CARDIOVASCULAR, PULMONARY, AND RENAL PATHOLOGY

Loss of Roundabout Guidance Receptor 2 (*Robo2*) in Podocytes Protects Adult Mice from Glomerular Injury by Maintaining Podocyte Foot Process Structure

Anna Pisarek-Horowitz,^{*} Xueping Fan,^{*} Sudhir Kumar,^{*} Hila M. Rasouly,^{*} Richa Sharma,^{*} Hui Chen,[†] Kathryn Coser,[‡] Crystal T. Bluet,[§] Dinesh Hirehallur-Shanthappa,[§] Sarah R. Anderson,[¶] Hongying Yang,[‡] Laurence H. Beck, Jr.,^{*} Ramon G. Bonegio,^{*} Joel M. Henderson,[†] Stephen P. Berasi,[‡] David J. Salant,^{*,†} and Weining Lu^{*,†}

From the Renal Section,^{*} Department of Medicine, and the Department of Pathology and Laboratory Medicine,[†] Boston University School of Medicine, Boston Medical Center, Boston, Massachusetts; the Centers for Therapeutic Innovation[‡] and Comparative Medicine,[§] Pfizer Inc., Cambridge, Massachusetts; and the Global Pathology, Drug Safety Research and Development,[¶] Pfizer Inc., Groton, Connecticut

Accepted for publication
December 17, 2019.

Address correspondence to
Weining Lu, M.D., Renal Section, Department of Medicine, and Department of Pathology and Laboratory Medicine, Evans Biomedical Research Center (EBRC) 538, Boston University School of Medicine, Boston Medical Center, 650 Albany St., Boston, MA 02118. E-mail: wlu@bu.edu.

Roundabout guidance receptor 2 (ROBO2) plays an important role during early kidney development. ROBO2 is expressed in podocytes, inhibits nephrin-induced actin polymerization, down-regulates non-muscle myosin IIA activity, and destabilizes kidney podocyte adhesion. However, the role of ROBO2 during kidney injury, particularly in mature podocytes, is not known. Herein, we report that loss of ROBO2 in podocytes [*Robo2* conditional knockout (cKO) mouse] is protective from glomerular injuries. Ultrastructural analysis reveals that *Robo2* cKO mice display less foot process effacement and better-preserved slit-diaphragm density compared with wild-type littermates injured by either protamine sulfate or nephrotoxic serum (NTS). The *Robo2* cKO mice also develop less proteinuria after NTS injury. Further studies reveal that ROBO2 expression in podocytes is up-regulated after glomerular injury because its expression levels are higher in the glomeruli of NTS injured mice and passive Heymann membranous nephropathy rats. Moreover, the amount of ROBO2 in the glomeruli is also elevated in patients with membranous nephropathy. Finally, overexpression of ROBO2 in cultured mouse podocytes compromises cell adhesion. Taken together, these findings suggest that kidney injury increases glomerular ROBO2 expression that might compromise podocyte adhesion and, thus, loss of *Robo2* in podocytes could protect from glomerular injury by enhancing podocyte adhesion that helps maintain foot process structure. Our findings also suggest that ROBO2 is a therapeutic target for podocyte injury and podocytopathy. (*Am J Pathol* 2020, 190: 799–816; <https://doi.org/10.1016/j.ajpath.2019.12.009>)

Podocytes are terminally differentiated epithelial cells that extend an elaborate net of foot processes to tightly wrap around and support the capillary vessels in kidney glomeruli. Foot processes from one podocyte interdigitate with foot processes from neighboring podocytes and connect to each other through a specialized cell junction called the slit diaphragm. Together with the fenestrated endothelium and glomerular basement membrane (GBM), podocytes constitute the last crucial layer of the blood-urine filtration barrier to retain essential plasma proteins in the blood. Because the podocyte cytoskeleton in foot processes

is mostly actin based,^{1,2} they exhibit a high degree of plasticity and structural changes in response to physiological and pathologic stimuli.^{1,3–5} Podocyte foot process structure positively correlates with its function as various forms of

Supported in part by NIH grants R01DK078226 (W.L.) and R01DK090029 (D.J.S.), a Pfizer Centers for Therapeutic Innovation research grant (W.L.), and a Massachusetts Life Sciences Center cooperative matching grant (W.L.).

A.P.-H., X.F., and S.K. contributed equally to this work.

Disclosures: W.L.'s work has been partially funded by a research grant from the Pfizer Centers for Therapeutic Innovation.

experimental podocyte injury induce podocyte foot process widening, simplification, and reorganization with increased foot process width, known as foot process effacement.^{6–10} Foot process effacement is recognized as a hallmark of numerous human glomerular diseases,^{11–15} and recently has also been reported as a predictive marker for renal outcomes after treatment.¹⁶

ROBOs are cell adhesion molecules that belong to the Ig superfamily of single-pass transmembrane receptors for secreted repulsive guidance cue SLITs.^{17,18} In vertebrates, there are four ROBO receptors (ROBO1 to ROBO4) and three SLIT ligands (SLIT1 to SLIT3). ROBO/SLIT was first described as a repulsive signaling pathway to regulate neuronal migration and axon path finding by means of regulating the neuronal cell cytoskeleton.^{17–19} Roundabout guidance receptor 2 (ROBO2) and its ligand slit guidance ligand 2 (SLIT2) also regulate Wolffian duct–nephrogenic cord separation²⁰ before embryonic kidney induction and are required for normal ureteric bud outgrowth.²¹ Disruption of either *Robo2* gene or *Slit2* gene in mice results in a failure of the normal separation of the anterior metanephric mesenchyme compartment from the Wolffian duct epithelium compartment, leading to supernumerary ureteric buds, duplex kidneys, and hydronephrosis phenotype.²⁰ In humans, mutations in either *ROBO2* or *SLIT2* cause urinary tract birth defects, such as vesicoureteral reflux and congenital anomalies of the kidney and urinary tract, due to abnormal ureteric bud outgrowth.^{22–24}

After ureteric bud outgrowth and nephron formation, the expression of ROBO2 is gradually restricted to the glomerulus, where it is predominantly localized to the foot processes and the basal side of the podocyte.²⁵ In podocytes, ROBO2 is able to form a complex with nephrin through the NCK adaptor protein.²⁵ Nephrin is a transmembrane protein in the glomerular slit diaphragm that plays a crucial role in maintaining the podocyte foot process structure and glomerular filtration barrier function.^{26,27} Nephrin phosphorylation induces actin polymerization in response to certain physiological and pathologic stimuli.^{28–30} The ROBO2/SLIT2 signaling pathway negatively regulates nephrin-induced actin polymerization.²⁵ ROBO2/SLIT2 signaling inhibits nonmuscle myosin IIA activity and reduces podocyte adhesion.³¹ However, the role of ROBO2 in adult kidney and podocyte foot process structure, particularly under injury conditions, is not known.

This study compared phenotypes between adult *Robo2* podocyte-specific knockout mice [*Robo2* conditional knockout (cKO)] and wild-type (WT) controls under two different glomerular injury conditions induced by protamine sulfate (PS) perfusion or nephrotoxic serum (NTS) injection. ROBO2 expression was also analyzed in the glomeruli of NTS injured mice, passive Heymann nephritis (PHN) rat, and membranous nephropathy (MN) and focal segmental glomerulosclerosis (FSGS) patients. These results suggest that podocyte injury up-regulates ROBO2 expression and *Robo2* deletion protects podocytes from

injury by enhancing podocyte adhesion, which preserves foot process structure.

Materials and Methods

Mouse PS Injury Model

Robo2 podocyte-specific knockout mice were generated as previously reported.^{23,25} Mice used in the PS terminal kidney injury studies were 8 to 12 weeks old of both sexes in C57BL/6 background. Mouse kidney perfusion with protamine sulfate was performed as previously described.³⁰ Briefly, mice were anesthetized with tribromoethanol (Sigma-Aldrich, St. Louis, MO) and kept at constant 37°C throughout the procedure using the heating pad. All solutions were delivered through the aortal perfusion. Mice were initially perfused with warm Hanks' balanced salt solution (HBSS; Mediatech, Inc., Tewksbury, MA) for 2 minutes to wash out the blood. Then, 2 mg/mL protamine sulfate solution (MP Biochemicals, Solon, OH) or the HBSS vehicle solution (MP Biochemicals) was delivered using the perfusion pump at 8 mL/minute rate for 15 minutes. After delivery of either solution, additional perfusion with HBSS was performed and individual kidneys were promptly dissected and preserved in Karnovsky's fixative for further electron microscopy (EM) analyses. To minimize any artifacts, all animals were perfused by the same operator (A.P.-H.), with the same equipment, and on the same day; all kidneys were visually examined to see if any unperfused areas were present. To minimize the spatial variability, great care was taken to use the cortical region, which was then cut into multiple small cubes. All those pieces were further processed for EM studies, and at least four pieces of tissue from each kidney were randomly selected for EM imaging.

Mouse NTS Injury Model

NTS used in this study was generated in the laboratory of D.J.S. by immunizing sheep with rat glomerular extracts as previously described.^{32–34} To induce podocyte injury, 1 mg of IgG1 fraction of NTS antibodies or 1 mg of innate sheep IgG1 (MP Biochemicals) in 200 µL volume was injected intravenously into 8- to 12-week-old WT and *Robo2*^{flox/flox}; *Nphs2*^{Cre+} (*Robo2* cKO) mice through the tail vein. Animals were allowed to recover with unrestricted access to food and water, and their urine samples were collected at the prespecified time points at 1 hour, 6 hours, 24 hours, and day 7. For gene and protein expression analyses, six animals in each experimental group were sacrificed on day 1 and day 7 after NTS injection and their kidney samples were isolated.

Rat PHN Injury Model

A modified PHN injury model is induced in Lewis rats (strain code 004; Charles River Laboratories, Wilmington,

MA) by one dose of i.v. injection [1.5 to 2 mL/kg sheep anti-rat Fx1A serum (PTX-002S; Probetex, San Antonio, TX)], according to Probetex and published protocol.³⁴ Urine samples were collected before anti-rat Fx1A serum injection (day 0) and at days 3, 7, 11, and 16 after anti-rat Fx1A serum injection. Rats were euthanized at day 16 after anti-rat Fx1A serum injection, and kidneys were collected and fixed in Karnovsky's fixative for EM analysis or fixed in 4% paraformaldehyde for immunofluorescence (IF) staining. Sterile 0.9% sodium chloride (buffered saline) was used in control rats. A total of six animals in each experimental group were studied.

