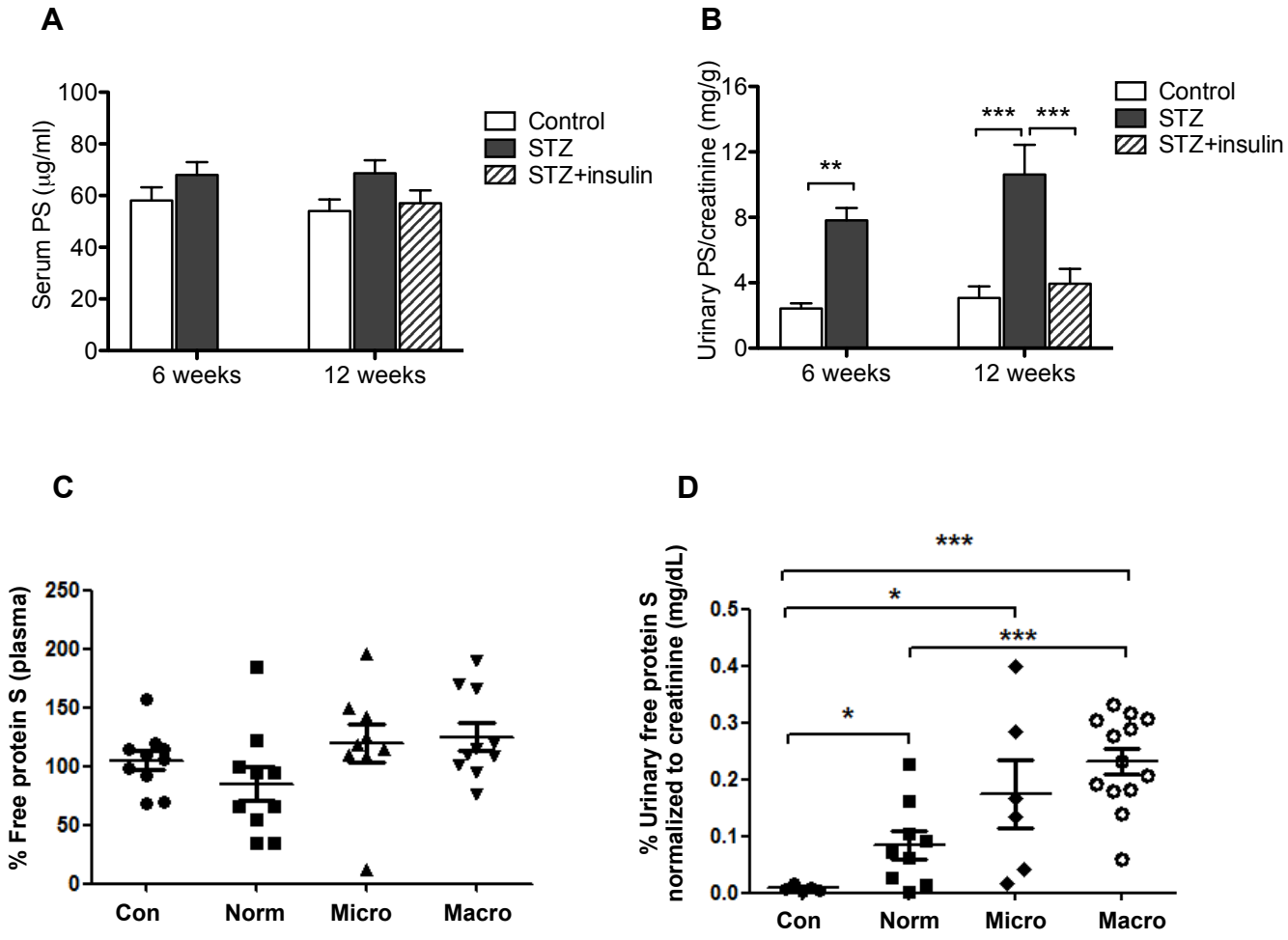
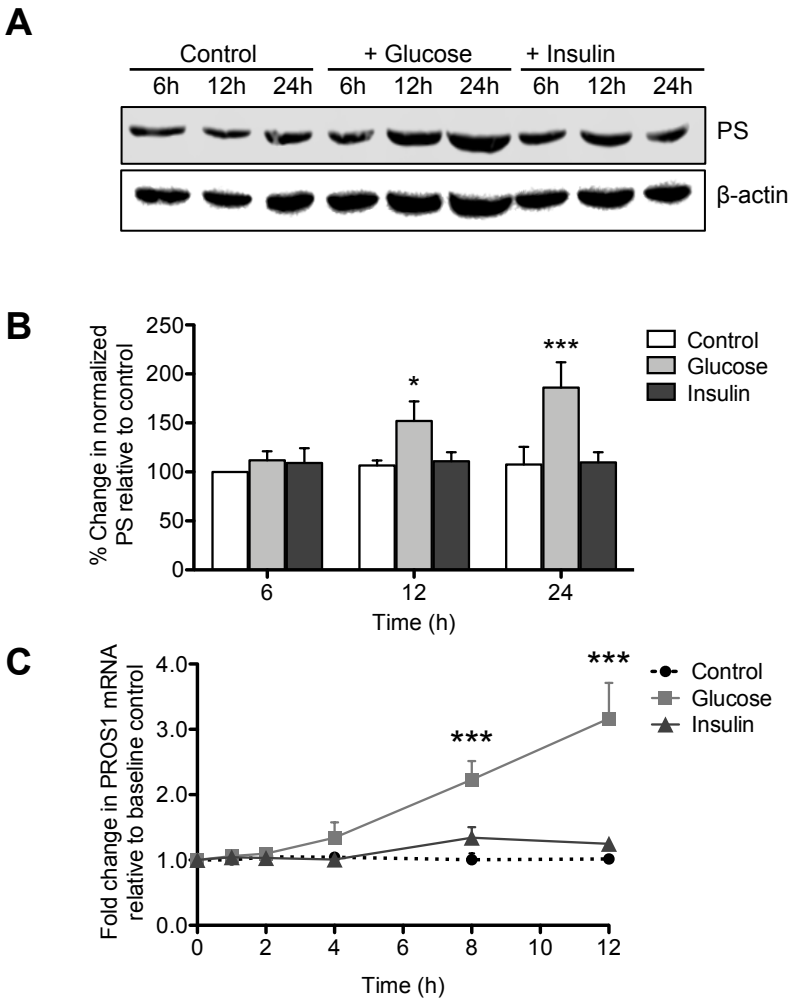
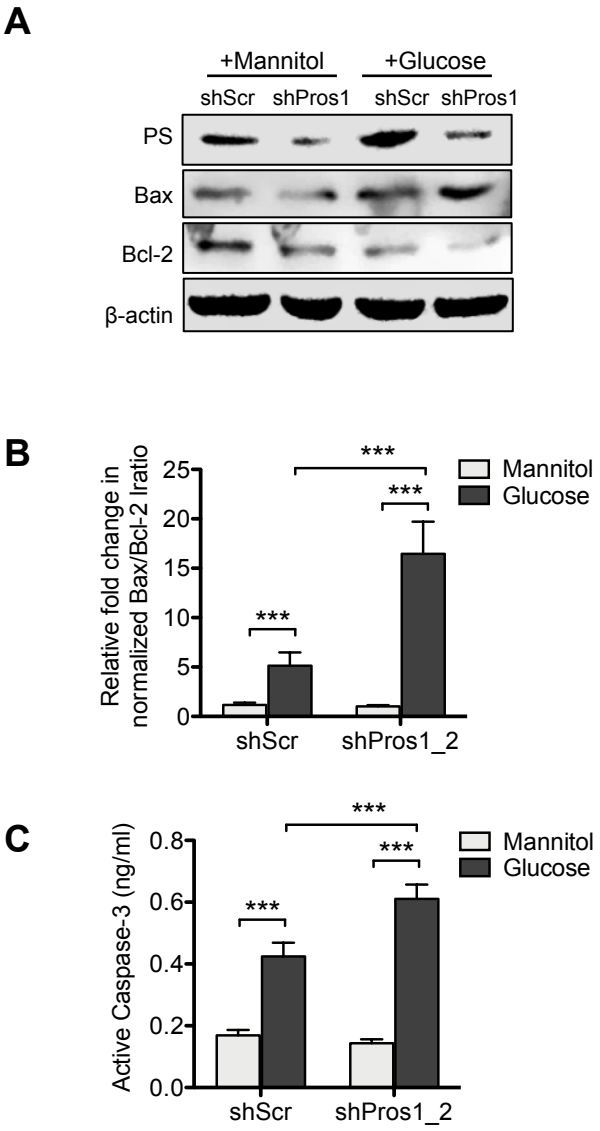
Supplementary Figures



