Podocyte-Specific Loss of Functional MicroRNAs Leads to Rapid Glomerular and Tubular Injury

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MicroRNAs (miRNAs) are in a class of endogenous, small, noncoding RNAs that exert their effects through posttranscriptional repression of specific target mRNAs. Although miRNAs have been implicated in the regulation of diverse biologic processes, little is known about miRNA function in the kidney. Here, mice lacking functional miRNAs in the developing podocyte were generated through podocyte-specific knockout of Dicer, an enzyme required for the production of mature miRNAs (Nphs2-Cre; Dicerflx/flx). Podocyte-specific loss of miRNAs resulted in significant proteinuria by 2 wk after birth, rapid progression of marked glomerular and tubular injury beginning at 3 wk, and death by 4 wk. Expression of the slit diaphragm proteins nephrin and podocin was decreased, and expression of the transcription factor WT1 was relatively unaffected. To identify miRNA–mRNA interactions that contribute to this phenotype, we profiled the glomerular expression of miRNAs; three miRNAs expressed in glomeruli were identified: mmu-miR-23b, mmu-miR-24, and mmu-miR-26a. These results suggest that miRNA function is dispensable for the initial development of glomeruli but is critical to maintain the glomerular filtration barrier.

transcriptional regulation of genes important for podocyte function. To test this hypothesis, we generated a mouse model that lacks mature miRNAs in podocytes using a conditionally floxed allele of Dicer. Here, we show that the loss of functional miRNAs in podocytes results in marked proteinuria, with rapid progression of glomerular and tubular damage, in association with aberrant apoptosis and proliferation. We characterize the expression of miRNAs in glomeruli and identify three miRNAs expressed in glomeruli: mmu-miR-23b, mmu-miR-24, and mmu-miR-26a.

To selectively delete functional miRNAs in podocytes, we bred transgenic mice with a podocyte-specific Cre recombinase allele (NPHS2-cre) to mice carrying a floxed Dicer allele. Cre-mediated DNA excision results in the loss of the second RNaseIII domain of Dicer, which was previously shown to be required for its activity, in podocytes from the capillary loop stage onward (Supplemental Figure 1). Mice of all genotypes were born in the expected Mendelian frequencies and seemed healthy until 3 wk of age. NPHS2-cre;DicerFlx/Flx mice developed significant proteinuria at 2 wk of age (Figure 1, B and C) and rapidly progressed to renal failure and death by 4 wk of age. Both DicerFlx/Flx and NPHS2-cre;DicerFlx/Flx mice are viable and fertile and display no overt abnormalities.

Histologic examination of the NPHS2-cre;DicerFlx/Flx kidneys from postnatal day 0 to 2 wk of age demonstrated normal glomerular development (data not shown). At 3 wk of age, the mutant kidneys displayed a corticomedullary gradient of glomerular abnormalities, with unremarkable subcapsular glomeruli and focal juxtamedullary glomerular changes consisting of extracap-

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Figure 1. Podocyte-specific loss of mature miRNAs results in marked proteinuria and glomerular and tubular injury. (A) Histologic analyses of control or NPHS2-cre;DicerFlx/Flx mice at 3 wk of age (H&E, hematoxylin and eosin stain; PAS, periodic acid-Schiff). Affected glomeruli from mutant kidneys display cellular crescents with glomerular tuft collapse. The renal tubules are mildly dilated with proteinaceous material. Bar = 50 μm. Electron microscopy demonstrates focal foot process effacement and wrinkling of the glomerular basement membrane. (B) Coomassie-stained SDS-PAGE gel of urine from control or mutant NPHS2-cre;DicerFlx/Flx mice. There is marked albuminuria (66 kD) in the mutant mice. MW, molecular weight. (C) Urinary albumin (mg/L), creatinine (mg/dL), and albumin-to-creatinine ratios (ACR) for control and mutant mice at 2 and 3 wk of age. Onset of proteinuria occurs by 2 wk of age in the mutant mice. Magnification, ×4800.
illary proliferation and glomerular tuft collapse (Figure 1A). This gradient may reflect the timing of cre-mediated recombination or, alternatively, heterogeneity in the somatic mutation. The renal tubules displayed mild dilation, with protein reabsorptive particles noted in the distal proximal tubule. Electron microscopy demonstrated focal foot process effacement and wrinkling of the GBM (Figure 1A). By 4 wk of age, almost all of the mutant glomeruli were severely affected, with glomerular tuft collapse and crescent formation (data not shown). At this stage, striking tubular injury was noted to be associated with prominent protein cast formation. Tubulointerstitial fibrosis was not evident on Masson’s trichrome stain (data not shown). The rapidity of the tubular damage was dramatic and may represent the nephrotoxic consequences of the marked proteinuria.

