Podocin Inactivation in Mature Kidneys Causes Focal Segmental Glomerulosclerosis and Nephrotic Syndrome

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ABSTRACT
Podocin is a critical component of the glomerular slit diaphragm, and genetic mutations lead to both familial and sporadic forms of steroid-resistant nephrotic syndrome. In mice, constitutive absence of podocin leads to rapidly progressive renal disease characterized by mesangiolysis and/or mesangial sclerosis and nephrotic syndrome. Using established Cre-loxP technology, we inactivated podocin in the adult mouse kidney in a podocyte-specific manner. Progressive loss of podocin in the glomerulus recapitulated albuminuria, hypercholesterolemia, hypertension, and renal failure seen in nephrotic syndrome in humans. Lesions of FSGS appeared after 4 wk, with subsequent development of diffuse glomerulosclerosis and tubulointerstitial damage. Interestingly, conditional inactivation of podocin at birth resulted in a gradient of glomerular lesions, including mesangial proliferation, demonstrating a developmental stage dependence of renal histologic patterns of injury. The development of significant albuminuria in this model occurred only after early and focal foot process effacement had progressed to diffuse involvement, with complete absence of podocin immunolabeling at the slit diaphragm. Finally, we identified novel potential mediators and perturbed molecular pathways, including cellular proliferation, in the course of progression of renal disease leading to glomerulosclerosis, using global gene expression profiling.


FSGS is a clinicopathologic syndrome characterized by podocyte injury and progressive scarring in the renal glomerulus. Epidemiologic studies have shown an increasing incidence of FSGS in the United States,1,2 particularly among black individuals, making it the most common cause of ESRD as a result of primary glomerular diseases in both white and black individuals.2 Given the rising prevalence of chronic kidney disease,3 efforts to understand the pathophysiologic and genetic mechanisms leading to FSGS are crucial for developing strategies aimed at prevention and therapy.

Recent discoveries have established the genetic bases of some familial forms of FSGS,4–8 as well as the role of genes in enhancing susceptibility to glo-
merular disease. The NPHS2 gene, which encodes the slit diaphragm protein podocin, not only accounts for 43% of familial and 10% of sporadic forms of nephrotic syndrome, but also some genetic variants may increase the risk for glomerular disease. Podocin acts as a structural scaffold in podocyte foot processes and interacts with slit diaphragm proteins to facilitate cellular signaling events.

Previously, we described the phenotypic consequences in mice of constitutive absence of podocin or expression of a podocin missense mutant and identified genetic and environmental modifiers of the renal disease. Podocin mutant mice present with albuminuria at birth and develop lesions of mesangiolysis and mesangial sclerosis. Their early death (within the first few weeks of life, depending on the genetic background) precludes extensive study into the mechanisms of glomerular disease. We therefore generated a novel murine model in which podocin was inactivated in the adult mouse kidney in a podocyte-specific manner, using established Cre-loxP technology, and here detail the physiologic, ultrastructural, and transcriptional changes leading to FSGS in this model.

RESULTS

Generation of Podocyte-Specific Nphs2 Knockout Mice

To study the effects of podocin inactivation in the mature kidney, we generated triallelic Nphs2lox2/lox2,Cre+ mice, bearing a floxed Nphs2 exon 2 allele (Figure 1A), a null Nphs2 allele, and a podocyte-expressed, tamoxifen-responsive Cre recombinase transgene (Supplemental Figures S1 and S2). Mice bearing a null allele in the heterozygous state do not demonstrate glomerular disease and neither do Nphs2lox2/lox2 mice, even after 8 wk of follow-up (data not shown). Administration of tamoxifen to phenotypically normal 6-wk-old Nphs2lox2/lox2, Cre+ mice resulted in nuclear translocation of Cre recombinase in approximately 70% of podocytes and in excision of exon 2. A progressive decrease in podocin expression was seen as early as 7 d after Cre induction, both at the mRNA level (shown using real-time PCR; Figure 1B) and at the protein level (demonstrated by Western blotting [Figure 1C] and by quantification of fluorescence signal intensity of podocin in glomeruli [Figure 1, D and E]).