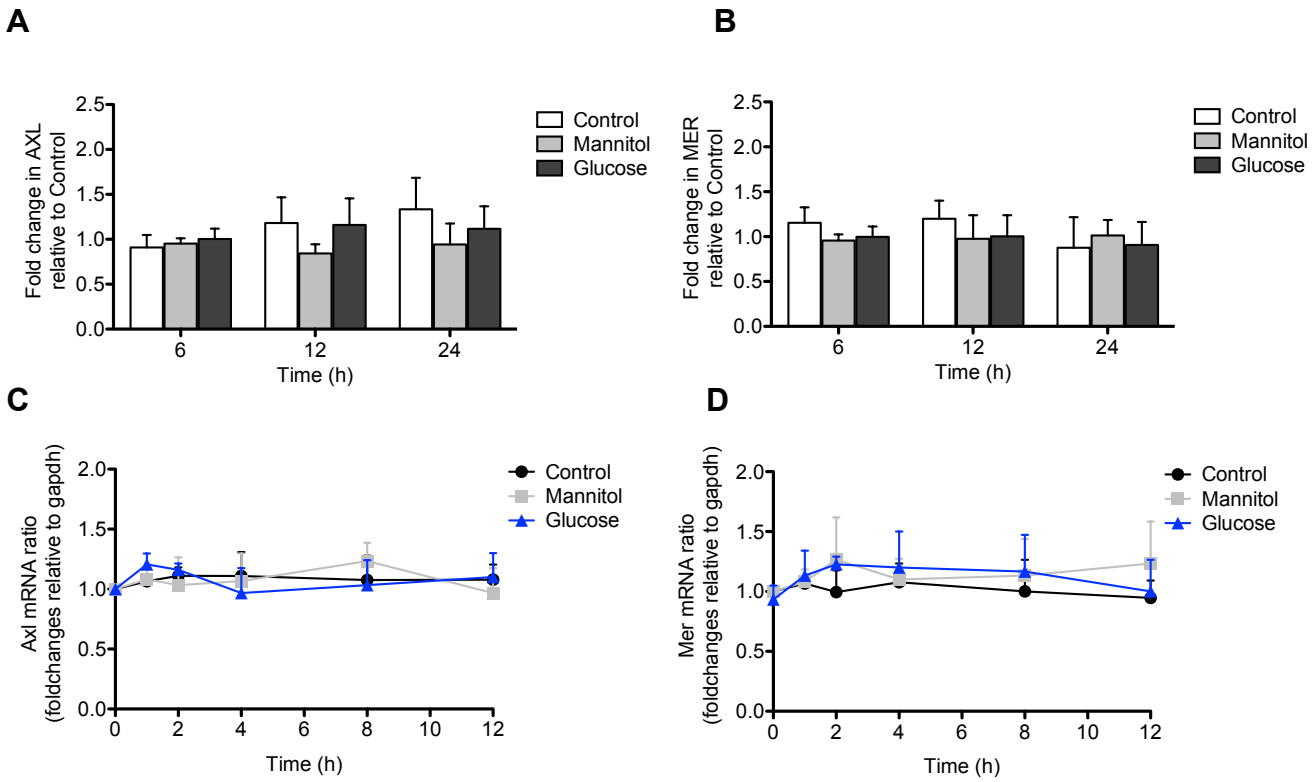
Supplementary Figure 1: ELISA measurements of PS concentrations in rat and human serum and urine. **A, B.** Serum and urine samples were obtained from diabetic and control rats. Total protein S levels were determined in these samples by ELISA (LifeSpan BioSciences Inc, Cat#:LS-F12427). **P<0.01 and ***P<0.001 when compared between groups. **C, D.** Human plasma samples were obtained from Shanghai 6th Hospital under an IRB-approved protocol, and free protein S levels were measured by using IMUCLONE Free Protein S ELISA kit (American Diagnostica Inc. Cat#:842). Free protein S concentrations are expressed as a relative change in % compared to the standard provided in the ELISA kit (pooled normal human serum). Con, healthy control subjects; Norm, type 2 diabetic patients with normoalbuminuria (UACR<30mg/g); Micro, with microalbuminuria (UACR<300mg/g); Macro, with macroalbuminuria (UACR>300mg/g). *P<0.05 and ***P<0.001 when compared between indicated groups. Patient characteristics are summarized in Supplementary Table 2.



