including collagen IV and fibronectin. Thus, this study supports the hypothesis that Nox5 plays an important role in the evolution of structural changes in DN, presumably by influencing fibrotic pathways in this state of chronic hyperglycemia. We also demonstrated Nox5 expression in the renal vasculature and cannot exclude a contribution of VSMC Nox5 in the renal phenotype observed in this study. However, animals did not develop a vascular phenotype, and it is more likely that glomerular mesangial Nox5 expression is the main mediator of glomerular injury in diabetic $SM22^{+}Nox5^{+}$ mice.

Mesangial expansion in early DN occurs not only because of ECM protein accumulation but also as a result of mesangial cell hypertrophy (26,27). The concept of mesangial cell hypertrophy evident in the diabetic renal cells is thought to be a cell cycle–dependent event and is partly mediated by cyclin-kinase inhibitors, such as p21 (27). It has been shown that diabetic $p21^{-/-}$ mice do not develop mesangial hypertrophy (26,28). We observed that in diabetes, glomerular p21 expression was increased, and importantly, it was further increased in diabetic mice expressing Nox5 in the mesangial cells, suggesting a role for Nox5 and/or derived ROS in augmenting mesangial cell growth in diabetes contributing to mesangial expansion. Renal α-SMA expression is increased in diabetes, is closely associated with renal fibrosis (29), and is considered a marker of activated glomerular mesangial cells (30,31). In this study, glomerular mesangial expansion and ECM protein accumulation correlated with increased α-SMA expression in the glomeruli in diabetes. Importantly, expression of Nox5 promoted a further increase in α-SMA expression in the diabetic mice.

Albuminuria reflects the changes in renal hemodynamics as well as changes within the glomerular filtration barrier due to structural alterations in key renal cells including endothelial cells and podocytes (8,9,32). Although selective Nox5 expression in mesangial cells was not directly expected to increase albuminuria, after 10 weeks of diabetes, mice expressing human Nox5 in mesangial cells demonstrated a trend toward increased albuminuria. Cross talk between mesangial cells and other renal cells including podocytes may explain this observation, as has been previously demonstrated in other contexts (33).

DN also involves a renal inflammatory response (34). Induction of diabetes was associated with a further increase in gene expression of the chemokine MCP-1 in association with increased glomerular macrophage infiltration in the mice expressing Nox5 in mesangial cells. Notably, nondiabetic Nox5-positive transgenic mice also showed increased expression of this proinflammatory chemokine. These findings suggest that Nox5-derived ROS not only aggravates renal fibrosis but also enhances proinflammatory pathways involved in diabetes.

We have previously shown that NADPH oxidase, particularly Nox4-derived ROS, can activate certain PKC isoforms including PKC-α within the kidney, thereby promoting renal injury in experimental diabetes (9,10). In addition, previous studies have shown that PKC mediates the phosphorylation of Nox5 (35,36). A recent study reported that PKC-α directly mediates Nox5 phosphorylation and activity, while PKC-ε and PKC-δ modulate Nox5-derived superoxide production through indirect mechanisms (37,38). Our study shows increased expression of PKC-α at both the gene and protein level in diabetic mice, which was significantly increased in Nox5 expressing mice after 10 weeks of diabetes. Importantly, nondiabetic Nox5-expressing mice also showed increased expression of PKC-α, with expression similar to levels observed in the wild-type diabetic mice that do not express Nox5.

Several cytokines and growth factors, including TGF-β, are involved in the pathophysiology of hyperglycemia-induced mesangial cell hypertrophy and subsequent glomerulosclerosis in diabetes (1,39,40). In addition, hyperglycemia markedly increases the production of ROS in mesangial cells through NADPH oxidase–dependent mechanisms (41). Given the importance of mesangial cells in morphological manifestations of DN, and to translate our findings from mice to the human context, and to specifically determine the potential mechanisms of renal injury at a cellular level, we performed complementary in vitro studies using human mesangial cells.

Upregulation of Nox5 was observed in mesangial cells in response to high glucose and TGF-β1. Silencing of Nox5 in human mesangial cells reduced high glucose plus TGF-β–induced ROS production, and this was associated with attenuation of critical pathways linked to renal fibrosis and inflammation, including reduced expression of collagen IV, fibronectin, CTGF, α-SMA, and MCP-1. These findings confirm a previous report where Nox5 was shown to play an important role in diabetes-induced ROS formation in podocytes (11). Importantly, our study extends these findings to mesangial cells as seen in the mouse model as well as in kidney biopsies from individuals with diabetes and individuals without diabetes and in human mesangial cells, emphasizing the impact of Nox5 on profibrotic and proinflammatory pathways.

