Urine Podocyte mRNAs Mark Progression of Renal Disease

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ABSTRACT

Because loss of podocytes associates with glomerulosclerosis, monitoring podocyte loss by measuring podocyte products in urine may be clinically useful. To determine whether a single episode of podocyte injury would cause persistent podocyte loss, we induced limited podocyte depletion using a diphtheria toxin receptor (hDTR) transgenic rat. We monitored podocyte loss by detecting nephrin and podocin mRNA in urine particulates with quantitative reverse transcriptase–PCR. Aquaporin 2 mRNA served as a kidney reference gene to account for variable kidney contribution to RNA amount and quality. We found that a single injection of diphtheria toxin resulted in an initial peak of proteinuria and podocyte mRNAs (podocin and nephrin) followed 8 d later by a second peak of proteinuria and podocyte mRNAs that were podocin positive but nephrin negative. Proteinuria that persisted for months correlated with podocin-positive, nephrin-negative mRNAs in urine. Animals with persistent podocyte mRNA in urine progressed to ESRD with global podocyte depletion and interstitial scarring. Podocytes in ectatic tubules expressed podocalyxin and podocin proteins but not nephrin, compatible with detached podocytes’ having an altered phenotype. Parallel human studies showed that biopsy-proven glomerular injury associated with increased urinary podocin:aquaporin 2 and nephrin:aquaporin 2 molar ratios. We conclude that a single episode of podocyte injury can trigger glomerular destabilization, resulting in persistent podocyte loss and an altered phenotype of podocytes recovered from urine. Podocyte mRNAs in urine may be a useful clinical tool for the diagnosis and monitoring of glomerular diseases.


The direct relationship between podocyte injury/loss and glomerulosclerosis is now established in both experimental models and human disease. It should therefore be clinically useful if podocyte loss could be monitored by measuring podocyte products in urine. Hara and colleagues detected podocytes and fragments of podocytes in the urine of humans with a variety of glomerular diseases using a podocalyxin antibody detection system. Lemley et al. demonstrated podocyturia in urine of humans with IgA nephropathy and systemic lupus erythematosus (SLE) in relation to disease activity. Patari et al. detected nephrin protein in urine of patients with diabetes by Western blotting. Vogelmann et al. and Petermann et al. reported that viable podocytes were detected in human and rat urine in health and kidney disease. Garovic et al. reported viable podocytes in urine in association with toxemia of pregnancy. We previously demonstrated nephrin mRNA in urine as a marker of podocyte loss in a rat PAN model. Szeto and colleagues reported podocyte mRNAs (nephrin, podocin, and synaptopodin) expressed in

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the urinary sediment of proteinuric diseases including diabetes and SLE. Yu et al.27 made the case that the identification of podocyte products in urine is a more specific measure of disease activity than is proteinuria.

To evaluate the potential impact of graded podocyte injury, we developed a transgenic (Tg) model [strain F344-Tg(DTR)C354Wig] in which the human diphtheria toxin receptor (hDTR) is expressed specifically on rat podocytes.6 In this model, stages of glomerular injury and sclerosis as defined by Kriz and colleagues2,4–6 can be identified in relation to the degree of podocyte loss. If podocyte depletion is indeed the major mechanism driving progression, then this should be reflected by detection of persistent podocyte products in the urine during long-term progression. To test this hypothesis, we caused a range of initial podocyte injury in heterozygous hDTR Tg rats and measured specific podocyte mRNA products in urine (nephrin and podocin mRNAs) using real-time quantitative PCR.

**RESULTS**

**hDTR Rat Model**

A range of podocyte injury was produced using low-dosage DT (40 ng/kg in 10 ml of normal saline intraperitoneally in 100-g rats) into 17 heterozygous hDTR Tg rats (seven female, 10 male). During the 6-mo observation period, eight rats progressed to end-stage kidney disease (ESKD; defined as “progressors”) and were identified clinically by an initial biphasic peak of proteinuria followed by persistent high-level proteinuria (Figure 1), high-volume urine output, and a scruffy appearance with weight loss as they developed uremia. ESKD was diagnosed by decreasing urine and fecal output and was confirmed by histologic analysis and serum creatinine measurements (Figure 1). Progressor rats were killed at 42, 42, 56, 62, 71, 78, 96, and 98 d after DT injection.