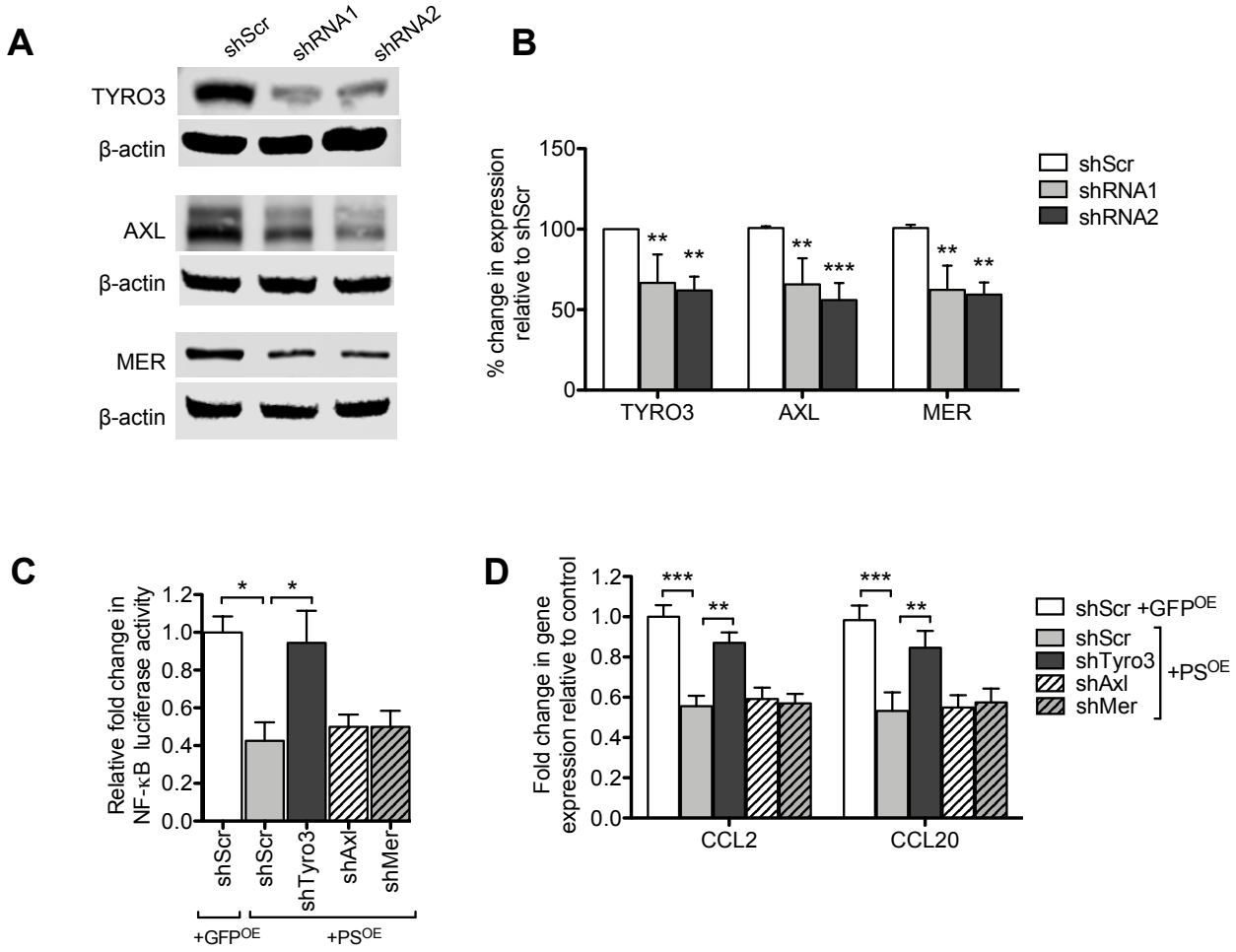
Supplementary Figure 2: Immortalized human podocytes were incubated with normal glucose (5mM glucose, control), high glucose (30mM) or normal glucose with insulin (10 ng/ml) for in the glucose-free RPMI medium for the indicated time intervals. Western blotting and real-time PCR were performed to assess PS expression levels. (A) Representative western blot of three independent experiments is shown. (B) Densitometric analyses were performed for western blots. (C) *PROS1* mRNA expression was measured by real-time PCR. (n=3, **P*<0.05 and ****P*<0.001 when compared to the control group).



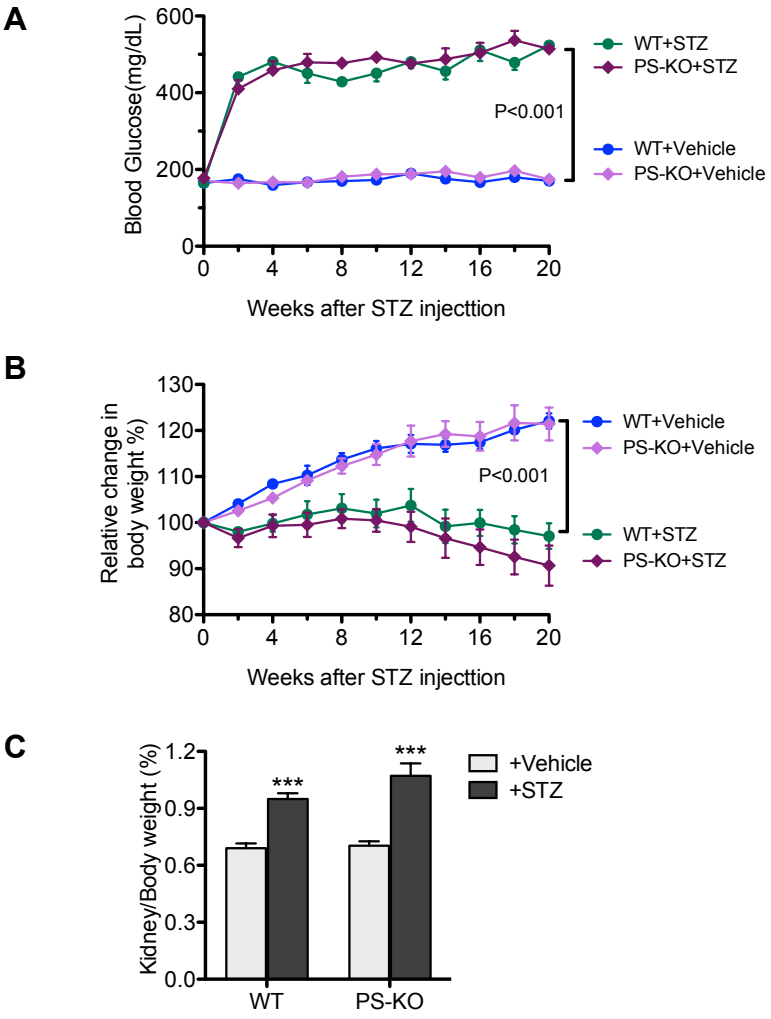
Supplementary Figure 3: Validation of data in Figure 4 using a second shRNA lentivector against Pros1 (shPros1_2). (A-C) Podocytes stably transduced with lentivirus expressing either scrambled shRNA (shScr) or Pros1 shRNA (shPros1_2) were exposed to high glucose (30mM) or high mannitol (5mM glucose ± 25mM mannitol) for 24 hours. Western blots were performed for PS, Bax, and Bcl-2. Representative blots of three independent experiments are shown (A). (B) Ratio of Bax to Bcl-2 levels normalized to β-actin. (C) Active Caspase-3 concentration was measured by ELISA. (n=3, ***P<0.001 when compared between indicated groups).