Proteinuria Measurement

Spot urine was collected according to the standard protocol.³⁵ To quantify proteinuria after NTS injury, a Coomassie Blue assay was used. To normalize urine samples from different animals, urine creatinine concentrations were measured in Jaffé reaction (Creatinine Companion kit; Exocell Inc., Philadelphia, PA). Urine samples were diluted with protein loading buffer (Boston BioProducts, Ashland, MA) based on creatinine concentrations. Urine samples were resolved on 12% SDS-PAGE gel, and each line of the gel was loaded with urine amount containing 1 mg creatinine. Coomassie Brilliant Blue reagent (Boston BioProducts) was then applied in accordance with the standard protocol to visualize total protein content. A total of 2 mg standard albumin (Thermo Fisher Scientific, Waltham, MA) was included in each gel as a reference control. Cleared gel images were digitally captured using the HP scanner (HP Inc., Palo Alto, CA), and densitometry measurements of urine albumin were taken using ImageJ software version 1.47v (NIH, Bethesda, MD; <https://imagej.nih.gov/ij>). The urine albumin densitometry measurements were normalized by urine creatinine and dilution factors using a calibration curve and were reported as urinary albumin/creatinine ratio (ACR).

Kidney Histology and Podocyte Quantification

For kidney histology, mouse kidneys were dissected and fixed in formalin overnight and followed with paraffin embedding according to standard procedure. Kidney paraffin sections were stained with standard periodic acid-Schiff staining kit (Polysciences, Inc., Warrington, PA; catalog number 24200-1). The periodic acid-Schiff stained slides were examined under an Olympus BHT light microscope (Olympus America Inc., Center Valley, PA) and analyzed for glomerular injury lesions, including glomerular matrix expansion and vacuolation. To quantify podocyte number, P57 was used as a podocyte nuclear marker and immunoperoxidase staining was performed on kidney sections using rabbit monoclonal anti-p57 Kip2 antibody (Abcam, Cambridge, MA; catalog number ab75974), followed by ImmPRESS HRP Anti-Rabbit IgG (Peroxidase)

Polymer Detection Kit (Vector Laboratories, Burlingame, CA; catalog number MP-7451) and ImmPACT DAB Peroxidase (HRP) substrate (Vector Laboratories; catalog number SK-4105).

SEM Data

Kidney samples were prepared following modified scanning EM (SEM) biological sample processing, as previously described.³⁶ Briefly, isolated kidney specimens designated for the SEM analysis were fixed for 2 hours in Karnovsky's fixative (2% paraformaldehyde, 2.5% glutaraldehyde, and 0.1 mol/L sodium phosphate buffer; Electron Microscopy Sciences, Hatfield, PA) and followed by post-fixation for 2 hours with 2% osmium tetroxide (Electron Microscopy Sciences) in deionized water. The specimens were subsequently dehydrated in a series of graded ethanol and chemically dried with hexamethyldisilazane infiltration (Electron Microscopy Sciences), which is a great alternative approach to critical point drying or freeze drying before SEM examination. After sputter coating of a thin layer of gold particles, SEM images of kidney glomeruli were captured using the Joel 6340F scanning electron microscope (JEOL USA Inc., Peabody, MA) equipped with a digital camera. The operator (A.P.-H.) was blinded as to the genotype and the injury status of the animals at the time of image collection and analyses.

Transmission EM

Fresh kidney samples were fixed in Karnovsky's fixative and processed according to the published protocol.³⁷ In brief, small pieces of kidney samples (<1 mm thick) were fixed in buffered 2.5% glutaraldehyde. Kidney specimens were then post-fixed in 1% osmium tetroxide (Electron Microscopy Sciences), dehydrated, embedded in propylene oxide/Epon resin, and sectioned (60 to 90 nm thick) using the ultramicrotome. Collected sections were post-stained with uranyl acetate and lead citrate (Electron Microscopy Sciences), according to the published protocol.³⁷ Images were obtained using the Joel JEM-1011 transmission electron microscope (JEOL USA Inc.) equipped with an Erlangshen ES100W digital camera (Gatan, Pleasanton, CA). The operator (A.P.-H.) was blinded as to the genotype and the injury status of the animals at the time of image collection and analyses.

Podocyte Foot Process Width and Slit-Diaphragm Density Measurement

The podocyte foot process width was manually traced and measured on $\times 25,000$ high-magnification transmission EM images using ImageJ software version 1.47v, according to published methods.^{38–40} Briefly, the podocyte foot process width was measured from one end of slit diaphragm to the other by drawing a line parallel to the

GBM. This was repeated for all the foot processes inside the image. These results were then pasted on Excel sheet (Microsoft Corporation, Redmond, WA) and adjusted for scale bar, and the harmonic mean for each image was calculated. To measure the slit-diaphragm density, number of slit diaphragms was counted and divided by the total length of the underlying GBM spanning the area of these slit diaphragms using ImageJ, according to published methods.^{40–42} If more than one GBM area was visible or the GBM was disconnected because of nonvisibility of the foot processes, the above procedure was repeated and the average of these measurements was used to calculate the slit-diaphragm density. More than three glomeruli per specimen and four or more areas per glomerulus were analyzed. Measurements were performed in six mice per group. All measurements were taken blindly (A.P.-H.), and groups were analyzed and compared using independent *t*-test (unpaired and parametric test).

IF and Immunohistochemistry Staining

IF staining was performed on mouse, rat, monkey, and human kidney sections, as previously described.³¹ Briefly, 4% paraformaldehyde, fixed or snap frozen, and OCT embedded kidney tissues were sectioned using a cryostat. The frozen rat or human tissue sections were post-fixed with freshly prepared ice-cold acetone-methanol (1:1) for 10 minutes at -20°C . Sections were then air dried for 30 minutes at room temperature. Subsequently, sections were washed with washing buffer (0.1% phosphate-buffered saline with Tween 20) and blocked with peptide blocking agent (Innovex Biosciences, Richmond, CA; catalog number NB306-50) for 1 hour at room temperature. Kidney sections were then stained with anti-ROBO2 primary antibody (catalog number NBP1-812399; Novus Biologicals, Centennial, CO; and catalog number SC-31607; Santa Cruz Biotechnology, Dallas, TX), followed with appropriate Alexa Fluor 488, Cy3, or Alexa Fluor 594 conjugated secondary antibodies. Tissue sections were washed and mounted with Vectashield antifade mounting medium (Vector Laboratories, Burlingame, CA; catalog number H-1000). Images were taken using a Zeiss LSM 700 confocal laser scanning microscope (Carl Zeiss Microscopy, White Plains, NY). Immunohistochemistry on human biopsy specimens using anti-ROBO2 antibody was performed on formalin-fixed and paraffin-embedded specimens by the IntelliPath Automated Slide Stainer (BioCare Medical, Concord, CA). In brief, paraffin slides were baked, deparaffinized, rehydrated, and blocked with Biocare peroxidase 1 solution (BioCare Medical) and Biocare Background Sniper (BioCare Medical). After the antigen retrieval step, the primary antibody was applied at room temperature. For secondary antibody, the Biocare Mach 4 Human Kit (BioCare Medical), was used, according to the manufacturer's recommendations. Diaminobenzidine (Thermo Fisher

Scientific) was used as a chromogenic substrate. Sections were counterstained with hematoxylin (Thermo Fisher Scientific).

TaqMan Real-Time RT-PCR Assay

Kidney samples were preserved in RNAlater RNA stabilization reagent from Life Technologies (Thermo Fisher Scientific). Total RNA was isolated using the Qiagen RNA extraction kit (RNeasy Mini Kit; Qiagen, Hilden, Germany), according to the manufacturer's instruction. RNA was measured using NanoDrop (Thermo Fisher Scientific), and equal amount of RNA was used for the conversion to cDNA (Verso cDNA Synthesis Kit; Thermo Fisher Scientific). TaqMan real-time PCR was used to compare the relative amount of ROBO2 and NEPHRIN transcripts. Mouse glyceraldehyde-3-phosphate dehydrogenase TaqMan probe was used as the housekeeping gene for relative quantification using the $\Delta\Delta\text{Ct}$ method. Data were obtained using the 7500Fast Real Time PCR instrument from Applied Biosystems (Thermo Fisher Scientific). For the rat kidney tissues from the PHN model study, rat Ppib TaqMan probe was used as the housekeeping gene for quantification using $\Delta\Delta\text{Ct}$ method, followed by comparing relative to vehicle control. Data were obtained using the ViiA 7 Real Time PCR instrument from Applied Biosystems.

Western Blot Assay

To measure the *in vivo* protein levels in kidneys, the cortical portions of fresh dissected kidney samples were used. Equal amount of starting specimen was used for each animal. Briefly, kidney tissues were homogenized in radio-immunoprecipitation assay buffer [50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, 1 mmol/L Na_3VO_4 , 1 mmol/L NaF, and $1\times$ protease inhibitor] on ice. Lysed tissues were centrifuged for 10 minutes at 4°C , and the supernatants were added with $6\times$ protein loading buffer (Boston BioProducts) and denatured at 95°C for 20 minutes before proteins were resolved on 10% SDS-PAGE gels. Proteins were transferred to membrane and blotted with mouse anti-ROBO2 (MABN122; Millipore Corp., Darmstadt, Germany), rabbit anti-NEPHRIN (sc-28192; Santa Cruz Biotechnology), and mouse anti-ACTIN (A1978; Sigma-Aldrich) antibodies. Appropriate horseradish peroxidase-conjugated secondary antibodies were used (GE Healthcare, Pittsburgh, PA), and the horseradish peroxidase signals were detected using the Amersham ECL Prime Western Blotting Detection Kit (Thermo Fisher Scientific) on radiography film (Denville Scientific, South Plainfield, NJ). Images were digitalized, and the intensity of the protein bands was measured using ImageJ software version 1.47v. For the rat kidney tissues from the PHN model study, lysates were resolved on a 4% to 12% SDS-PAGE gel and then transferred onto a nitrocellulose membrane and blotted with rabbit anti-ROBO2

(NBP1-81399; Novus Biologicals, Centennial, CO) and rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (Cell Signaling Technology, Danvers, MA) antibodies, followed by a goat anti-rabbit IgG IRDye 800CW secondary antibody (926-32211; LI-COR Biosciences, Lincoln, NE). Blot was scanned by an infrared imager Odyssey CLx and quantified by using Image Studio version 5.2.9 (LI-COR Biosciences).