Podocin Loss Leads to Nephrotic Syndrome

Inactivation of podocin resulted in death of Nphs2lox2/lox2, Cre+ mice at a median time of 11 wk after the start of tamoxifen administration (Figure 2A). Albuminuria was detected on a Coomassie blue–stained protein gel (Figure 2B) after a mean of 10 d (range 8 to 13 d). This progressed to massive, nonselective proteinuria by 4 wk (Figure 2B). Tailcuff plethysmography measurements, carried out 4 wk after podocin inactivation, showed a modest but significant increase in the BP of null mice (Figure 2C). Finally, plasma levels of
Figure 2. Conditional Nphs2 knockout mice have various phenotypes. (A) Kaplan-Meier survival curve of control and Nphs2lox2/− mice upon Cre activation by tamoxifen shows median survival of 11 wk upon podocin loss. (B) Mice develop detectable albuminuria at 2 wk by Coomassie staining of 12% SDS-PAGE. This progresses to massive nonspecific proteinuria by 4 wk. (C) Tail-cuff plethysmography of Nphs2lox2/− mice 4 wk after Cre activation shows mild hypertension. Experiments were performed on 12 control and 12 Nphs2lox2/− mice. §P < 0.05 after two-tailed t test. (D) Elevations in serum levels of cholesterol were significantly elevated by 4 wk, whereas plasma urea and creatinine levels began to rise only at 6 wk (Figure 2D).

Mice Develop FSGS and Tubular Damage

Despite downregulation of podocin expression, the renal histology of Nphs2lox2/−,Cre+ mice at the light microscopic level 1 wk (data not shown) after induction of Cre recombinase activity was similar to those of control (Nphs2lox2/−,Cre− and Nphs2lox2/+,Cre+) mice (Figure 3A). After 2 wk, occasional podocyte hypertrophy was noted, with minimal mesangial matrix expansion in some glomeruli (Figure 3B). Four weeks after Cre induction, FSGS was observed in many glomeruli, with varying degrees of severity (Figure 3C). Glomerular pseudocrescents were observed in at least 30% of glomeruli beginning at 4 wk (Figure 3D).

Glomerulosclerosis continued to worsen by 6 wk, with increasing segmental involvement (Figure 3E). At or near the time of death, global sclerosis was evident in the majority of glomeruli (Figure 3F). Tubulointerstitial injury characterized by diffuse tubular dilation, tubular atrophy and necrosis, and the presence of proteinaceous casts (Figure 3, E and F) was consistently present only 6 wk after Cre recombinase induction. Mice that were killed after 9 wk demonstrated progression of tubular injury, with basement membrane thickening and interstitial fibrosis (Figure 3F).

Developmental Stage Effects on Renal Disease

Given the discrepancy between the renal phenotypes resulting from constitutive and conditional inactivation of podocin, we investigated the effects of Nphs2 inactivation at birth, during which nephrogenesis is not completed in mice. After administration of tamoxifen to nursing mothers for 3 d after birth, resulting in tamoxifen delivery via milk, albuminuria developed in Nphs2lox2/−, Cre− pups after 2 wk (data not shown). When massive proteinuria was present at 4 wk by Coomassie blue staining, mice were killed. The renal histology showed a gradient of lesions. Superficial cortical glomeruli, which were not present at the time of birth, were normal and podocin expression was intact (Figure 4, A and B). In midcortical and the majority of juxtamedullary glomeruli, in which Cre recombinase was active and podocin was diminished (Figure 4A), lesions of mesangial proliferation were observed (Figure 4B). In addition, in the most severely affected juxtamedullary glomeruli, podocin was absent (Figure 4A) and lesions of FSGS were present (Figure 4B).