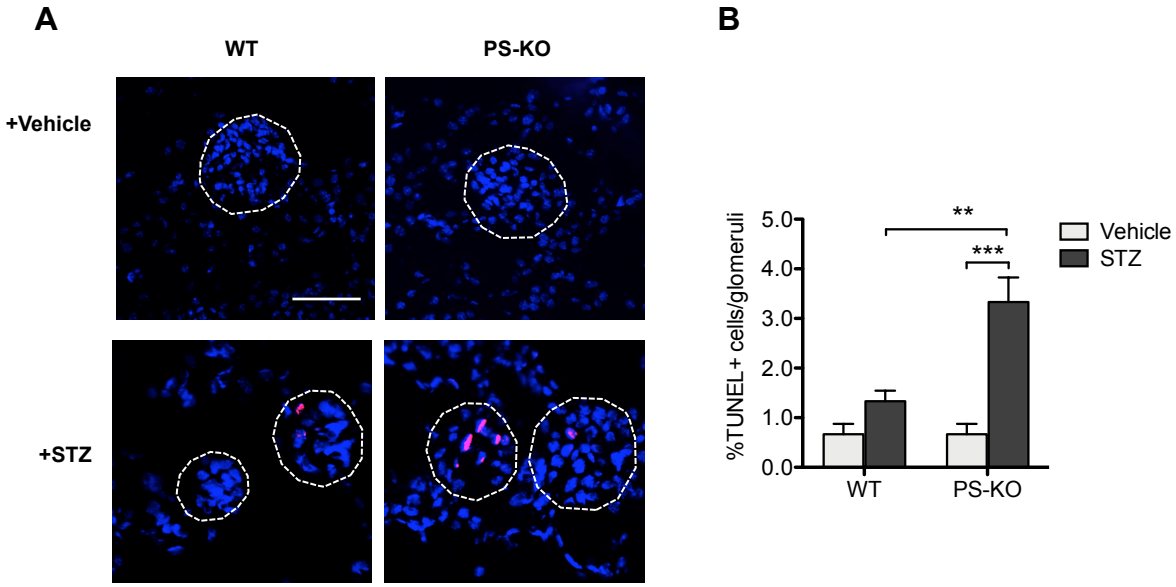
Supplementary Figure 4: (A-B) Densitometric analysis of western blotting for AXL (A) and MER (B) included in Figure 5A are shown. (C-D) mRNA expression of AXL and MER in podocytes exposed to control media, high mannitol or high glucose.



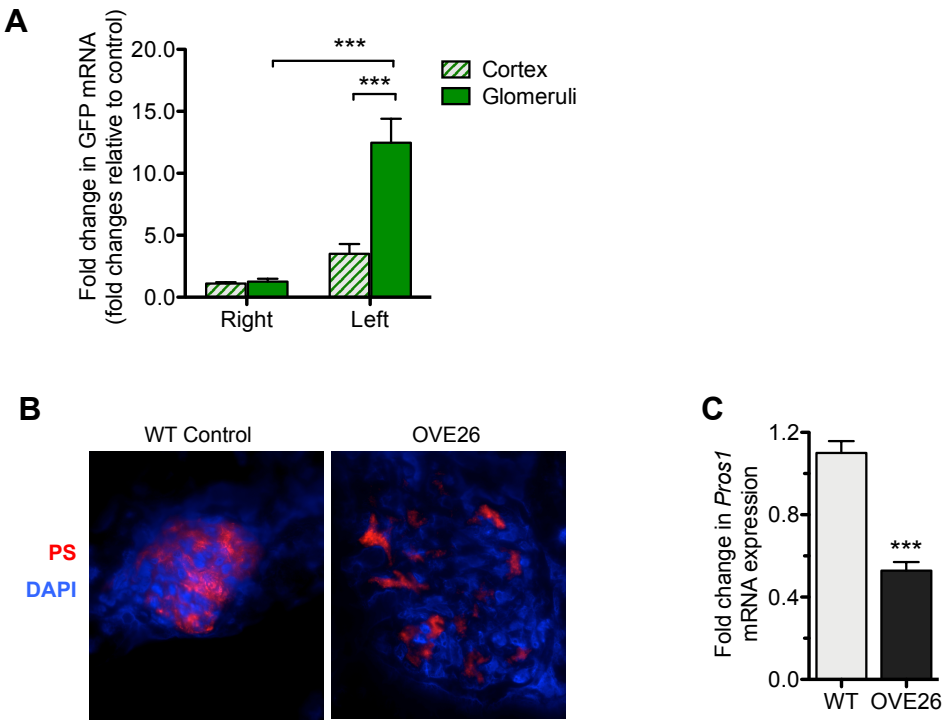
Supplementary Figure 5: shRNA-mediated knockdown of individual TAM receptors. (A) Western blots were performed to confirm the efficiency of knockdown for Tyro3, Axl, and Mer, respectively in podocytes transduced with two different shRNA lentiviral vectors (shRNA1 and shRNA2) specific for each TAM receptors or scrambled shRNA control (shScr). (B) Densitometric analysis of western blots (n=3, **p<0.01 and ***p<0.001 when compared to shScr control). (C-D) Replication of Figure 5D-E using a second set of shRNA lentivectors (shRNA2). Podocytes stably transduced with lentivirus expressing either scrambled shRNA (shScr), or shRNA for Tyro3 (shTyro3), Axl (shAxl), and Mer (shMer) were transfected with NF-κB luciferase reporter and renilla luciferase, together with either control GFP overexpression vector (GFP^{OE}) or PS overexpression vector (PS^{OE}). (C) NF-κB luciferase reporter activity is shown as fold change to GFP^{OE}/shScr control. (D) Real-time PCR analysis of selected NF-κB-targeted gene expression was performed in these cells. (n=3, *P<0.05, **P<0.01 and ***P<0.001 when compared between groups).