Podocyte Cell Culture

Cell culture dishes were coated with SLIT2N of 0, 40, and 80 nmol/L at 37°C, 5% CO₂, for 2 hours. SLIT2N was removed, and differentiated mouse podocyte cells (gift from Dr. Jochen Reiser) were seeded onto coated dishes overnight. Cells were then fixed with 4% paraformaldehyde (Electron Microscopy Sciences; catalog number 15710) at room temperature for 30 minutes. F-actin cytoskeleton was labeled using Alexa Fluor 488 phalloidin (Invitrogen, Carlsbad, CA; catalog number A12379), according to manufacturer's instruction. Images were obtained by fluorescence confocal microscopy. Single cell area and phalloidin signal were measured using ImageJ. Analysis of variance was used for group statistical analysis.

Statistical Analysis

Different treatment groups were compared using the unpaired *t*-test, two tailed. The unpaired nonparametric *U*-test was used for the albuminuria, PHN mRNA, and PHN ROBO2 protein analysis. Two-way analysis of variance with all pairwise multiple comparison procedures (Holm-Sidak method) was used for the podocyte foot process effacement analysis. All statistics were performed using Microsoft Excel, GraphPad Prism (GraphPad Software, San Diego, CA), or SigmaPlot 12.5 (Systat, San Jose, CA), with *P* < 0.05 considered statistically significant.

Study Ethical Approval

Deidentified human kidney tissues were obtained from discarded biopsy samples (such as MN and FSGS samples) at Boston Medical Center (Boston, MA). The human subject research was approved by the institutional review board at Boston University Medical Center (protocol number H-31111). Animal studies were approved by the Institutional Animal Care and Use Committee at Boston University Medical Center (protocol numbers 14388, 15328, and 15368). All procedures performed on animals in the PHN model studies were in accordance with regulations and established guidelines and were reviewed and approved by an Institutional Animal Care and Use Committee or through an ethical review process.

Results

Loss of *Robo2* in Podocytes Alleviates Kidney Podocyte Injury Induced by Protamine Sulfate

Perfusion of rodent kidneys with PS is a well-established model to study inducible podocyte injury that affects foot process structure.^{7,43,44} Protamine sulfate is a polycation molecule that neutralizes negatively charged glycocalyx covering the cell membranes, leading to podocyte foot process effacement.⁴³ PS is also toxic to the cellular components of the glomeruli,⁴³ and results in calcium influx,⁴⁵ increased tyrosine phosphorylation,³⁰ and actin cytoskeleton rearrangement.^{44,46–48} Because ROBO2 inhibits actin polymerization,²⁵ PS perfusion was applied to *Robo2* podocyte-specific cKO mice to determine if loss of ROBO2 affects podocyte injury and foot process structure in adult mice.

To examine podocyte morphology, SEM was performed on kidney samples after perfusion with either PS or control HBSS. In agreement with previously reported studies,^{7,45,49} PS perfusion caused podocyte foot process effacement and disorganized foot process interdigitating pattern in WT mice (Figure 1A). In contrast, the *Robo2* cKO mice showed little change in global podocyte foot process arrangement after PS perfusion (Figure 1A).

Podocyte foot process width and slit-diaphragm density are surrogate markers to evaluate podocyte injury, foot process effacement, and the integrity of the glomerular filtration barrier.^{7,40,50} Increased foot process width and reduced slit-diaphragm density predict the severity of podocyte injury in several human glomerular diseases.^{16,38,41} To evaluate podocyte injury and foot process effacement, foot process width and slit-diaphragm density were measured by transmission EM.^{40,42} As expected, foot process width was significantly increased in the WT mice after PS injury in comparison to HBSS-treated controls ($0.41 \pm 0.02 \mu\text{m}$ in WT mice treated with PS versus $0.34 \pm 0.01 \mu\text{m}$ in WT mice treated with HBSS control solution; *P* = 0.026) (Table 1 and Figure 1, B and C). Interestingly, the foot process width remained unchanged in the *Robo2* cKO after PS injury compared with HBSS-treated controls ($0.36 \pm 0.01 \mu\text{m}$ in *Robo2* cKO mice treated with PS versus $0.37 \pm 0.01 \mu\text{m}$ in *Robo2* cKO treated with HBSS control solution; *P* = 0.6) (Table 1 and Figure 1, B and C). Consistent with this result, the slit-diaphragm density in the WT mice showed a significant 29.5% reduction after the PS insult (2.83 ± 0.18 slit-diaphragm density number per $1 \mu\text{m}$ length of the glomerular basement membrane in HBSS control WT mice versus 1.99 ± 0.09 in the PS-injured WT mice; *P* = 2.22×10^{-5}), whereas this dramatic slit-diaphragm density damage by PS was not observed in the *Robo2* cKO mice (2.56 ± 0.16 in HBSS control *Robo2* cKO mice versus 2.7 ± 0.14 in PS-injured *Robo2* cKO mice; *P* = 0.2) (Table 1 and Figure 1, B and D). These findings suggest that loss of ROBO2 makes podocytes more resilient to PS insults.

Figure 1 Loss of roundabout guidance receptor 2 (*Robo2*) in mouse podocytes reduces podocyte foot process (FP) damage caused by protamine sulfate (PS) injury. **A:** Representative scanning electron micrographs showing the foot process morphology in the wild-type (WT) and *Robo2* conditional knockout (cKO) mice after control (Hanks' balanced salt solution buffer) or PS perfusions. **Arrows** indicate representative individual foot processes; **asterisks**, the effaced foot processes. Six kidneys were analyzed in each group. **B:** Representative transmission electron micrographs in the wild-type and *Robo2* cKO mice after control or PS perfusion. **Arrows** indicate the foot processes; **asterisk**, foot process effacement. Six kidneys were analyzed in each group. **C:** Quantification of the FP width in the wild-type and *Robo2* cKO mice after control or PS perfusion. **D:** Quantification of the density of slit diaphragms (SDs). Data are expressed as means ± SEM (**C** and **D**). *n* = 6 kidneys (**C** and **D**). **P* < 0.05. Original magnification, ×7000 (**A**); ×25,000 (**B**). GBM, glomerular basement membrane.

Loss of *Robo2* in Podocytes Attenuates Glomerular Injury Induced by Nephrotoxic Serum in Adult Mice

Because protamine sulfate perfusion is a short-term terminal procedure,⁴³ it precludes longitudinal analysis of urine albumin in injured mice. To evaluate albuminuria level and determine if the observed renoprotective effect on foot process structure in *Robo2* cKO mice could be reproduced in a different glomerular injury model, sheep anti-rat NTS

injection was used in adult mice. NTS (alias anti-GBM antibody) includes a spectrum of glomerular antibodies.³⁴ Systemic injection of the NTS into mice or rats has been shown to cause proteinuria and direct injury to podocytes accompanied by podocyte foot process effacement.^{33,51–53}

To evaluate the effect of direct podocyte injury caused by NTS in the context of *Robo2* deletion, an i.v. injection of NTS in *Robo2* cKO and wild-type control mice was administered and urine albumin level was analyzed at 1, 6,

Table 1 Podocyte Foot Process Width and Slit-Diaphragm Density in Wild-Type Controls and Roundabout Guidance Receptor 2 (*Robo2*) Conditional Knockout Mice after Protamine Sulfate Injury

Variable	Wild-type FP width, μm	<i>Robo2</i> cKO FP width, μm	Wild-type SD density, SD, n/μm GBM	<i>Robo2</i> cKO SD density, SD, n/μm GBM
Control HBSS perfusion	0.34 ± 0.01	0.37 ± 0.01	2.83 ± 0.18	2.56 ± 0.16
PS perfusion	0.41 ± 0.02	0.36 ± 0.01	1.99 ± 0.09	2.7 ± 0.14

Data are expressed as means ± SEM. *n* = 6 in each group. cKO, conditional knockout; FP, foot process; GBM, glomerular basement membrane; HBBS, Hanks' balanced salt solution; PS, protamine sulfate; SD, slit-diaphragm.

and 24 hours and 7 days after injection (Figure 2A). First, it was ascertained if NTS antibodies could efficiently reach the glomeruli after i.v. injection in both wild-type and *Robo2* cKO using immunofluorescence (IF) staining (Figure 2B). Similar IF pattern and intensity were observed, demonstrating that NTS antibody was equally delivered and distributed in the glomeruli of both *Robo2* cKO and wild-type mice (Figure 2B). Spot urine was then collected at 1, 6, and 24 hours and day 7 after NTS injection and the urine albumin level was evaluated (Table 2 and Figure 2C). One hour after NTS injury, the wild-type mice developed minimal detectable albuminuria (0.33 ± 0.02 mg/mg ACR), which continued increasing at 6 hours (0.8 ± 0.06 mg/mg ACR), 24 hours (9.76 ± 1.14 mg/mg ACR), and day 7 (93.24 ± 12.44 mg/mg ACR) (Table 2 and Figure 2, C and D). Interestingly, *Robo2* cKO mice developed significantly milder albuminuria at 1 hour (0.19 ± 0.01 mg/mg ACR), 6

hours (0.13 ± 0.02 mg/mg ACR), 24 hours (1.34 ± 0.01 mg/mg ACR), and day 7 (7.88 ± 5.26 mg/mg ACR) (Table 2 and Figure 2, C and D). To ensure reproducibility, mice were injected with different batch of NTS and confirmed proteinuria reduction in *Robo2* cKO mice at day 3 and day 5 after NTS injection compared with wild-type controls (Supplemental Figure S1). These data suggest that loss of *Robo2* in podocytes protects mice from proteinuria induced by NTS injury.