Ultrastructural Studies

We performed serial ultrastructural studies in adult Nphs2lox2/−,Cre+ mice, after Cre induction, to understand the role of foot process effacement (FPE) in the development of proteinuria. Whereas foot processes in control mice were evenly spaced and separated by a slit diaphragm (Figure 5A), foot processes in Nphs2lox2/−,Cre+ mice were focally effaced at 1 and 2 wk after Cre induction (Figure 5B and C). Morphometric measurements revealed a near doubling of the mean foot process width as early as 1 wk (Figure 5E). The degree of FPE progressed to diffuse effacement 4 wk (Figure 5D) after tamoxifen administration. Progression of FPE corresponded to the worsening of albumin leak, eventually to massive, nonselective proteinuria. Contrary to the findings of others implicating podocyte detachment and subsequent glomerular basement membrane (GBM) denudation as a mechanism of glomerular sclerosis, ultrastructural studies did not reveal areas of denuded GBM in Nphs2lox2/−,Cre+ mice at any of the time points investigated.

Using immunogold electron microscopic labeling, we localized podocin at the slit diaphragm in controls (Figure 6A) and in areas where foot processes were maintained in Nphs2lox2/−, Cre+ mice at 1 and 2 wk (Figure 6B). No labeling was seen in parietal epithelial, endothelial, or mesangial cells. In some ar-
bas of focal effacement at 1 and 2 wk, gold particles were displaced within the cytoplasm of effaced podocytes, sometimes clustering along the plasma membrane facing the urinary space (Figure 6C). Only after 4 wk was podocin completely absent in diffusely effaced foot processes (Figure 6D).

Global Gene Expression Profiling of Mutant Glomeruli
To identify molecular pathways perturbed upon podocin loss, we performed global gene expression profiling of glomeruli isolated from mutant and control mice at weeks 1, 2, and 4. We validated microarray results using quantitative real-time PCR (Supplemental Figure S3; Supplemental Table S1) and revealed a complex array of transcriptional changes (Supplemental Table S2) and canonical pathway perturbations (Supplemental Table S3). Functional annotation, using the Ingenuity Pathway Analysis Program, showed early and significant perturbations of cell-cycle regulation and cellular proliferation pathways. Proliferation of resident cells in the glomerulus during the development of glomerulosclerosis has been inconsistently demonstrated in humans and animal models. In Nphs2<sup>lox2/lox</sup>, Cre<sup>+</sup> mice, immunostaining for Ki-67 identified a significant increase in the percentage of proliferating resident glomerular cells (Figure 7A), involving podocytes and endothelial and mesangial cells (Figure 7B). Immunostaining for CD133 and CD24, markers of progenitor cells, were negative, suggesting that proliferative cells were intrinsic to the glomerulus (data not shown).

DISCUSSION
Animal models of podocyte injury, using toxic agents, immunologic damage, renal ablation, gene targeting, and transgenesis or via expression of HIV component proteins, have offered tremendous insights into the pathophysiologic mechanisms leading to glomerulosclerosis. In addition, dysregulation of podocin expression was described in an experimental model of membranous nephropathy, in FSGS, and in other forms of acquired proteinuric diseases, therein highlighting its pivotal role in the pathogenesis of glomerular disease development.

The early demise of two previous mouse models of podocin inactivation and the presence of histologic lesions of mesangiolysis and mesangial sclerosis, which are not characteristic of human disease because of podocin mutations, impelled us to generate a model of podocin inactivation in the mature kidney. Using an established Cre deleter line, expression and nuclear translocation of Cre recombinase in approximately 70% of podocytes resulted in a 50% downregulation of podocin expression as early as 1 wk after induction. By 4 wk, podocin was nearly absent in all glomeruli, suggesting that podocin loss was secondarily achieved through a vicious cycle of damaged podocytes’ damaging neighboring podocytes. This may explain immunogold labeling studies showing displaced podocin in some focally effaced foot processes, reflecting podocytes in which Cre recombinase was not activated. Alternatively, these particles may represent residual podocin in the process of cytosolic degradation. Indeed, failure of podocin