Supplementary Figure 6: Blood glucose monitoring of control and diabetic mice (A) and body weight (B) after induction of diabetes. (C) Kidney-to-body weight ratio at 20 weeks post-induction (*P<0.001 vs. vehicle-treated mice, n=6).



Supplementary Figure 7: (A) Representative image of TUNEL stained glomeruli are shown. (B) Quantification of TUNEL+ cells are shown as % of TUNEL+ cell number/total DAPI+ number per glomerular cross section. (60 glomeruli per group, n=6 mice per group, ***P*<0.01 and ****P*<0.001 when compared between indicated groups).



Supplementary Figure 8: (A) Transient overexpression of EGFP by intra-renal arterial injection into the left was confirmed by real-time PCR of isolated glomeruli or in kidney cortices in comparison to the contra-lateral right kidney (n=4, **P<0.001 compared between indicated groups). (B) Representative image of PS immunofluorescence in age-matched control and in OVE26 kidneys. (C) Real-time PCR analysis of *Pros1* mRNA in isolated glomeruli (n=4 mice, ***P<0.001 compared to wildytpe (WT) littermate).

Supplementary Tables

	Control-6w (n=5)	DM-6w (n=5)	Control-12w (n=5)	DM-12w (n=5)	DM-12+INS (n=5)
Body Weight (g)	475.8±10.9	294.3±20.3*	590.3±32.9	379.2±40.7**	508.3±38.7$^{\#}$
Blood Glucose (mmol/L)	137.4±32.2	551.6±128.9*	129.7±6.6	583.8±117.3**	133.7±39.1$^{\#}$
Kidney-body weight ratio (%)	0.67±0.05	1.17±0.13*	0.74±0.13	1.32±0.17**	0.88±0.05$^{\#}$
Albumin/ Creatinine (mg/g)	5.5±2.0	21.0±6.4*	7.5±4.8	25.8±10.1***	11.0±6.4$^{\#\#}$

Supplementary Table 1: Summary of blood glucose, body weight, kidney/body weight ratio in diabetic rats and their controls. Both diabetic (DM), diabetic with insulin treatment (DM+INS), and control rats were sacrificed at 6 weeks or 12 weeks after the induction of diabetes. Blood, urine and kidney samples were collected at the time of sacrifice (n = 5, *p<0.01 compared to Control-6w, **p<0.01 compared to Control-12w, ***p<0.05 compared to Control-12w, $^{\#}$p<0.01 compared to DM-12w, $^{\#\#}$p<0.05 compared to DM-12w). Data are expressed as mean ± SEM.

	Controls	Type 2 diabetes		
		Normoalbuminuria	Microalbuminuria	Macroalbuminuria
Case (M/F)	10 (5/5)	10 (5/5)	10 (5/5)	10 (5/5)
Age (years)	59.6±8.87	56.4±15.56	59.7±14.2	60.2±8.1
Duration of diabetes (years)	----	3.4±5.2	7.6±4.2	12.5 ± 8.7
SBP(mmHg)	133±23	129±14	128±14	141±10
DBP(mmHg)	82±10	79±9	78±9	81±9
BMI(Kg/m2)	22.5±2.4	25.5±2.5*	24.4±2.9	25.3±3.9*
HbA1c(%)	5.4±0.2	9.7±2.4**	7.6±0.8*	8.5±1.7**
TC(mmol/l)	4.7±0.6	4.6±0.7	5.2±1.0*	5.3±1.0
LDL-c(mmol/l)	3.2±0.8	3.4±0.8	3.5±0.8*	2.7±0.8
TG(mmol/l)	1.2±0.5	1.4±0.7	2.4±1.5*	2.6±2.6
HDL-c(mmol/l)	1.4±0.4	1.1±0.3**	1.1±0.2**	1.2±0.1**
Cr(mmol/l)	62.2±7.7	76.5±11.4	69.2±8.3	82.9±39.2
AER(μg/min)	7.1(4.1-9.6)	8.1(7.2-11.0)	61.3(46.3-97.1)**††	1350.7(561.2-2461.5) **††‡‡
eGFR (ml•min-1•1.73m2)	100.6±13.9	88.5±12.3	93.2±19.5	101.7±45.4
Hypertention (%)	0	6(60%)	6(60%)	10 (100%)
DR(non/simplex/ proliferative)	----	0/0/0	0/0/0	0/0/1

Supplementary Table 2: General and clinical parameters of healthy control subjects and type 2 diabetic patients. Data are expressed as means ± SD or geometric means [95% confidence interval (CI)]. Data were analyzed using one-way analysis of variance (ANOVA). BMI: body-mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; TC: total cholesterol; LDL-c: low-density lipoprotein-cholesterol; TG: triglyceride; HDL-c: high density lipoprotein-cholesterol; Cr: serum creatinine; AER: albumin excretion rate; and DR: diabetic retinopathy. *$P < 0.05$; **$P < 0.01$ vs. control; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ vs. normoalbuminuria; $^{\ddagger}P < 0.05$, $^{\ddagger\ddagger}P < 0.01$ vs. microalbuminuria.