To further characterize the NPHS2-cre;DicerFlx/Flx phenotype, we performed immunofluorescence staining on the kidneys of 3-wk-old mice (Figure 2). The earliest difference noted in the mutant kidneys was decreased expression of the slit diaphragm proteins nephrin and podocin. In keeping with the histologic findings, the deeper glomeruli were the most affected, with relatively normal expression in the superficial cortical glomeruli. The pattern of nephrin and podocin expression changed from a linear distribution to a granular pattern, as has been described in patients with minimal-change disease, FSGS, and membranous nephropathy. In contrast, the linear pattern of α3 integrin was preserved, although there was segmental loss of α3 integrin in the more severely affected glomeruli. Immunostaining for the transcription factor Wt1 was present in almost all of the mutant glomeruli. Immunofluorescence staining for the endothelial marker platelet/endothelial cell adhesion molecule 1 (PECAM-1, CD31) was increased in the mutant glomeruli (Figure 3), suggesting that the glomerular endothelium may be responding to changes in gene expression in podocytes. Expression of desmin and α-smooth muscle actin was increased in the more severely affected mutant glomeruli, suggesting that mesangial cell activation may be occurring secondary to the podocyte injury. Taken together, these observations are consistent with the idea that the loss of functional miRNAs in podocytes results in abnormalities in the slit diaphragm of podocyte foot processes and thus gives rise to the significant proteinuria in the NPHS2-cre;DicerFlx/Flx mice; however, given the nature of the mutation, the proteinuria is likely to be multifactorial, and it remains possible that changes in the GBM or podocyte–endothelial interactions contribute to this phenotype.

Because miRNAs were previously implicated in the regulation of cellular proliferation and apoptosis, we performed immunostaining for the proliferative marker Ki-67 and the terminal deoxynucleotidyl transferase–mediated digoxigenin-deoxyuridine nick-end labeling (TUNEL) assay on 3-wk-old kidneys from control and mutant mice (data not shown). To identify glomeruli with TUNEL-positive cells, we performed co-staining for Wt1. In the control kidneys,
there was one TUNEL-positive glomerulus/199 Wt1-positive glomeruli, and in the NPHS2-cre; DicerFlx/Flx kidneys, there were nine TUNEL-positive glomeruli/208 Wt1-positive glomeruli. Although Ki-67 \(^{+}\) cells were present in a proportion of the cellular crescents of the NPHS2-cre; DicerFlx/Flx kidneys, these did not represent a large proportion of the crescents. These results suggest that aberrant proliferation and apoptosis do not play a significant role in the phenotype of these mice.