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Figure 3. Renal lesions progress upon Nphs2 inactivation in the mature kidney. (A) Control mice show normal glomerular and tubular morphologies. (B) At 2 wk, there were no significant glomerular changes besides mild glomerular enlargement and occasional podocyte hypertrophy. (C) There is segmental accumulation of collagen (arrow) in many glomeruli 4 wk after Cre induction. (D) In at least 30% of glomeruli starting at 4 wk, pseudocrescents were observed. (E) There is progression of lesions of FSGS 6 wk later, accompanied by tubular dilation and protein casts. (F) At 9 wk, diffuse global sclerosis was present, along with marked tubular dilation, atrophy, and interstitial fibrosis. Bars = 50 µm. Slides were stained with periodic acid-Schiff stain. Magnification: ×400 in A, B, C, and E; ×1000 in D and F.
targeting to sites of action on the plasma membrane has been shown both in vitro and in vivo to result in loss of function.18,32,33

Podocyte injury in our model leads to progressive renal disease, ultimately recapitulating features of nephrotic syndrome in humans, such as hyperlipidemia, hypertension, and renal insufficiency. Heterogeneity between mutant mice in the evolution of renal disease may partly be due to individual differences resulting from their underlying mixed genetic background. Less phenotypic variability has been seen in ongoing studies on congenic mice (C.A., unpublished observations).

In contrast to our previously described podocin knockout model in which foot processes are effaced at birth,17 podocyte FPE evolved from focal to diffuse involvement with inactivation in the mature kidney; however, despite the presence of focally effaced foot processes at 1 wk, we found no significant albuminuria until the second week. The absence of albuminuria at 1 wk may reflect the limits of detection of our assay or, alternatively, may represent a time when the capacity of compensatory proximal tubular reabsorption of albumin has not yet been saturated. Studies to investigate the tubular mechanisms of albumin handling in these mice are ongoing.

Our findings contrast with models in which proteinuria has been described in the absence of FPE but are in agreement with two previous studies conducted in human samples36 and in the puromycin aminoglycoside nephrosis model37 showing absence of proteinuria despite extensive FPE. Finally, the appearance of massive, nonselective albuminuria coincided in our study with diffuse FPE, but we cannot exclude concomitant changes in GBM composition, although no gross abnormalities in the GBM were noted by electron microscopy.

Our results demonstrate that the evolution of renal phenotypes of mice in which podocin has been inactivated in the mature kidney is different from those of the previous Nphs2 models in which functional podocin was absent during kidney development. Rather than displaying lesions of mesangiosis

Figure 4. Inactivation at birth reveals developmental stage dependence of phenotypes. (A) At 4 wk of life, podocin expression (in red) demonstrated a gradient from the outer cortical (OC) to midcortical (MC) and to juxtamedullary (JM) glomeruli, marked with nidogen (in green). Because OC glomeruli are not formed at birth, podocin expression is intact, whereas MC glomeruli, which are immature at birth, have decreased podocin expression. At birth, JM glomeruli are mature and show near absence of podocin. (B) Histologic analysis showed normal OC glomeruli, glomerular hypertrophy and lesions of mesangial proliferation in MC glomeruli and lesions of FSGS in severely affected JM glomeruli. Slides were stained with periodic acid-Schiff. Magnification, ×400.