Gene	Forward	Reverse
GAPDH	5'-AAGCCTGCCGGTGACTAAC	5'-GTTAAAAGCAGCCCTGGTGAC
PROS1	5'-GCAGTTTGCAGGGGTTGTTT	5'-TCTGCGTACAGTATCACGCC
TRYO3	5'-GCCACTGGTGGTCTCTTCTC	5'-CGTTAGCACACCAAGGACCA
AXL	5'-CTGCAGTCGCACTTACAAGAC	5'-GTGACCCCCTTGGCGTTATG
MER	5'-TGGGGTCCAGAACCATGAGA	5'-GGGATCGGTTCTCCAGCAAG
CCL2	5'-CTCTCGCCTCCAGCATGAAA	5'-TTGGGGAATGAAGGTGGCTG
CCL20	5′-TTGCTCCTGGCTGCTTTG	5′-ACCCTCCATGATGTGCAAG
CXCL11	5′-TGTCTTTGCATAGGCCCTGG	5′-TAAGCCTTGCTTGCTTCGAT
TRAF1	5′-GTAGGCGGTGGCGGAG	5′-TGGAAATCCCCAGGGTGTTG
Gapdh(rats)	5'-GCAAGTTCAACGGCACAG	5'-GCCAGTAGACTCCACGACAT
Pros1(rats)	5'- GCCATCCCAGACCAATGTGA	5'- CTCCTTGCCAGCCTGGTTTA
Gapdh	5'-GCCATCAACGACCCCTTCAT	5'-ATGATGACCCGTTTGGCTCC
Pros1	5'-CAATACAAGGCAAAGGGAATGAGG	5'-GAGCTCTCAGAGACTAAAC
Nphs1	5'-GTGCCCTGAAGGACCCTACT	5'-CCTGTGGATCCCTTTGACAT
Wt1	5'-GAGAGCCAGCCTACCATCC	5'-GGGTCCTCGTGTTTGAAGGAA
Podocyxlin	5'-CCATAAGGCCAGATGAGGAA	5'-GATTCTCTTCACTGCCACCG

Supplementary Table 3: Primers used for real-time PCR

SUPPLEMENTAL METHODS

Diabetic rat model

Male Sprague-Dawley (SD, 8 weeks old) rats were obtained from Jackson Research Laboratories (Bar Harbor, ME). Care, use, and treatment of all animals in this study were approved by the Institute of Animal Care and Use Committee at Icahn School of Medicine at Mount Sinai. Diabetes was induced in SD rats (8 weeks of age) by an intraperitoneal injection of streptozotocin (STZ) (60mg/kg in 10mmol/L of citrate buffer, pH 4.5) (Sigma, St. Louis, MO) after an overnight fast. Control rats received an injection of citrate buffer. Blood glucose levels were measured at 48 hours after the injection and monitored every 3 days thereafter. Only the animals with blood glucose concentrations >16.7mmol/l were considered diabetic. A group of diabetic rats received Neutral Insulin (16U/kg; Novo Nordisk, Copenhagen, Denmark) via subcutaneous injection twice a day from 6 to 12 weeks of diabetes for hyperglycemic control. Non-fasting glucose was monitored before the insulin injection. Rats were euthanized and their kidneys were harvested for glomerular isolation and histology at 20 weeks post diabetes onset. Glomeruli were isolated by serial sieving in ice-cold PBS and were used for proteomic analysis. Purity of the glomerular preparation was confirmed by light microscopy as described [1]. 12-hour-urine samples were collected on the day before euthanasia by housing the animals in metabolic cages to determine the urine albumin excretion rate (Houston, TX).

Proteomics

Proteomic study was performed as described previously [1]. Briefly, glomeruli were lysed by sonication in lysis buffer [8 M urea, 100 mM TEAB, 1.0% octyl β-D-glucopyranoside (pH 8.5)] containing protease and phosphatase inhibitors. A total of 100 µg of proteins was used for each sample. Proteins were digested into peptides, and peptides were labeled with iTRAQ tags as per the manufacturer's instructions. Resulting peptides were fractionated and desalted. Liquid chromatography (LC)–MS/MS analyses were performed with an UltiMate 3000 nano LC system (Dionex) and LTQ Orbitrap Velos mass spectrometer. Desalted peptides from SCX fractions were first captured onto a reversed-phase (0.3-mm × 5-mm) trapping column and then resolved on a capillary PepMap column with a 180-min gradient of solvent. Eluted peptides were introduced directly to LTQ Orbitrap Velos. Full-scan MS spectra were acquired in the positive ion mode with a scanning mass range of mass/charge ratio (*m/z*) 350 to 2000 and a resolution of 60,000 full-width at half maximum (FWHM). The higher-energy collision dissociation (HCD) MS/MS spectra were acquired in a data-dependent manner. The 10 most abundant ions were selected for HCD fragmentation per MS scan in the Orbitrap at a resolution of 7500 FWHM. The normalized collision energy was set to 45. The lock mass feature was engaged for accurate mass measurements. The MS/MS spectra from the analyses were searched against rat protein sequences of SwissProt protein database with Mascot (version 2.3) and Sequest search engines via the Proteome Discoverer platform (version 1.3, Thermo Scientific). The resulting *.dat files from Mascot and *.msf files from Sequest search were filtered with Scaffold (version 3.3.2, Proteome Software Inc.) for protein identification and quantification analyses. For additional validation of identification, X!Tandem search was engaged in Scaffold with same modification as described for Mascot and Sequest. All peptides were identified with at least 95% confidence interval value as specified by the PeptideProphet algorithm and less than 1% false discovery rate (FDR) based on forward/reverse database searches. Proteins were considered confidently identified with at least two unique peptides, and an experiment-wide FDR of no more than 1.0% at both the protein and the peptide levels. Proteins that share the same peptides and could not be differentiated on the basis of MS/MS analysis alone were grouped together to reduce the redundancy using Scaffold [2]. Relative quantification of proteins was determined with Scaffold Q+ module in a normalized $\log_2$-based relative iTRAQ ratio format, with iTRAQ 113 tag as the reference denominator. Statistical comparisons were performed using a Z-test followed by Benjamini-Hochberg multiple hypothesis correction. A corrected α value of 0.05 was accepted as statistically significant. Proteomic results from three rats were combined for each comparison.

Cell culture

Human podocytes were obtained from Dr. Moin Saleem and cultured as described [3]. Cells were serum starved in 1% serum containing medium for 12 hours followed by treatment with the medium containing either normal glucose (5mM), high mannitol (5mM glucose ± 25mM mannitol) or high glucose (30mM) for the indicated time intervals.