The study next evaluated the podocyte morphology after NTS injury by SEM. Similar to PS-induced injury, a progressively worsening foot process effacement was found in wild-type mice at 1, 6, and 24 hours after NTS injection (Figure 3A). In contrast, podocyte foot processes in *Robo2* cKO mice showed minimal effacement after NTS injury (Figure 3A). By transmission EM, it was found that wild-type mice had a significant progressive increase in foot

Figure 2 Loss of roundabout guidance receptor 2 (*Robo2*) in podocytes attenuates proteinuria induced by nephrotoxic serum (NTS) injury. **A:** Experimental design for the NTS injury via tail vein injection with urine and kidney sample collections in both the wild-type (WT) and *Robo2* conditional knockout (cKO) mice. **B:** Representative images of NTS deposition in the WT and *Robo2* cKO mouse glomeruli after detection by anti-sheep IgG antibody staining at 1, 6, and 24 hours after NTS tail vein injection. **C:** Coomassie Blue–stained gel showing the amount of urine albumin (Alb.) from the WT and *Robo2* cKO mice at the indicated time points after NTS injection. The urine samples at day 7 (D7) after NTS injection are diluted 50-fold (1:50) before loading. **D:** Urine albumin/creatinine ratios (ACRs) in the WT and *Robo2* cKO mice at each time point after NTS injection. Data are expressed as means $\pm$ SEM (**D**). $n = 5$ (**C** and **D**). $**P < 0.01$. Original magnification, $\times 600$ (**B**).

Table 2 Urine Albumin Level in Wild-Type Controls and Roundabout Guidance Receptor 2 (*Robo2*) Conditional Knockout (cKO) Mice after Nephrotoxic Serum (NTS) Injury

Variable	1 Hour after NTS	6 Hours after NTS	24 Hours after NTS	7 Days after NTS
Wild-type, ACR mg/mg	0.33 ± 0.02	0.8 ± 0.06	9.76 ± 1.14	93.24 ± 12.44
<i>Robo2</i> cKO, ACR mg/mg	0.19 ± 0.01	0.13 ± 0.02	1.34 ± 0.01	7.88 ± 5.26

Data are expressed as means ± SEM. $n = 5$ in each group.

ACR, albumin/creatinine ratio.

process width at 1 hour ($0.61 \pm 0.04 \mu\text{m}$; $n = 6$), 6 hours ($0.66 \pm 0.02 \mu\text{m}$; $n = 6$), and 24 hours ($0.82 \pm 0.08 \mu\text{m}$; $n = 6$) after NTS injection (Table 3 and Figure 3, B and C). Similar to the findings in PS injury, the foot process width in *Robo2* cKO mice did not change significantly after NTS injury (Table 3 and Figure 3, B and C). At 24 hours after injury, the difference in foot process width between *Robo2* cKO ($0.475 \pm 0.02 \mu\text{m}$) and wild-type controls ($0.82 \pm 0.08 \mu\text{m}$) is most striking—with only approximately 11% increase in podocyte foot process width in *Robo2* cKO compared with 163% increase in wild-type control mice (Table 3 and Figure 3C). Consistent with the changes in the foot process width, the slit-diaphragm density remained unchanged in the *Robo2* cKO mice at 24 hours after NTS injury ($2.13 \pm 0.08 \text{ n}/\mu\text{m GBM}$ versus $2.34 \pm 0.04 \text{ n}/\mu\text{m GBM}$ after control IgG injection; $n = 6$ per group; $P > 0.05$), whereas the density was significantly reduced in the WT mice under the same condition ($1.25 \pm 0.1 \text{ n}/\mu\text{m GBM}$ at 24 hours after NTS injection versus $3.2 \pm 0.05 \text{ n}/\mu\text{m GBM}$ after control IgG injection; $n = 6$ per group; $P < 0.05$) (Table 3 and Figure 3D). Histopathologic analysis using periodic acid-Schiff staining further demonstrated significantly less glomerular lesions at day 7 after NTS injury in *Robo2* cKO compared with wild-type controls (Supplemental Figure S2). As only short-term glomerular injury was studied in the heterologous phase of NTS injury, a podocyte loss phenotype was not observed in both *Robo2* cKO and wild-type controls (Supplemental Figure S3). Taken together, these data further demonstrate that loss of *Robo2* in podocytes protects the glomerular filtration barrier during podocyte injury in adult mice by maintaining podocyte foot process structure.

ROBO2 Expression Is Up-Regulated in Wild-Type Mice after Nephrotoxic Serum–Induced Glomerular Injury

Podocyte injury often leads to abnormal expression of podocyte proteins, such as nephrin.^{54–57} Down-regulation of nephrin has been reported in animal models and human kidney disease with podocyte injury.^{56,58–61} ROBO2 has been shown to form a complex with nephrin in podocytes and inhibits nephrin-induced actin polymerization.²⁵ To understand the molecular mechanism underlying the renoprotective effect of loss of ROBO2 on glomerular injury, the mRNA and protein levels of nephrin and ROBO2 in the kidney of *Robo2* cKO mice and wild-type littermate controls

were analyzed before and after NTS injury using TaqMan real-time RT-PCR and Western blot analyses (Figure 4).

At 24 hours after NTS injury, the mRNA levels of nephrin were down-regulated in the kidneys of both wild-type controls and *Robo2* cKO mice (Figure 4A), and the nephrin expression was restored on day 7 after NTS injury (Figure 4A). Similar to the nephrin expression, *Robo2* mRNA expression was also down-regulated 24 hours after NTS injection (Figure 4B) but was significantly up-regulated in wild-type kidney on day 7 after NTS injury (Figure 4B). Consistent with the mRNA changes, Western blot analyses of nephrin and ROBO2 in mouse kidney cortical lysates revealed a down-regulation of nephrin and ROBO2 expressions in the kidney 24 hours after NTS injection (Figure 4, C–E). However, significant increase of *Robo2* protein level was detected on day 7 after NTS injury in the kidney of wild-type mice (Figure 4, C and E). As expected, *Robo2* protein levels in *Robo2* cKO mice were barely detectable at all time points (Figure 4, C and E). Because nephrin is uniquely expressed in kidney podocytes and *Robo2* is also specifically localized in these cells in the kidney, these data suggest that *Robo2* expression is up-regulated in wild-type mice after nephrotoxic serum–induced glomerular injury.

Increased *Robo2* Expression in the Glomeruli of Passive Heymann Nephritis Rats and MN Patients

Because the expression of *Robo2* is up-regulated in antibody-mediated NTS injury in mice, this study investigated if the *Robo2* expression is also increased in the kidney of another animal model of antibody-mediated glomerular injury. PHN in rats is a proteinuric model of human MN with antibody (anti-Fx1A) and antigen (megalalin) immune complex depositions in the glomerular sub-epithelial space between podocyte foot processes and the GBM.^{34,62,63} By immunostaining, it was first confirmed that *Robo2* is expressed in adult rat glomeruli and is colocalized with nephrin in podocytes as in mice (Figure 5, A–C). PHN was then generated by anti-Fx1A i.v. injection (Figure 5D). These PHN rats developed severe proteinuria starting on day 3 after anti-Fx1A injection (Figure 5E). The PHN rats were sacrificed on day 16 after anti-Fx1A injection and the kidney glomerular ultrastructure was analyzed by transmission EM. Severe podocyte injury, extensive foot process effacement and irregularity,

Figure 3 Loss of roundabout guidance receptor 2 (*Robo2*) in podocytes preserves podocyte foot process (FP) architecture after nephrotoxic serum (NTS) injury. **A:** Representative scanning electron micrographs showing the foot process morphology at indicated time points after NTS injection in wild-type (WT) and *Robo2* conditional knockout (cKO) mice. At least five different regions of each glomerulus and three glomeruli per animal were analyzed. **Arrows** indicate foot processes; **asterisks**, areas of effacement. **B:** Representative transmission electron micrographs showing podocyte foot process reorganization and foot process effacement in both WT and *Robo2* cKO groups at indicated time points after NTS injection. At least five different regions in each glomerulus and three glomeruli per animal were analyzed. **Arrows** indicate foot processes; **asterisks**, effaced foot processes. **C:** Quantification of foot process width in **B** in both WT and *Robo2* cKO groups. Foot process width was measured at indicated time points. **D:** Quantification of slit diaphragms (SDs) per μm length of underlying glomerular basement membrane (GBM) was measured in both WT and *Robo2* cKO groups at indicated time points after NTS injection versus control IgG injection. Data are expressed as means $\pm$ SEM (**C** and **D**). $n = 6$ animals (**C** and **D**). * $P < 0.05$ versus control. Original magnification: $\times 7000$ (**A**); $\times 25,000$ (**B**).