Figure 5. FPE develops upon podocin loss. (A) Ultrastructural studies in a control mouse revealed elaboration of podocytes (P) into regularly spaced foot processes (FP), on the opposite side of the GBM as the fenestrated endothelial cells (En) lining the capillary lumen (L). (B and C) At 1 (B) and 2 wk (C) after Cre induction, FPE was focal. (D) After 4 wk, foot processes were globally effaced. (E) Morphometric studies revealed progressive increase in foot process width over10(129,705),(676,846)
Intriguingly, we found that genes involved in cell-cycle regulation were the earliest genes to be transcriptionally activated in our model. Several other mouse models of glomerular disease failed to reveal a prominent role of proliferation of resident cells of the glomerulus, besides parietal epithelial cells.46–48 Conversely, we found proliferation not only of podocytes, as seen in models of HIV-associated nephropathy,49 but also of mesangial and endothelial cells. This may represent dedifferentiation of terminally differentiated cells in the glomerulus in response to podocyte injury in our model. It will be interesting to determine whether cellular proliferation is a compensatory or a deleterious event in glomerulosclerosis.

Conditional inactivation of podocin in the adult murine kidney represents a novel model system of nephrotic syndrome as a result of FSGS, thereby recapitulating human disease resulting from mutations in the podocin gene and providing a vehicle for better understanding key pathophysiologic mechanisms of glomerular disease development. Furthermore, it revealed important phenotypic differences from previous constitutive knockout models of podocin, therein highlighting the importance of conditional targeting of other genes implicated in nephrotic syndrome.

**CONCISE METHODS**

**Generation of Mice**

The Nphs2 mutant mouse line was established at the Mouse Clinical Institute–Institut Clinique de la Souris (http://www.mci.u-strasbg.fr; Illkirch, France). The mouse genome was modified to allow for exci-
Phenotypic Characterization
Survival was assessed by following a cohort of mice until death. Thereafter, five control and five null mice were killed at weeks 1, 2, 4, and 6 after tamoxifen administration. Blood was obtained via cardiac puncture, centrifuged at 5000 rpm for 5 min to obtain plasma, and blood urea nitrogen, creatinine, and cholesterol were measured using an Olympus AU-400 multiparametric analyzer. Time of onset of albuminuria was determined by daily collection of spontaneously voided urine after initiation of tamoxifen induction, and 2 μl of urine was examined on a 12% SDS-PAGE gel stained with Coomassie blue. One kidney was extracted and snap-frozen in liquid nitrogen for protein and RNA analysis. When podocin was inactivated at birth, pups were genotyped at day 5 and albuminuria was tested thereafter. Litters were killed at 4 wk and characterized.

Four weeks after initiation of tamoxifen administration, mice weighing 20 to 25 g were habituated to tail-cuff plethysmography over a 2-d period. Thereafter, repeated BP measurements (at least 10 per session per mouse) were obtained over 3 d, during which the arterial pulse was detected by a piezoelectric detector. Signal acquisition and processing were performed by PowerLab 4sp instruments using Chart 4.1.1 (ADInstruments, Spechbach, Germany). Kidneys fixed in alcoholic Bouin solution were paraffin-embedded, and 3-μ thick sections were stained with periodic acid-Schiff and Masson-Trichrome.

Protein and RNA Analysis
See supplemental data for detailed protocols on Western blotting, immunofluorescence, real-time PCR, and microarray analysis.

Transmission and Immunogold Electron Microscopy
Methods for tissue preparation for electron microscopy may be found in supplemental data. Electron micrographs were obtained using a Jeol JEM-100CX II transmission electron microscope. Eight to 10 photographs, covering at least 15 open random capillary loops in several glomeruli per mouse were taken; negatives were digitized, and images with a final magnification of ×15,000 were obtained. With the use of ImageJ software (National Institutes of Health; http://rsweb.nih.gov/ij/features.html), the length of the peripheral GBM was measured; the number of slit pores overlying this GBM length was then manually counted. The average foot process width, expressed in microns, was calculated by dividing the total GBM length measured in the single glomerulus by the total number of slits counted. The value obtained was multiplied by π/4, a correction factor for the random orientation in which the foot processes were sectioned.

Statistical Analysis
All data are presented as means ± SEM and compared using two-tailed t test or Mann-Whitney test for independent samples, using GraphPad Prism. P < 0.05 was considered statistically significant.

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DISCLOSURES
None.

REFERENCES


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