Luciferase Reporter assays
To measure the effects of PS on TNFα-induced NF-κB activation in podocytes, cells were transfected with PROS1 overexpressiong vector and NF-κB luciferase and renilla luciferase plasmids (Stratagene, La Jolla, CA). 48-hours post transfections, cells were incubated with or without of TNFα for an additional 24 h. Cells transfected with GFP plasmid were used as a negative overexpression control. Reporter activity was detected by using a luciferase assay system (Promega Corp., Madison, WI) and read on a 20/20n Luminometer (Turner Biosystems, Sunnyvale, CA).

Overexpression and knockdown PS and TAM receptors with shRNA-lentivirus
A PROS1 clone was purchased from Thermo Scientific (Huntsville, AL) and inserted into a *gag-*, *pol-*, and *env*-deficient lentivector construct, *VVE/BBW* (gift of Dr. Luca Gusella). Lentiviral particles were generated and used for infection of podocytes. Podocytes with stable PROS1 expression were selected using blasticidin. Cultured murine and human podocytes infected with the empty *VVE/BBW* lentivector served as controls. *PROS1, TYRO3, AXL,* and *MERTK* knockdown in human podocytes was performed using an Expression Arrest GIPZ Lentiviral shRNAmir system (Thermo Scientific, Huntsville, AL). Two sets of shRNAs against PROS1 and each of the TAM receptors were used in the experiments (Thermo Fisher Catalog#: PROS1 RHS4531-EG5627 (2 different clones); TYRO3 shRNA clones S14544 and S14545; MERTK shRNA clones S20472 and S20473; AXL shRNA clones S1845 and S1846). Lentiviral particles were produced by transfecting 293T cells with a combination of lentiviral expression plasmid, pCD/NL-BH ΔΔΔ packaging plasmid, and VSV-G–encoding pLTR-G plasmid. For viral transduction of human podocyte cell line, viral supernatants were supplemented with 8μg/ml polybrene and incubated with cells for a 24-hour period. Cells expressing shRNA were selected with puromycin for 2 to 3 weeks prior to use in all studies. Western blot analysis was performed to confirm the efficiency of knockdown (Supplementary Figure 3).

Western blot
Cells were homogenized in lysis buffer containing protease inhibitor cocktail. Equal amounts of protein samples were separated on SDS polyacrylamide gel, transferred to PVDF membranes (Millipore) and probed with primary antibodies (Rabbit anti-PROS1 from MYBIOSOURCE, rabbit anti-TYRO3 from Abcam, and rabbit anti-Axl and anti-Mer from Cell Signaling). Membranes were then washed with PBST and incubated with a secondary antibody (horseradish peroxidase conjugated antibodies to mouse IgG or to rabbit IgG). Blots were developed with the enhanced chemiluminescence system. Densitometry analysis for quantification was performed as described previously [4].

Generation of conditional Pros1 knockout Mice
Pros1$^{fl/fl}$ mice in C57BL/6 background [5] were provided by Dr. Greg Lemke from the Salk Institute, La Jolla, California. *Pod-Cre* transgenic animals were obtained from Jackson Research Laboratories (Bar Harbor, ME). Genotyping for podocyte-specific *Pros1* knockout mice (KO) performed using the following primers: forward, 5'-caa tac aag gca aag gga atg agg-3; reverse, 5'-gag ctct cag aga cta aac-3'.

Diabetic mouse model
WT and PS-KO mice used in this study were on a congenic C57BL/6 background. At 8 weeks of age, male WT and PS-KO mice were injected for 5 consecutive days with either STZ (50μg/g) (Sigma-Aldrich, St Louis, MO) per day intraperitoneally or sodium-citrate vehicle. Fasting blood glucose levels were monitored weekly by using a glucometer. Diabetic OVE26 mice [FVB(Cg)-Tg(Cryaa-Tag,Ins2-CALM1)26Ove/PneJ] were obtained from Jackson Laboratory and age-matched normal FVB/N mice were used as healthy controls. Diabetic OVE26 mice were maintained by daily monitoring of blood glucose and long-acting insulin injections as needed. These mice developed significant proteinuria at age of 6-8 weeks and proteinuria peaked at age of 10-12 weeks. OVE26 and their control mice received rAAV9 injection at age of 10 weeks using a protocol as described below. All mice were housed and cared for in the Animal Care Facility at out Institution with free access to food and water. All protocols were approved by the Animal Care Committee at Icahn School of Medicine at Mount Sinai. Mice were sacrificed at 20 weeks after STZ injection.

Cloning of hPROS1, rAAV9 production and renal Injection
To produce the recombinant adeno-associated virus (rAAV9) encoding human PROS1 under the control of

a CMV/chicken beta actin hybrid promoter, the cDNA for human PROS1 was excised from pCMV-SPORT6-hPROS1 (Harvard Plasmid Repository, Boston, MA) by double digestion with SmaI/XhoI and gel purified. pTR-UF11 (ATCC, Manassas, VA) was digested with SbfI and blunted with T4 DNA polymerase, followed by a digestion with SalI. The hPROS1 fragment was ligated with pTR-UF11 gel purified vector backbone, yielding the plasmid pTR-hPROS1. E. coli cells (New England Biolabs, Ipswich, MA) were transformed and expanded at 30 °C in LB broth, and DNA was purified with Zymopure Plasmid Maxi Prep Kit (ZymoResearch, Irvine, CA). The recombinant rAAV9 virus was produced by co-transfection of pTR-hPROS1 and pDG9 into HEK293T cells. The transfection and purification of rAAV9.hPROS1 by iodixanol gradient purification was performed as described [6]. The gradient purified virus was then injected into renal vein of adult mice as previously described [7]. Briefly, mice were anesthetized with ketamine/xylazine and the left kidney was exposed with a dorsal incision. The renal vein was clamped distally and a total volume of 100µl saline with final viral titer of $1x10^{12}$ PFU/ml was slowly injected into the kidneys. After approximately 15 more seconds, the clamp was released. The whole procedure lasted approximately 30 seconds minimizing renal ischemia. For the mice receiving bilateral injection of rAAV9, both kidneys were exposed for injection. rAAV9 expressing eGFP was used as the control.

Measurement of Urine Albumin and Creatinine

Urine albumin was quantified by ELISA using a kit from Bethyl Laboratories, Inc. (Houston, TX). Urine creatinine levels were measured in the same samples using QuantiChrom™ creatinine assay kit (DICT-500) (BioAssay Systems) according to the manufacturer's instruction. The urine albumin excretion rate was expressed as the ratio of albumin to creatinine. 12-hour urine collections in the metabolic cages were also used for determination of urinary albumin excretion.