To identify the miRNAs that are expressed in glomeruli, we performed miRNA locked nucleic acid (LNA) microarray expression profiling using total RNA from glomeruli isolated with magnetic beads from wild-type mice at 3 wk of age (Supplemental Table 1). \(^{18,19}\) The most highly expressed miRNAs in the microarray were compared with a recently published large-scale small RNA cloning atlas in mammals for miRNAs cloned from glomeruli and a podocyte cell line. \(^{20}\) From these miRNAs, we selected a subset for further study by Northern blot and LNA in situ hybridization (ISH). We confirmed these miRNAs to be expressed in the adult kidney by Northern blot (Figure 4A). Whereas mmu-let-7c had widespread expression in the adult and embryonic tissues examined, the remainder of the miRNAs showed more specific expression patterns in a subset of the panel of adult tissues. These miRNAs were also expressed in embryos at day 14 (E14) and E14 kidneys (Figure 4A). Consistent with recent findings for other miRNAs, the expression of these miRNAs displays tissue specificity and developmental regulation. \(^{21,22}\) Of note, mmu-miR-23b and mmu-miR-24, which are present in the same primary transcript, display similar patterns of expression. By LNA ISH, three miRNAs displayed a pan-glomerular expression pattern (mmu-miR-23b, mmu-miR-24, and mmu-miR-26a), two miRNAs showed a tubular pattern (mmu-miR-10a and mmu-miR-30c), and one was ubiquitous (mmu-let-7c; Figure 4B). LNA ISH with a scrambled negative control confirmed the specificity of the technique (data not shown).

Of the four miRNAs expressed in glomeruli, relatively little is known regarding their biologic function. miR-23b has previously been described to downregulate the Hairy/Enhancer of split protein (Hes1), a basic helix-loop-helix transcriptional repressor, in an embryonic carcinoma cell line and to be upregulated in human bladder cancer. \(^{23,24}\) Hes1 is a downstream target of activated Notch signaling and is expressed in specific nephron segments during development. \(^{25,26}\) miR-26a has previously been shown to be expressed in lung epithelia during development and has been suggested to regulate SMAD1 during terminal osteoblast differentiation in an in vitro model system. \(^{27,28}\) Finally, miR-24 has been implicated in inhibition of the human activin type I receptor ALK4 during erythropoiesis. \(^{29}\) Ectopic expression of miR-24 results in a delay in activin-induced maturation of hematopoietic cells in culture. Although mmu-let-7c is ubiquitously expressed in the kidney, let-7c may also play a key role in the glomerulus. The function of these miRNAs and their specific targets in the context of the podocyte remains unclear.

In summary, we have shown that the loss of mature miRNAs in the podocyte in NPHS2-cre; DicerFlx/Flx mice results in marked proteinuria, with rapid progression of glomerular and tubular damage. The proteinuria is likely related to structural abnormalities in the slit diaphragm and foot processes, given the decreased nephrin and podocin expression, in association with foot process effacement; however, the nature of the mutation is such that the phenotype is probably multifactorial, so other processes, such as podocyte loss, may be important contributors to the phenotype. To begin to identify the specific miRNA–mRNA interactions that contribute to this phenotype, we characterized the expression of miRNAs in glomeruli and identified three miRNAs expressed in glomeruli: mmu-miR-23b, mmu-miR-24, and mmu-miR-26a. Together, our results provide evidence that regulation of multiple cellular processes by miRNAs in podocytes plays an important role in the maintenance of the glomerular filtration barrier.

**CONCISE METHODS**

**Mouse strains**

Transgenic NPHS2-cre mice were generated using a construct obtained from Dr. Susan

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**Figure 3.** Immunofluorescence staining for PECAM, desmin, and α-smooth muscle actin for control (top) and NPHS2-cre; DicerFlx/Flx (bottom) kidneys. PECAM staining is unchanged in the mutant glomeruli, whereas desmin and α-smooth muscle actin display increased expression in the mesangium. Bar = 25 μm.
Quaggin (University of Toronto, Toronto, Ontario, Canada). Expression of the NPHS2-cre transgene in podocytes was verified using the Z/EG reporter mice (Supplemental Figure 1). The Dicer conditional allele (Dicer<sup>Flx</sup>) was generated in Dr. Clifford Tabin’s laboratory (Harvard Medical School, Boston, MA) and was previously described. In brief, the conditional Dicer allele contains two loxP sites flanking a portion of the second RNaseIII domain of Dicer, which is required for its activity. Mice that were homozygous for the Dicer<sup>Flx</sup> allele were crossed to mice carrying the NPHS2-cre allele to generate NPHS2-cre;Dicer<sup>Pols/+</sup> mice. The offspring were genotyped with the following PCR primers: Dicer<sup>5</sup>‘-CCT GAC AGT GAC GGT CCA AAG-3‘ and 5‘-CAT GAC TCT TCA ACT CAA ACT-3‘ (product sizes 420 bp for the Dicer<sup>Pols</sup> allele and 351 bp for the wild-type allele); Cre, 5‘-ATG TCC AAT TTA CTG ACC GT-3‘ and 5‘-GC CCG ATA ACC AGT GAA AC-3‘ (product size 370 bp). The NPHS2-cre mice were maintained on a 129/SvEv background, and the Dicer<sup>Flx</sup> mice were maintained on a mixed background. The animal experiments were carried out in accordance with the policies of the Institutional Animal Care and Use Committee at Children’s Hospital Boston.