In addition to fibrosis and inflammation, we also examined the involvement of key intracellular signaling pathways implicated in DN such as TRPC6, PKC-α, and PKC-β. In human mesangial cells, glucose and/or TGF-β1–induced increased gene expression of both PKC-α and PKC-β was seen, and furthermore, suppression of Nox5 using an shRNA approach attenuated expression of both PKC isoforms. Interestingly, Nox5 has been shown to be activated

as a result of phosphorylation by either PKC-α or PKC-β (38,42). These findings could represent a feedback loop whereby Nox5 and certain PKC isoforms interact in a bidirectional manner to amplify renal injury. Indeed, there is a significant body of data demonstrating that PKC-α or PKC-β plays a key role in the pathogenesis of albuminuria and ECM accumulation, respectively, in DN (43–45).

TRPC6 is a Ca^{2+}-permeable cation channel that regulates the intracellular Ca^{2+}entry. An interrelationship between Nox-derived ROS and the regulation of Ca^{2+} channels by TRPC6 has previously been shown, including in a model of podocyte injury (46–49). Since the Nox5 isoform contains four N-terminal Ca^{2+}-binding EF-hand regulatory domains that serve to enhance the catalytic production of superoxide (50), it is possible that elevation of the intracellular Ca^{2+} concentration may also activate Nox5 in renal cells, particularly in diabetes. Indeed, we found increased expression of TRPC6 in human mesangial cells in response to hyperglycemia, and silencing of Nox5 attenuated this effect. These findings suggest that a positive-feedback loop or a bidirectional potentiation between TRPC6 and Nox5 may exist (51). Hence, our findings in human mesangial cells suggest that Nox5 is a key upstream regulator of PKC-α, PKC-β, and TRPC6 expression in the setting of hyperglycemia. We postulate that inhibition of Nox5 could represent a new approach to target the deleterious effects of these key intracellular signaling molecules including PKC-α, PKC-β, and TRPC6 in the diabetic kidney.

Finally, we examined the effect of Nox5 expression on other Nox isoforms in the isolated glomeruli. The integral roles of other Nox isoforms have been extensively examined in experimental models of diabetes complications including nephropathy (7–9,23,52). While most of these studies have been performed using rodents, the observed structural and functional changes may not be recapitulated in human DN, as Nox5 is absent in these animals. Thus, it is important to investigate whether an interaction exists among Nox isoforms, including Nox5. Indeed, in this study, Nox4 was upregulated in the glomerular fraction of diabetic mice. Interestingly, the transgenic expression of human Nox5 in diabetic mice did not increase the gene expression of Nox4. We have recently shown that deficiency of Nox4 is renoprotective in the setting of diabetes (8,9). However, in the current study renal injury was still apparent even in the absence of Nox4 upregulation in the diabetic Nox5 transgenic mice. This suggests that Nox5 is most likely a major source of renal ROS in this setting and leads to renal injury. However, gene expression of Nox isoforms does not always correlate with protein levels and/or activity (53). Thus, further studies are required to explore the relative importance of these two isoforms, Nox4 and Nox5, in DN.

The presence of diabetes also led to increased expression of Nox2 in the glomeruli, but there was no further increase in diabetic Nox5 transgenic mice. Nox2 has been extremely well characterized with respect to its essential role in phagocytic defense and inflammation. It is possible that the effects on Nox2 expression may also be related to associated macrophage infiltration, since these mononuclear cells are an important source of Nox2 including in the kidney. In addition, no significant difference in Nox1 expression was found in Nox5 transgenic mice, consistent with previous studies demonstrating that Nox1 does not play a significant role in diabetic kidney disease (8).

In conclusion, we postulate that Nox5 may be the preferred target in terms of the various NADPH oxidase isoforms for the prevention of the progression of human DN, with these studies further strengthening the case to develop a Nox5-specific inhibitor.

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Author Contributions. J.C.J. collected research data, contributed to discussion, and wrote, reviewed, and edited the manuscript. C.B., J.O., S.P.G., T.H., B.S.M.C., V.T.-B., L.D.V., and C.E.H. collected research data and contributed to the manuscript. M.T.C., D.A.P., A.S., and E.I.E. collected human kidney biopsies, prepared the samples for processing, and contributed to discussion of the manuscript. M.E.C. contributed to discussion and wrote, reviewed, and edited the manuscript. R.M.T. contributed to discussion and reviewed and edited the manuscript. C.R.K. contributed to discussion and reviewed and edited the manuscript. K.J.-D. contributed to discussion and wrote, reviewed, and edited the manuscript. J.C.J. and K.J.-D. are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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