Kidney histology

Kidneys were removed and fixed with 4% paraformaldehyde 16 hours at 4 °C. The 4µm sections were cut from paraffin-embedded kidney tissues. Sections were stained with periodic acid–Schiff (PAS) for histology analysis. Assessment of the mesangial and glomerular cross-sectional areas was performed by pixel counts on a minimum of 10 glomeruli per section in a blinded fashion, under 400x magnification (Zeiss AX10 microscope, Carl Zeiss Canada Ltd, Toronto, ON, Canada).

Electron Microscopy

Tissues were fixed in 2.5% glutaraldehyde with 0.1M sodium cacodylate (pH 7.4) for 72 hr at 4°C. Samples were further incubated with 2% osmium tetroxide and 0.1M sodium cacodylate (pH 7.4) for 1 hr at room temperature. Ultrathin sections were stained with lead citrate and uranyl acetate and viewed on a Hitachi H7650 microscope. Briefly, negatives were digitized, and images with a final magnitude of up to X10,000 were obtained. ImageJ 1.26t software (National Institutes of Health, rsb.info.nih.gov) was used to measure the length of the peripheral GBM, and the number of slit pores overlying this GBM length was counted. The arithmetic mean of the foot process width (W_{FP}) was calculated as shown below:

$$W_{FP} = \frac{\pi}{4} \times \frac{\sum GBM\ LENGTH}{\sum silts}$$

where Σslits indicates the total number of slits counted; ΣGBM LENGTH indicates the total GBM length measured in one glomerulus, and π/4 is the correction factor for the random orientation by which the foot processes were sectioned [8].

Isolation of mouse glomeruli

Mouse glomeruli were isolated as described [9]. Briefly, animals were perfused with Hanks' buffered salt solution containing 2.5mg/ml iron oxide and 1% bovine serum albumin. At the end of perfusion, kidneys were removed, decapsulated, minced into $1mm^3$ pieces, and digested in Hanks' buffered salt solution containing 1mg/ml collagenase A and 100units/ml deoxyribonuclease I. Digested tissue was then passed through a 100µm cell strainer and collected by centrifugation. The pellet was resuspended in 2 ml of Hanks' buffered salt solution, and glomeruli were collected using a magnet. The purity of glomerular was verified under microscopy. Total RNA was isolated from kidney glomeruli of mice using TRIzol (Invitrogen).

Isolation of primary podocytes
Briefly, isolated glomeruli were transferred onto a 6-cm tissue culture dish coated with type I collagen and cultured in RPMI 1640 medium (Life Technologies) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Glomeruli and cells were allowed to attach to the plate for 5 days in a 37°C incubator without any agitation. Five days later, glomeruli and outgrowth cells were detached from the plate using a 0.12% trypsin-EDTA solution. Trypsinized cells [primary glomerular epithelial cells (PGECs)] were strained using a 40-μm cell strainer and replated onto collagen-coated dishes. PGECs were allowed to grow to 80% confluence before passaging at a ratio of 1:3. PGECs are cells derived from decapsulated glomeruli, isolated by perfusion of magnetic particles. PGECs consist mostly of podocytes, with approximately 70% of PGECs being positive for podocalyxin staining.

Real-time PCR
Total RNA was extracted by using TRIzol (Invitrogen). First strand cDNA was prepared from total RNA (2.0 μg) using the Superscript ^{TM} III first strand synthesis kit (Invitrogen) and cDNA (1 μl) was amplified in triplicate using SYBR GreenER qPCR Supermix on an ABI PRISM 7900HT (Applied Biosystems, Foster City, CA). The primer sequences are listed in Supplementary Table 3. Light Cycler analysis software was used to determine crossing points using the second derivative method. Data were normalized to housekeeping genes (GAPDH) and presented as fold increase compared with RNA isolated from WT animals using the $2\text{-}\Delta\Delta^{CT}$ method.

Immunofluorescence
Kidney sections from all mice were prepared in an identical fashion. Immunostaining was performed using rabbit anti-podocalyxin (R&D Systems), rabbit anti-nephrin (a gift from Dr. Larry Holzman), and mouse anti-WT1 antibodies (Santa Cruz Biotechnology). After washing, sections were incubated with a fluorophore-linked secondary antibody (Alexa Fluor 488 anti-rabbit IgG and Alexa Fluor 568 anti-mouse IgG from Invitrogen). After staining, slides were mounted in Aqua Poly/Mount (Polysciences Inc.) and photographed under an AxioVision IIe microscope with a digital camera.

Immunohistochemistry
Archival human biopsy specimens of healthy donor nephrectomies and diabetic nephropathy were collected at Icahn School of Medicine at Mount Sinai under a protocol approved by the Institutional Review Board. Specimens were initially baked for 20 minutes in 55–60 °C oven and then processed as described below. Briefly, formalin-fixed and paraffin-embedded sections were deparaffinized, and endogenous peroxidase was inactivated with H_2O_2. Sections were then blocked in 2% goat serum in phosphate-buffered saline (PBS) for 1 hour at room temperature and then incubated with a rabbit anti-PS antibody (1:1000, GenScript) at 4°C overnight. The next day, sections were washed three times with PBS and then incubated with secondary antibody for 30 minutes. Positive staining was revealed by peroxidase-labeled streptavidin and diaminobenzidine substrate with a fixed exposure time of 3 minutes for all experiments among the groups. The control included a section stained with only secondary antibody. TUNEL staining was performed according to the manufacturer's instructions (Abcam #ab6610).

Quantification of Immunostaining
After sections were stained with anti-PS antibody, negatives were digitized, and images with a final magnitude of approximately X400 were obtained. ImageJ 1.26t software was used to measure the level of immunostaining in the glomeruli. First, the images were converted to 8-bit grayscale. Glomerular regions were selected for measurement of area and integrated density, and background intensity was measured by selecting three distinct areas in the background with no staining. The corrected optical density (COD) was determined as shown below:

$$\mathbf{COD} = \boldsymbol{ID} - (\boldsymbol{A} \times \boldsymbol{MGV})$$

where ID is the integrated density of the selected glomerular region, A is the area of the selected glomerular region, and MGV is the mean gray value of the background readings) [10].

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