A total of 17 NPHS2-cre;Dicer<sup>Pols/+</sup> and 16 control (eight Dicer<sup>Pols</sup>; eight NPHS2-Cre; Dicer<sup>Pols/+</sup>) mice were studied at various time points. The majority of the analysis was done at 3 wk of age (five mutant kidneys and five control kidneys, including Dicer<sup>Pols/+</sup> or the NPHS2-cre;Dicer<sup>Pols/+</sup> kidneys). For each of the histologic analyses and immunofluorescence studies, we examined a minimum of three mutant kidneys and three control kidneys.

**Proteinuria and Albuminuria**

Urine samples from 2- and 3-wk-old control littermates (NPHS2-cre;Dicer<sup>Pols/+</sup> or Dicer<sup>Pols/Pols</sup>) and mutant (NPHS2-cre; Dicer<sup>Pols/Pols</sup>) mice were run on a 10% SDS-PAGE gel, which was subsequently stained with Coomassie to visualize the urine protein. Urinary albumin (mg/L) and creatinine (mg/dl) levels were measured using the DCA 2000 microalbumin/creatinine reagent kit, as per the manufacturer’s directions (Bayer Healthcare LLC, Elkhart, IN).

**Histopathology**

Kidneys were dissected from control littermates (NPHS2-cre;Dicer<sup>Pols/+</sup> or Dicer<sup>Pols/Pols</sup>) and mutant (NPHS2-cre;Dicer<sup>Pols/Pols</sup>) mice at 3 wk of age. The kidneys were fixed in 4% paraformaldehyde overnight; paraffin-embedded; and stained with hematoxylin and eosin, periodic acid-Schiff, or Masson’s trichrome stain. The sections were visualized with a Nikon Eclipse 80i microscope and photographed with a Qimaging Retiga 2000R Fast 1394 camera using NIS-Elements Basic Research 2.34 software (Micro Video Instruments, Avon, MA). Kidney tissue for electron microscopy was fixed in 6.25% glutaraldehyde (Electron Microscopy Sciences, Hatfield, PA) in 0.1 M sodium cacodylate buffer and embedded in Epon. The specimens were examined with a Tecnai G2 Spirit BioTWIN electron microscope and photographed with an AMT 2k CCD camera at the Conventional Electron Microscopy Core, Harvard Medical School.