Table 3 Podocyte FP Width and SD Density in Wild-Type Controls and Roundabout Guidance Receptor 2 (*Robo2*) cKO Mice after NTS Injury

Variable	Wild-type FP width, μm	<i>Robo2</i> cKO FP width, μm	Wild-type SD density, SD, $n/\mu\text{m}$ GBM	<i>Robo2</i> cKO SD density, SD, $n/\mu\text{m}$ GBM
Control IgG	0.31 ± 0.003	0.43 ± 0.02	3.2 ± 0.05	2.34 ± 0.04
1 Hour after NTS	0.61 ± 0.04	0.41 ± 0.01	1.66 ± 0.1	2.42 ± 0.06
6 Hours after NTS	0.66 ± 0.02	0.45 ± 0.02	1.53 ± 0.04	2.27 ± 0.1
24 Hours after NTS	0.82 ± 0.08	0.475 ± 0.02	1.25 ± 0.1	2.13 ± 0.08

Data are expressed as means $\pm$ SEM. $n = 6$ in each group.
cKO, conditional knockout; FP, foot process; GBM, glomerular basement membrane; NTS, nephrotoxic serum; SD, slit diaphragm.

and subepithelial immune complex deposition were observed in the PHN rat glomeruli but not in vehicle-injected sham control rats (Figure 5, F and G), confirming immune-mediated glomerular injury. Immunostaining of *Robo2* was then performed on the kidney sections from PHN rats and controls and enhanced ROBO2 expression was observed in the glomeruli of PHN rats compared with vehicle-injected controls (Figure 5, H and I). Quantitative

RT-PCR and Western blot analyses also showed up-regulation of *Robo2* mRNA (Figure 5J) and *Robo2* protein (Figure 5, K and L) levels in PHN rat kidney tissues compared with vehicle-injected controls. These data suggest that *Robo2* expression is up-regulated in the glomeruli of rats on immune-mediated podocyte injury. To determine if these findings of *Robo2* expression in the rodent models could be replicated in human diseases of

Figure 4 Nephlin and roundabout guidance receptor 2 (*Robo2*) expression in nephrotoxic serum (NTS) injured mouse kidney. **A** and **B**: Quantitative real-time RT-PCR assay shows the mRNA levels of nephlin and *Robo2* on day (D)0, day 1, and day 7 after NTS injection. **C**: Western blot analysis showing protein levels of nephlin and *Robo2* in the kidney cortex of wild-type (WT) and *Robo2* conditional knockout (cKO) mice on D0, D1, and D7 after NTS injection. β -Actin was used as loading control. **D** and **E**: Quantification of Western blot results presented in **C**. Data are expressed as means $\pm$ SEM (**A**, **B**, **D**, and **E**). $n = 5$ animals in each group (**A** and **B**); $n = 3$ animals (**D** and **E**). * $P < 0.05$ versus D0.

Figure 5 Roundabout guidance receptor 2 (ROBO2) expression is up-regulated in rat passive Heymann nephritis (PHN) kidney. **A–C:** ROBO2 and nephlin colocalization immunofluorescence staining shows specific glomerular podocyte expression in adult rat glomeruli. **D:** Experimental design for passive Heymann nephritis rat model. Anti-Fx1a and vehicle were injected in rats via tail vein. Urine samples were collected at the indicated days (green arrowheads). Rats were sacrificed at day 16 after anti-Fx1a or vehicle injection, and kidney samples were collected for analysis. **E:** Urine albumin/creatinine ratio (UACR) in the PHN (anti-Fx1a-injected) and vehicle (sterile 0.9% NaCl buffered saline-injected) rats. At least five different regions in each glomerulus and three glomeruli per animal were analyzed. **Arrows** indicate foot processes; **asterisk**, foot process effacement; **arrowheads**, subepithelial immune complex deposition. **H and I:** Robo2 immunofluorescence staining in the glomeruli of control (vehicle-injected) and PHN (anti-Fx1a-injected) rats. **J:** Quantitative RT-PCR assay showed increased *Robo2* mRNA expression in kidneys of rats intravenously injected with 1.5 mL/kg anti-Fx1a (PHN) compared with that of vehicle-injected (control) rats. **K:** Western blot analysis showed increased levels of Robo2 protein in kidneys of rats intravenously injected with 1.5 mL/kg anti-Fx1a (PHN) compared with that of vehicle-injected (control) rats. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a loading control. **L:** Quantification of Western blot results in **K**. Data are expressed as means $\pm$ SEM (**J** and **L**). $n = 4$ animals for each group (**J** and **L**). * $P < 0.05$, ** $P < 0.01$ versus control. Original magnification: $\times 200$ (**A–C**, **H**, and **I**); $\times 25,000$ (**F** and **G**).

immune-mediated glomerular injury, kidney biopsy samples were examined from patients diagnosed with MN, an antibody-mediated glomerular immune complex deposition disease similar to PHN in rats.⁶⁴ First, the ROBO2 localization was examined in normal human kidney tissues. It was found that they displayed similar expression patterns as in mice and rats (Supplemental Figure S4, A and B). As expected, ROBO2 in the glomeruli of cynomolgus monkey also formed the same expression pattern (Supplemental Figure S4, C and D), confirming evolutionary conservation of ROBO2 expression in the glomeruli of both rodents and primates. IF staining was then performed to evaluate the level of ROBO2 in frozen human kidney biopsy samples from three MN patients. Frozen kidney biopsy samples from three patients with thin basement membrane disease (a non-antibody-mediated glomerular basement membrane disease) were used as controls. ROBO2 is expressed at relatively low level in adult podocytes in thin basement membrane disease patients (Figure 6A). By contrast,

ROBO2 levels are higher in adult podocytes of membranous nephropathy kidneys (Figure 6B). By costaining ROBO2 and the podocyte-specific protein podocin, it was confirmed that ROBO2 expression is up-regulated in MN patient podocytes (Figure 6C). The up-regulation of ROBO2 expression was further confirmed by immunohistochemistry on formalin-fixed, paraffin-embedded kidney biopsy samples after antigen retrieval (Supplemental Figure S5, A and B). By measuring ROBO2-positive area in the podocyte (Supplemental Figure S5C), a significant increase of ROBO2 levels was found in the MN kidneys compared with control samples (2.17 ± 0.14 versus 0.45 ± 0.06 ratio of ROBO2-positive area/cell nuclear area; $P = 0.013$) (Supplemental Figure S5D). Taken together, these data suggest that ROBO2 expression is up-regulated in the kidney glomeruli of MN patients, which is consistent with the observations in the NTS-injured mice and rat PHN models described above. Interestingly, ROBO2 expression is also up-regulated in frozen kidney biopsy samples from patients

Figure 6 Roundabout guidance receptor 2 (ROBO2) expression in human frozen kidney biopsy samples from control and membranous nephropathy (MN) patients. **A:** Representative images of ROBO2 immunofluorescence staining in control frozen kidney biopsy samples. Subjects 1 to 3 are control kidneys with thin basement membrane disease (TBMD; a non—antibody-mediated glomerular basement membrane disease). A secondary (2nd) antibody only image without primary anti-ROBO2 antibody on TBMD biopsy sample showing background fluorescence signal. **B:** Representative images of ROBO2 immunofluorescence staining in frozen human MN kidney biopsy samples. Subjects 4 to 6 are diagnosed with MN (an autoantibody-mediated podocytopathy). A 2nd antibody only image without primary anti-ROBO2 antibody on MN biopsy sample showing background fluorescence signal. **C:** Representative images of ROBO2 and podocin coexpression in human MN confirm ROBO2 expression is up-regulated in MN patient podocytes. The podocin signal is relatively weak because of podocyte injury in MN. Original magnifications, $\times 200$ (**A** and **B**); $\times 400$ (**C**).

with primary FSGS, a different podocytopathy (Supplemental Figure S6).

ROBO2 Overexpression Enhances Podocyte Detachment on SLIT2N-Coated Cell Culture Plates

In a previously generated stable ROBO2-overexpressing mouse podocyte cell line, the addition of SLIT2N (functional recombinant SLIT2 N terminus) in the culture media can reduce podocyte adhesion to collagen I-coated plates.³¹ Loss of podocyte adhesion and podocyte depletion is a central mechanism of irreversible damage in many glomerular diseases.⁶⁵ Given these findings of ROBO2 up-regulation in animal models of podocyte injury and human podocytopathies, it was sought to determine if ROBO2 overexpression alone can affect podocyte adhesion on SLIT2N-coated culture plates in a dose-dependent manner; ROBO2-overexpressing podocytes and control podocytes were seeded onto culture plates coated with 0, 40, and 80 nmol/L SLIT2N and cultured them for 24 hours. The

numbers of podocytes remaining on the culture plates were then quantified after removing the nonadherent podocytes. Although there were similar numbers of ROBO2-overexpressing podocytes and control podocytes on the culture plates without SLIT2N coating, significant fewer ROBO2-overexpressing podocytes were found on SLIT2N-coated culture plates at both 40 and 80 nmol/L concentration compared with wild-type control podocytes (Figure 7, A and B). In an analysis of podocyte F-actin cytoskeleton and podocyte surface area, it was found that ROBO2 overexpression causes significant reduction of podocyte spreading and F-actin intensity (Figure 7, C–E). These data suggest that ROBO2 up-regulation in podocytes can reduce podocyte adhesion and enhance podocyte detachment in the presence of its ligand SLIT2.

Discussion

Podocytes are the last layer of the glomerular filtration barrier preventing plasma proteins, such as albumin, from

Figure 7 Overexpression of roundabout guidance receptor 2 (ROBO2) enhances podocyte detachment. **A:** Representative images of control and ROBO2 overexpressed mouse podocytes remaining on the cell culture plates coated with 0, 40, and 80 nmol/L recombinant slit guidance ligand 2 (SLIT2) after 24-hour culture. **B:** Podocyte quantification of **A**, showing lower ROBO2 overexpressed mouse podocytes remaining on the cell culture plates coated with recombinant SLIT2. **C:** Representative images of vector control and ROBO2 overexpressed single podocyte stained with phalloidin (green) to label F-actin cytoskeleton at cell culture condition with different SLIT2 concentrations, as in **A**. **D** and **E:** Single-cell area/spreading (**D**) and single-cell F-actin intensity (**E**) were measured and quantified using ImageJ software version 1.47v. * $P < 0.05$ versus control; † $P < 0.05$ versus vector. Scale bar = 1 mm (**A**). Original magnification, $\times 400$ (**C**).

leaking into the urinary space from glomerular capillaries. Because of their physical location and terminally differentiated status, podocytes are particularly vulnerable to injury and detachment/loss, which can lead to a leaky glomerular filtration barrier, significant albuminuria, and glomerulosclerosis. Many studies have shown that podocyte injury and eventual detachment/loss is a common pathway in

glomerular diseases of diverse etiology, including diabetic nephropathy,^{66,67} Alport syndrome,^{68,69} transplant glomerulopathy,^{70–72} and aging kidney.^{68,73} To maintain the integrity of the glomerular filtration barrier, podocytes are capable of remarkable foot process structure remodeling in adaptation to injurious signals, manifested as increased foot process width known as podocyte foot process effacement

and accompanying reduced slit-diaphragm density.^{4,9,38} Maintaining podocyte foot process width and slit-diaphragm density is beneficial for the podocyte and is associated with good clinical outcomes in many glomerular kidney diseases and experimental models.^{7,15,16,40} This study analyzed adult *Robo2* podocyte-specific knockout mice and wild-type control mice after two different podocyte injury animal models induced by either PS perfusion or NTS injection. Significantly less changes were observed to foot process width and slit-diaphragm density in *Robo2* cKO mice compared with wild-type controls in both models (Figure 1, C and D, and Figure 3, C and D). These results support the conclusion that loss of ROBO2 protects kidney from glomerular injury by maintaining podocyte ultrastructure.