**Immunofluorescence**

Kidneys from control (NPHS2-cre;Dicer<sup>Pols/+</sup> or Dicer<sup>Pols/Pols</sup>) and mutant (NPHS2-cre; Dicer<sup>Pols/Pols</sup>) mice were frozen in Tissue-TEK OCT (Sakura Finetek, Torrance, CA) in a liquid nitrogen–cooled isopentane bath and cryosectioned at 5 μm for immunofluorescence. Tissues were fixed for 10 min in cold methanol at −20°C, washed in PBS, and blocked for 1 h in 5% normal goat serum in...
PBS at room temperature. The primary antibodies used were as follows: Rabbit anti-nephrin 1:100 (gift from Dr. Lawrence B. Holzman, University of Michigan, Ann Arbor, MI), rabbit anti-podocin 54:1 (1:100; Dr. Peter Mundel, University of Miami School of Medicine, Miami, FL), rabbit anti-α-smooth muscle actin clone 1A4 (1:500; Sigma, St. Louis, MO), rat anti-PECAM 1:100 (BD Pharmingen, Franklin Lakes, NJ), and rabbit anti-Ki-67 (Vector Laboratories, Burlingame, CA). Sections were incubated overnight at 4°C with the primary antibodies, washed with PBS, and incubated in the appropriate secondary antibodies at room temperature for 1 h. The secondary antibodies used were donkey anti-rabbit Texas Red or donkey anti-mouse FITC at 1:200 (Jackson ImmunoResearch Laboratories, West Grove, PA). The slides were washed with PBS and mounted with Prolong Gold antifade reagent with DAPI (Molecular Probes, Invitrogen Corp, Carlsbad, CA). TUNEL staining was performed with the Apoptag Plus Fluorescein In situ Apoptosis Detection kit, as per the manufacturer’s directions (Millipore (formerly from Chemicon Int.), Billerica, MA). Co-staining with Wt1 was performed after the TUNEL assay, as described already. The slides were visualized with a Nikon Eclipse 80i microscope as described already.

The number of Wt1-positive glomeruli and TUNEL-positive glomeruli was counted in sections from three control and three mutant kidneys. These were expressed as #TUNEL-positive glomeruli/#Wt1-positive glomeruli.

**miRNA Expression**

Glomeruli were isolated from CD-1 mice at 3 wk of age using Dynabead perfusion as described previously. Total RNA was extracted and pooled from the glomeruli using the Qiagen miRNeasy Mini kit (Qiagen, Valencia, CA). The expression of glomerular miRNAs was profiled using Exiqon’s miRCURY LNA Arrays miRNA profiling services (Exiqon, Vedbaek, Denmark). In brief, RNA samples were checked for RNA integrity on the Bioanalyser2100, labeled with Hy5, and hybridized in triplicate to miRCURY LNA Arrays 8.1. Total RNA samples from E14 kidneys were labeled with Hy3 and hybridized to these arrays as well (embryonic kidney data to be described elsewhere). The quantified signals were normalized with the global Lowess regression algorithm to minimize intensity-dependent differences between the two dyes. Spike-in controls confirmed that labeling and hybridization to the arrays were successful. The miRNAs expressed in glomeruli were listed in descending order on the basis of the average signal intensities for each miRNA from the three replicates (Supplemental Table 1). In addition, the fold increase in signal intensity over background was calculated, and a two-fold change in Hy5 signal intensity above background was used as the lower cutoff for the listed miRNAs.

The expression of individual miRNAs was subsequently confirmed by Northern blot and LNA ISH. In brief, total RNA was isolated from adult tissues with Trizol (Invitrogen), and 15 μg of each sample was run on a 15% TBE-urea polyacrylamide gel (BioRad, Hercules, CA). The RNA was transferred to an Amersham Hybond N+ nylon membrane (GE Life Sciences, Piscataway, NJ). The membrane was hybridized overnight with P32-labeled oligonucleotides complementary to the mature miRNA sequence in 10X Denhardt’s/6X SSC/0.1% SDS at 42°C (Supplemental Table 2). The membrane was subsequently washed and exposed to Kodak BioMax MS film (Sigma).

LNA probes complementary to the mature miRNA with approximately 30% LNA incorporation of 50% formamide, 1.3X SSC, 5 mM EDTA, 50 mg/mL yeast tRNA, 0.2% Tween-20, 0.5% CHAPS, and 100 mg/mL heparin. Hybridization was performed at 20°C below the Tm of the LNA probe overnight. The antibody blocking solution was 5% heat-inactivated sheep serum, 2% Blocking Reagent (Roche Diagnostics Corporation, Indianapolis, IN) in 0.1 M Tris pH 7.5, 0.15 M NaCl. The color reagent used was BM Purple AP substrate, precipitating (Roche Diagnostics Corporation, Indianapolis, IN). A scrambled LNA oligo was used as a negative control (Exiqon).

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**DISCLOSURES**

None.

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