Podocyte adhesion is crucial for normal foot process structure and glomerular filtration barrier function.^{2,74–76} Both positive and negative signals of actin cytoskeleton regulators at podocyte slit diaphragms and focal adhesions are required to maintain podocyte adhesion and morphology under physiological and disease conditions.^{2,74–76} The balance between these positive (eg, nephrin and nonmuscle myosin IIA) and negative (eg, ROBO2) actin cytoskeleton regulators and focal adhesion components in podocytes is essential to maintain podocyte adhesion and normal foot process structure.^{25,31,77} Previous studies show that ROBO2 signaling functions as a negative regulator of nephrin-induced actin polymerization²⁵ and podocyte adhesion,³¹

and enhanced ROBO2 signaling can destabilize podocyte adhesion.³¹ Consistent with these reports, the current study shows that ROBO2 expression is up-regulated in wild-type mice during podocyte injury after NTS injection. As high ROBO2 expression levels in wild-type mice are expected to enhance ROBO2 signaling and reduce podocyte adhesion, this may force podocytes to change their morphology (eg, increasing foot process width and reducing slit-diaphragm density) to maintain attachment of podocytes to the GBM during injury (Figure 8A). In *Robo2* cKO mice, however, loss of Robo2 enhances nephrin-induced actin polymerization and promotes podocyte adhesion to the GBM,^{25,31} which may protect podocytes from significant morphologic damage during podocyte injury (Figure 8B). Consistent with this finding, a moderately increased foot process width and microalbuminuria phenotypes have been detected in *Robo2* cKO mice without podocyte injury.²⁵ These studies thus provide insights to the molecular and cellular mechanisms for ROBO2 in regulating podocyte adhesion and plasticity under physiological and pathologic conditions (Figure 8).

This study also confirmed that ROBO2 expression in podocytes is evolutionarily conserved among different mammalian species, including mice, rats, cynomolgus monkeys, and humans. In addition to mouse NTS model, up-regulation of ROBO2 expression was also found in the glomeruli of rat model of PHN and kidney biopsy samples from patients with MN and primary FSGS. However, the

Figure 8 Proposed model: Loss of roundabout guidance receptor 2 (*Robo2*) in podocytes reduces podocyte injury by enhanced podocyte adhesion that maintains foot process (FP) structure. **A:** In wild-type (WT) mice, podocyte injury leads to up-regulation of ROBO2 expression, which reduces podocyte adhesion with severe foot process effacement, increased foot process width, reduced slit-diaphragm (SD) density, and high proteinuria. **B:** In *Robo2* conditional knockout (cKO) mice, loss of *Robo2* enhances actin polymerization and podocyte adhesion, which cause slightly broad foot process structure initially in *Robo2* cKO. During podocyte injury, however, enhanced actin polymerization and podocyte adhesion in *Robo2* cKO prevents further foot process effacement with preserved podocyte ultrastructure and less proteinuria. GBM, glomerular basement membrane.

mechanism of up-regulation of ROBO2 expression after podocyte injury is little understood. One possibility is that ROBO2 expression is closely linked to nephrin expression in the podocyte during injury and recovery phase. Previous studies show that both nephrin and ROBO2 expression is regulated by WT1, a podocyte-specific transcription factor.⁷⁸ Many published reports also show that nephrin is crucial in maintaining podocyte structure and function.^{26,27} Loss or down-regulation of nephrin has been extensively documented in animal models of podocyte injury and human proteinuric diseases.^{57–59,79–81} During the injury phase, podocytes down-regulate nephrin expression, which is correlated with foot process effacement and a proteinuric phenotype.⁵⁶ During the podocyte injury recovery phase (eg, by treatment), however, nephrin expression is often up-regulated,⁸² which may enhance podocyte actin polymerization and podocyte adhesion to restore podocyte foot process structure and slit-diaphragm density. It was previously demonstrated that ROBO2 complexes with nephrin through the adaptor protein NCK.²⁵ ROBO2 signaling acts as a negative regulator on nephrin-induced actin polymerization to decrease the F-actin cytoskeleton and podocyte adhesion in podocytes, and loss of *Robo2* alleviates podocyte foot process defects in nephrin null mice.²⁵ Increased nephrin expression during podocyte recovery phase could potentially stimulate ROBO2 expression through common transcriptional factors (eg, WT1)⁷⁸ to maintain the balance of positive (eg, nephrin) and negative (eg, ROBO2) signaling in regulating actin dynamics in podocytes. Consistent with this possibility, down-regulation of both nephrin and *Robo2* expression was observed during podocyte injury phase 1 day after NTS injection, but subsequently up-regulation of both mRNA and protein expression of nephrin and *Robo2* in the recovery phase at day 7 after NTS injury (Figure 4).

Similar to mouse NTS injury, both PHN rats and MN patients are characterized by direct antibody-mediated podocyte injury and localized formation of immune complexes. It is hypothesized that the up-regulation of ROBO2 expression in PHN rats and MN patients may also reflect a ROBO2-specific activity during podocyte injury recovery phase that often occurs in antibody- and immune complex-mediated glomerular diseases. For example, MN patients often have spontaneous remission during the course of their disease,^{83,84} which may suggest a recovery phase when ROBO2 expression is up-regulated. Interestingly, ROBO2 protein expression is up-regulated in the glomeruli of frozen kidney biopsy samples from patients with primary FSGS. Further studies are needed to confirm this finding and to determine if ROBO2 signaling is also enhanced in other podocytopathies, such as minimal change disease and genetic or secondary forms of FSGS, which involve podocyte injury in the absence of immune complex formation and immune-mediated injury.

In summary, this study demonstrates that loss of ROBO2 in podocytes protects adult mice from two forms of

podocyte injury (ie, PS perfusion and NTS injection) by maintaining foot process structure and slit-diaphragm density. ROBO2 expression is up-regulated in the glomeruli after NTS injury in mice, PHN model in rats, and human membranous nephropathy and primary FSGS biopsy samples; and ROBO2 overexpression in podocytes results in reduced podocyte adhesion, cell spreading, and podocyte F-actin intensity. These data suggest that ROBO2 signaling plays a deleterious role in a specific subset of chronic kidney diseases characterized by significant podocytopathy (eg, membranous nephropathy and primary FSGS). As ROBO2 podocyte expression is well conserved among different mammalian species, blocking ROBO2 signaling pharmacologically might enhance podocyte adhesion, preserve podocyte foot process structure, and maintain glomerular filtration barrier function, which, in turn, may reduce proteinuria and slow kidney function decline in patients or animals with podocytopathies.

Acknowledgments

We thank Dr. Janet Buhlmann for help with ROBO2-overexpressing cells; Joseph Tashjian for kindly providing focal segmental glomerulosclerosis biopsy samples; Dr. Jochen Reiser for kindly providing podocyte cell lines; Dr. Herbert Cohen for advice and discussion; Stefanie Chan, John Kreeger, Lindsay Tomlinson, Cedo Bagi, and Catherine Andresen for technical support; and Lily Lu for help with artwork in Figure 8.

Author Contributions

A.P.-H. and W.L. conceived and designed the experiments; A.P.-H., X.F., S.K., H.M.R., H.C., K.C., C.T.B., D.H.-S., S.R.A., H.Y., and R.G.B. performed the experiments and acquired the data; A.P.-H., X.F., S.K., R.S., R.G.B., and W.L. analyzed the data; L.H.B., J.M.H., S.P.B., D.J.S., and W.L. provided reagents/materials/analysis tools; A.P.-H. and W.L. wrote the manuscript; all authors reviewed the manuscript.

Supplemental Data

Supplemental material for this article can be found at <http://doi.org/10.1016/j.ajpath.2019.12.009>.

References

1. Faul C, Asanuma K, Yanagida-Asanuma E, Kim K, Mundel P: Actin up: regulation of podocyte structure and function by components of the actin cytoskeleton. *Trends Cell Biol* 2007, 17:428–437
2. Perico L, Conti S, Benigni A, Remuzzi G: Podocyte-actin dynamics in health and disease. *Nat Rev Nephrol* 2016, 12:692–710
3. Teng B, Lukasz A, Schiffer M: The ADF/cofilin-pathway and actin dynamics in podocyte injury. *Int J Cell Biol* 2012, 2012:320531

4. Mundel P, Shankland SJ: Podocyte biology and response to injury. *J Am Soc Nephrol* 2002, 13:3005–3015
5. Endlich N, Kress KR, Reiser J, Uttenweiler D, Kriz W, Mundel P, Endlich K: Podocytes respond to mechanical stress in vitro. *J Am Soc Nephrol* 2001, 12:413–422
6. Brahler S, Yu H, Suleiman H, Krishnan GM, Saunders BT, Kopp JB, Miner JH, Zinselmeyer BH, Shaw AS: Intravital and kidney slice imaging of podocyte membrane dynamics. *J Am Soc Nephrol* 2016, 27:3285–3290
7. George B, Verma R, Soofi AA, Garg P, Zhang J, Park TJ, Giardino L, Ryzhova L, Johnstone DB, Wong H, Nihalani D, Salant DJ, Hanks SK, Curran T, Rastaldi MP, Holzman LB: Crkl/2-dependent signaling is necessary for podocyte foot process spreading in mouse models of glomerular disease. *J Clin Invest* 2012, 122:674–692
8. Bertani T, Poggi A, Pozzoni R, Delaini F, Sacchi G, Thoua Y, Mecca G, Remuzzi G, Donati MB: Adriamycin-induced nephrotic syndrome in rats: sequence of pathologic events. *Lab Invest* 1982, 46:16–23
9. Kriz W, Shirato I, Nagata M, LeHir M, Lemley KV: The podocyte's response to stress: the enigma of foot process effacement. *Am J Physiol Renal Physiol* 2013, 304:F333–F347
10. Garg P, Verma R, Cook L, Soofi A, Venkatarreddy M, George B, Mizuno K, Gurniak C, Witke W, Holzman LB: Actin-depolymerizing factor cofilin-1 is necessary in maintaining mature podocyte architecture. *J Biol Chem* 2010, 285:22676–22688
11. Tondel C, Kanai T, Larsen KK, Ito S, Politei JM, Warnock DG, Svarstad E: Foot process effacement is an early marker of nephropathy in young classic Fabry patients without albuminuria. *Nephron* 2015, 129:16–21
12. Kfoury H: Epithelial cell foot process effacement in podocytes in focal and segmental glomerulosclerosis: a quantitative analysis. *Ultrastruct Pathol* 2014, 38:303–308
13. Chang JW, Pardo V, Sageshima J, Chen L, Tsai HL, Reiser J, Wei C, Ciano G, Burke GW 3rd, Fornoni A: Podocyte foot process effacement in postreperfusion allograft biopsies correlates with early recurrence of proteinuria in focal segmental glomerulosclerosis. *Transplantation* 2012, 93:1238–1244
14. Tewari R, Nada R, Rayat CS, Boruah D, Dudeja P, Joshi K, Sakhuja V: Correlation of proteinuria with podocyte foot process effacement in IgA nephropathy: an ultrastructural study. *Ultrastruct Pathol* 2015, 39:147–151
15. Wiggins RC: The spectrum of podocytopathies: a unifying view of glomerular diseases. *Kidney Int* 2007, 71:1205–1214
16. Ichinose K, Kitamura M, Sato S, Fujikawa K, Horai Y, Matsuoka N, Tsuboi M, Nonaka F, Shimizu T, Fukui S, Umeda M, Koga T, Kawashiri SY, Iwamoto N, Igawa T, Tamai M, Nakamura H, Origuchi T, Nishino T, Kawakami A: Podocyte foot process width is a prediction marker for complete renal response at 6 and 12 months after induction therapy in lupus nephritis. *Clin Immunol* 2018, 197:161–168
17. Blockus H, Chedotal A: Slit-Robo signaling. *Development* 2016, 143:3037–3044
18. Dickson BJ, Gilestro GF: Regulation of commissural axon pathfinding by slit and its Robo receptors. *Annu Rev Cell Dev Biol* 2006, 22:651–675
19. Ypsilanti AR, Zagar Y, Chedotal A: Moving away from the midline: new developments for Slit and Robo. *Development* 2010, 137:1939–1952
20. Wainwright EN, Wilhelm D, Combes AN, Little MH, Koopman P: ROBO2 restricts the nephrogenic field and regulates Wolffian duct-nephrogenic cord separation. *Dev Biol* 2015, 404:88–102
21. Grieshammer U, Le M, Plump AS, Wang F, Tessier-Lavigne M, Martin GR: SLIT2-mediated ROBO2 signaling restricts kidney induction to a single site. *Dev Cell* 2004, 6:709–717
22. Bertoli-Avella AM, Conte ML, Punzo F, de Graaf BM, Lama G, La Manna A, Polito C, Grassia C, Nobili B, Rambaldi PF, Oostra BA, Perrotta S: ROBO2 gene variants are associated with familial vesicoureteral reflux. *J Am Soc Nephrol* 2008, 19:825–831
23. Lu W, van Eerde AM, Fan X, Quintero-Rivera F, Kulkarni S, Ferguson HL, Kim H, Fan Y, Xi Q, Li QG, Sanlaville D, Andrews W, Sundaresan V, Bi W, Yan J, Giltay JC, Wijmenga C, de Jong TPVM, Feather S, Woolf A, Rao Y, Lupski JR, Eccles MR, Quade BJ, Gusella JF, Morton CC, Maas RL: Disruption of ROBO2 is associated with urinary tract anomalies and confers risk of vesicoureteral reflux. *Am J Hum Genet* 2007, 80:616–632
24. Wang H, Li Q, Liu J, Mendelsohn C, Salant DJ, Lu W: Noninvasive assessment of antenatal hydronephrosis in mice reveals a critical role for Robo2 in maintaining anti-reflux mechanism. *PLoS One* 2011, 6:e24763
25. Fan X, Li Q, Pisarek-Horowitz A, Rasouly HM, Wang X, Bonegio RG, Wang H, McLaughlin M, Mangos S, Kalluri R, Holzman LB, Drummond IA, Brown D, Salant DJ, Lu W: Inhibitory effects of Robo2 on nephrin: a crosstalk between positive and negative signals regulating podocyte structure. *Cell Rep* 2012, 2:52–61
26. Welsh GI, Saleem MA: Nephrin: signature molecule of the glomerular podocyte? *J Pathol* 2010, 220:328–337
27. Patrakka J, Tryggvason K: Nephrin: a unique structural and signaling protein of the kidney filter. *Trends Mol Med* 2007, 13:396–403
28. New LA, Martin CE, Scott RP, Platt MJ, Keyvani Chahi A, Stringer CD, Lu P, Samborska B, Eremina V, Takano T, Simpson JA, Quaggin SE, Jones N: Nephrin tyrosine phosphorylation is required to stabilize and restore podocyte foot process architecture. *J Am Soc Nephrol* 2016, 27:2422–2435
29. Lahdenpera J, Kilpelainen P, Liu XL, Pikkariainen T, Reponen P, Ruotsalainen V, Tryggvason K: Clustering-induced tyrosine phosphorylation of nephrin by Src family kinases. *Kidney Int* 2003, 64:404–413
30. Verma R, Kovari I, Soofi A, Nihalani D, Patrie K, Holzman LB: Nephrin ectodomain engagement results in Src kinase activation, nephrin phosphorylation, Nck recruitment, and actin polymerization. *J Clin Invest* 2006, 116:1346–1359
31. Fan X, Yang H, Kumar S, Tumelty KE, Pisarek-Horowitz A, Rasouly HM, Sharma R, Chan S, Tyminski E, Shamashkin M, Belghasem M, Henderson JM, Coyle AJ, Salant DJ, Berasi SP, Lu W: SLIT2/ROBO2 signaling pathway inhibits nonmuscle myosin IIA activity and destabilizes kidney podocyte adhesion. *JCI Insight* 2016, 1:e86934
32. Salant DJ, Belok S, Madaio MP, Couser WG: A new role for complement in experimental membranous nephropathy in rats. *J Clin Invest* 1980, 66:1339–1350
33. O'Meara YM, Natori Y, Minto AW, Goldstein DJ, Manning EC, Salant DJ: Nephrotoxic antiserum identifies a beta 1-integrin on rat glomerular epithelial cells. *Am J Physiol* 1992, 262:F1083–F1091
34. Salant DJ, Cybulsky AV: Experimental glomerulonephritis. *Methods Enzymol* 1988, 162:421–461
35. Kurien BT, Scofield RH: Mouse urine collection using clear plastic wrap. *Lab Anim* 1999, 33:83–86
36. Friedman PL, Ellisman MH: Enhanced visualization of peripheral nerve and sensory receptors in the scanning electron microscope using cryofracture and osmium-thiocarbohydrazide-osmium impregnation. *J Neurocytol* 1981, 10:111–131
37. Graham L, Orenstein JM: Processing tissue and cells for transmission electron microscopy in diagnostic pathology and research. *Nat Protoc* 2007, 2:2439–2450
38. Deegens JK, Dijkman HB, Borm GF, Steenbergen EJ, van den Berg JG, Weening JJ, Wetzels JF: Podocyte foot process effacement as a diagnostic tool in focal segmental glomerulosclerosis. *Kidney Int* 2008, 74:1568–1576
39. Inoki K, Mori H, Wang J, Suzuki T, Hong S, Yoshida S, Blattner SM, Ikenoue T, Ruegg MA, Hall MN, Kwiatkowski DJ, Rastaldi MP, Huber TB, Kretzler M, Holzman LB, Wiggins RC, Guan KL: mTORC1 activation in podocytes is a critical step in the development of diabetic nephropathy in mice. *J Clin Invest* 2011, 121:2181–2196

40. Veron D, Reidy KJ, Bertuccio C, Teichman J, Villegas G, Jimenez J, Shen W, Kopp JB, Thomas DB, Tufro A: Overexpression of VEGF-A in podocytes of adult mice causes glomerular disease. *Kidney Int* 2010, 77:989–999
41. Lahdenkari AT, Lounatmaa K, Patrakka J, Holmberg C, Wartiovaara J, Kestila M, Koskimies O, Jalanko H: Podocytes are firmly attached to glomerular basement membrane in kidneys with heavy proteinuria. *J Am Soc Nephrol* 2004, 15:2611–2618
42. Shea SM, Morrison AB: A stereological study of the glomerular filter in the rat: morphometry of the slit diaphragm and basement membrane. *J Cell Biol* 1975, 67:436–443
43. Messina A, Davies DJ, Ryan GB: Protamine sulphate-induced proteinuria: the roles of glomerular injury and depletion of polyanion. *J Pathol* 1989, 158:147–156
44. Asanuma K, Kim K, Oh J, Giardino L, Chabanis S, Faul C, Reiser J, Mundel P: Synaptopodin regulates the actin-bundling activity of alpha-actinin in an isoform-specific manner. *J Clin Invest* 2005, 115: 1188–1198
45. Rudiger F, Greger R, Nitschke R, Henger A, Mundel P, Pavenstadt H: Polycations induce calcium signaling in glomerular podocytes. *Kidney Int* 1999, 56:1700–1709
46. Subramanian B, Sun H, Yan P, Charoonratana VT, Higgs HN, Wang F, Lai KV, Valenzuela DM, Brown EJ, Schlondorff JS, Pollak MR: Mice with mutant *Inf2* show impaired podocyte and slit diaphragm integrity in response to protamine-induced kidney injury. *Kidney Int* 2016, 90:363–372
47. Takeda T, McQuistan T, Orlando RA, Farquhar MG: Loss of glomerular foot processes is associated with uncoupling of podocalyxin from the actin cytoskeleton. *J Clin Invest* 2001, 108: 289–301
48. Kerjaschki D: Polycation-induced dislocation of slit diaphragms and formation of cell junctions in rat kidney glomeruli: the effects of low temperature, divalent cations, colchicine, and cytochalasin B. *Lab Invest* 1978, 39:430–440
49. Kojima K, Davidovits A, Poczewski H, Langer B, Uchida S, Nagy-Bojarski K, Hovorka A, Sedivy R, Kerjaschki D: Podocyte flattening and disorder of glomerular basement membrane are associated with splitting of dystroglycan-matrix interaction. *J Am Soc Nephrol* 2004, 15:2079–2089
50. Hinamoto N, Maeshima Y, Yamasaki H, Nasu T, Saito D, Watatani H, Ujike H, Tanabe K, Masuda K, Arata Y, Sugiyama H, Sato Y, Makino H: Exacerbation of diabetic renal alterations in mice lacking vasohibin-1. *PLoS One* 2014, 9:e107934
51. Hoppe JM, Vielhauer V: Induction and analysis of nephrotoxic serum nephritis in mice. *Methods Mol Biol* 2014, 1169:159–174
52. Chugh S, Yuan H, Topham PS, Haydar SA, Mittal V, Taylor GA, Kalluri R, Salant DJ: Aminopeptidase A: a nephritogenic target antigen of nephrotoxic serum. *Kidney Int* 2001, 59:601–613
53. Lin F, Emancipator SN, Salant DJ, Medof ME: Decay-accelerating factor confers protection against complement-mediated podocyte injury in acute nephrotoxic nephritis. *Lab Invest* 2002, 82: 563–569
54. Menne J, Meier M, Park JK, Boehne M, Kirsch T, Lindschau C, Occhipka R, Leitges M, Rinta-Valkama J, Holthofer H, Haller H: Nephron loss in experimental diabetic nephropathy is prevented by deletion of protein kinase C alpha signaling in-vivo. *Kidney Int* 2006, 70:1456–1462
55. Matsusaka T, Sandgren E, Shintani A, Kon V, Pastan I, Fogo AB, Ichikawa I: Podocyte injury damages other podocytes. *J Am Soc Nephrol* 2011, 22:1275–1285
56. Yuan H, Takeuchi E, Taylor GA, McLaughlin M, Brown D, Salant DJ: Nephron dissociates from actin, and its expression is reduced in early experimental membranous nephropathy. *J Am Soc Nephrol* 2002, 13:946–956
57. Luimula P, Ahola H, Wang SX, Solin ML, Aaltonen P, Tikkanen I, Kerjaschki D, Holthofer H: Nephron in experimental glomerular disease. *Kidney Int* 2000, 58:1461–1468
58. Gagliardini E, Benigni A, Tomasoni S, Abbate M, Kalluri R, Remuzzi G: Targeted downregulation of extracellular nephrin in human IgA nephropathy. *Am J Nephrol* 2003, 23:277–286
59. Doublier S, Salvidio G, Lupia E, Ruotsalainen V, Verzola D, Deferrari G, Camussi G: Nephrin expression is reduced in human diabetic nephropathy: evidence for a distinct role for glycated albumin and angiotensin II. *Diabetes* 2003, 52:1023–1030
60. Wernerson A, Duner F, Pettersson E, Widholm SM, Berg U, Ruotsalainen V, Tryggvason K, Hultenby K, Soderberg M: Altered ultrastructural distribution of nephrin in minimal change nephrotic syndrome. *Nephrol Dial Transplant* 2003, 18:70–76
61. Koop K, Eikmans M, Baelde HJ, Kawachi H, De Heer E, Paul LC, Bruijn JA: Expression of podocyte-associated molecules in acquired human kidney diseases. *J Am Soc Nephrol* 2003, 14:2063–2071
62. Cybulsky AV, Quigg RJ, Salant DJ: Experimental membranous nephropathy redux. *Am J Physiol Renal Physiol* 2005, 289:F660–F671
63. Salant DJ, Quigg RJ, Cybulsky AV: Heymann nephritis: mechanisms of renal injury. *Kidney Int* 1989, 35:976–984
64. Beck LH Jr, Salant DJ: Membranous nephropathy: from models to man. *J Clin Invest* 2014, 124:2307–2314
65. Fukuda A, Wickman LT, Venkatarreddy MP, Sato Y, Chowdhury MA, Wang SQ, Shedden KA, Dysko RC, Wiggins JE, Wiggins RC: Angiotensin II-dependent persistent podocyte loss from destabilized glomeruli causes progression of end stage kidney disease. *Kidney Int* 2012, 81:40–55
66. Toyoda M, Najafian B, Kim Y, Caramori ML, Mauer M: Podocyte detachment and reduced glomerular capillary endothelial fenestration in human type 1 diabetic nephropathy. *Diabetes* 2007, 56:2155–2160
67. Weil EJ, Lemley KV, Yee B, Lovato T, Richardson M, Myers BD, Nelson RG: Podocyte detachment in type 2 diabetic nephropathy. *Am J Nephrol* 2011, 33 Suppl 1:21–24
68. Ding F, Wickman L, Wang SQ, Zhang Y, Wang F, Afshinnia F, Hodgin J, Ding J, Wiggins RC: Accelerated podocyte detachment and progressive podocyte loss from glomeruli with age in Alport syndrome. *Kidney Int* 2017, 92:1515–1525
69. Wickman L, Hodgin JB, Wang SQ, Afshinnia F, Kershaw D, Wiggins RC: Podocyte depletion in thin GBM and Alport syndrome. *PLoS One* 2016, 11:e0155255
70. Yang Y, Hodgin JB, Afshinnia F, Wang SQ, Wickman L, Chowdhury M, Nishizono R, Kikuchi M, Huang Y, Samaniego M, Wiggins RC: The two kidney to one kidney transition and transplant glomerulopathy: a podocyte perspective. *J Am Soc Nephrol* 2015, 26: 1450–1465
71. Haas M: Transplant glomerulopathy: the view from the other side of the basement membrane. *J Am Soc Nephrol* 2015, 26:1235–1237
72. Naik AS, Afshinnia F, Aqeel J, Cibrik DM, Samaniego M, Wickman L, Wang SQ, Chowdhury M, Wiggins RC: Accelerated podocyte detachment early after kidney transplantation is related to long-term allograft loss of function. *Nephrol Dial Transplant* 2018, 34:1232–1239
73. Hodgin JB, Bitzer M, Wickman L, Afshinnia F, Wang SQ, O'Connor C, Yang Y, Meadowbrooke C, Chowdhury M, Kikuchi M, Wiggins JE, Wiggins RC: Glomerular aging and focal global glomerulosclerosis: a podometric perspective. *J Am Soc Nephrol* 2015, 26:3162–3178
74. Lennon R, Randles MJ, Humphries MJ: The importance of podocyte adhesion for a healthy glomerulus. *Front Endocrinol* 2014, 5:160
75. Sachs N, Sonnenberg A: Cell-matrix adhesion of podocytes in physiology and disease. *Nat Rev Nephrol* 2013, 9:200–210
76. Sever S, Schiffer M: Actin dynamics at focal adhesions: a common endpoint and putative therapeutic target for proteinuric kidney diseases. *Kidney Int* 2018, 93:1298–1307
77. Abdelhak S, Kalatzis V, Heilig R, Compain S, Samson D, Vincent C, Weil D, Cruaud C, Sahly I, Leibovici M, Bitner-Glindzic M, Francis M, Lacombe D, Vigneron J, Charachon R, Boven K, Bedbeter P, Van Regemorter N, Weissenbach J, Petit C: A human homologue of the *Drosophila* eyes absent gene underlies branchio-

- oto-renal (BOR) syndrome and identifies a novel gene family. *Nat Genet* 1997, 15:157–164
78. Lefebvre J, Clarkson M, Massa F, Bradford ST, Charlet A, Buske F, Lacas-Gervais S, Schulz H, Gimpel C, Hata Y, Schaefer F, Schedl A: Alternatively spliced isoforms of WT1 control podocyte-specific gene expression. *Kidney Int* 2015, 88:321–331
 79. Li X, Chuang PY, D'Agati VD, Dai Y, Yacoub R, Fu J, Xu J, Taku O, Premrurit PK, Holzman LB, He JC: Nephin preserves podocyte viability and glomerular structure and function in adult kidneys. *J Am Soc Nephrol* 2015, 26:2361–2377
 80. Kandasamy Y, Smith R, Lumbers ER, Rudd D: Nephin: a biomarker of early glomerular injury. *Biomark Res* 2014, 2:21
 81. Kim BK, Hong HK, Kim JH, Lee HS: Differential expression of nephin in acquired human proteinuric diseases. *Am J Kidney Dis* 2002, 40:964–973
 82. Moysiadis DK, Perysinaki GS, Bertias G, Stratakis S, Kyriacou K, Nakopoulou L, Boumpas DT, Daphnis E: Early treatment with glucocorticoids or cyclophosphamide retains the slit diaphragm proteins nephin and podocin in experimental lupus nephritis. *Lupus* 2012, 21:1196–1207
 83. Polanco N, Gutierrez E, Covarsi A, Ariza F, Carreno A, Vigil A, Baltar J, Fernandez-Fresnedo G, Martin C, Pons S, Lorenzo D, Bernis C, Arrizabalaga P, Fernandez-Juarez G, Barrio V, Sierra M, Castellanos I, Espinosa M, Rivera F, Olier A, Fernandez-Vega F, Praga M; Grupo de Estudio de las Enfermedades Glomerulares de la Sociedad Espanola de Nefrologia: Spontaneous remission of nephrotic syndrome in idiopathic membranous nephropathy. *J Am Soc Nephrol* 2010, 21:697–704
 84. Polanco N, Gutierrez E, Rivera F, Castellanos I, Baltar J, Lorenzo D, Praga M; Grupo de Estudio de las Enfermedades Glomerulares de la Sociedad Espanola de Nefrologia: Spontaneous remission of nephrotic syndrome in membranous nephropathy with chronic renal impairment. *Nephrol Dial Transplant* 2012, 27:231–234