A “limited proteinuric” (n = 4) group, a “nonproteinuric” group (n = 5), and a control group that received no diphtheria toxin (n = 4; two male, two female) were followed for the 175-d time course (Figure 1) and used for comparison with the progres sor group. On the basis of the proteinuria, profiles we divided the progression process into an acute injury phase (lasting 21 d) followed by a chronic progression phase defined as beginning at day 22 and lasting until death at ESKD (Figure 1).

**Histologic and Morphometric Analysis**

All eight rats that diagnosed clinically as ESKD (progressors) showed >90% loss of podocytes from glomeruli as judged by GLEPP1 immunoperoxidase (Figures 2 and 3). Using WT1 as a podocyte nuclear marker, progressor rats had 8.4 ± 2.5 remaining podocytes per glomerulus compared with 121 ± 25 podocytes per normal glomerulus, a reduction of 93%. Other podocytes markers (podocin, nephrin, and podocalyxin) were similarly markedly reduced or absent from glomeruli at ESRD (Figure 4). There was also widespread interstitial fibrosis and glomerular scarring in progressor rat kidney as judged by Mason’s Trichrome staining (Figure 2). Conversely, the limited proteinuric group of rats that showed transient proteinuria had focal and segmental sclerosis and associated focal and segmental absence of podocytes (Figures 2 and 3) and patchy interstitial scarring. The nonproteinuric group had occasional segmental glomerular lesions as shown in Figure 2. Control rats did not show segmental sclerosis or interstitial scarring.

**Urine RNA Amount and Quality**

Figure 5A shows the average amounts of RNA excreted per day for the various groups. Progressors had significantly increased urine RNA excretion in both the acute injury phase and the chronic progression phase. The quality of RNA recovered from urine was variable as judged by Agilent Technologies capillary electrophoresis. The average RNA integrity number (RIN) of urine RNA samples was 4.9 ± 1.8 (range 2.4 to 7.8; n = 18 samples tested); the range of RIN is 1 to 10 with 1 being lowest and 10 being highest (see the Concise Methods section). For comparison, two RNA samples purified from isolated rat glomeruli by the same method gave RINs of 8.8 and 8.9; therefore, as expected, the RNA obtained from urine is variably degraded. There was no correlation between amount of RNA excreted and RIN ($r^2 = 0.05$) or the amount of proteinuria and RIN ($r^2 = 0.0005$).

![Figure 1](https://www.jasn.org)
Aquaporin 2 as a Kidney-Derived Reference Gene

To account for variability in amount and quality of recovered urine RNA and contamination from other sources and to facilitate the use of spot urine samples for measurement, we sought a robustly expressed kidney-specific reference gene to represent kidney RNA from the nonglomerular compartment. Immunofluorescent analysis using antibodies to various markers in the normal and end-stage rat kidney showed that aquaporin 2 (AQP2) continued to be expressed by the collecting ducts of functioning nephrons in end-stage kidneys (data not shown). AQP2 mRNA was detectable in 70.3% of control rat urine samples (n=11005276). Figure 5B shows that AQP2 expression was increased in association with progression and to a lesser but still significant extent in rats with limited proteinuria for both the acute and chronic phases. A similar result was observed for uromodulin mRNA (data not shown). We conclude that both the acute injury and chronic progression processes were associated with increased loss of nonpodocyte kidney RNA species into the urine in both the acute and chronic phases.

Urine Podocyte mRNA Excretion

Figure 5, C and D, shows mean nephrin and podocin mRNA excretion during the acute injury and chronic progression phases. Figure 6 shows the time course for the podocyte mRNAs in the various groups. There was significantly increased nephrin and podocin mRNA excretion in rats that progressed. Podocin mRNA was robustly expressed in urine during the chronic progression period. There was markedly less urine nephrin and podocin mRNA in the limited proteinuria group and barely detectable or undetectable increases in nephrin and podocin mRNAs in the nonproteinuric and control groups (Figures 5, C and D, and 6).

Because urine AQP2 mRNA also increased in progressors, we expressed podocyte mRNAs as a ratio to AQP2 to examine the relative increase in podocyte mRNAs detected over and above that for AQP2 (Figure 7). The nephrin:AQP2 ratio was increased in the acute phase for progressors; however, for the chronic phase, there was no statistical increase in nephrin:AQP2 ratio in progressors compared with other groups. In contrast, the podocin:AQP2 ratio was significantly increased in progressors compared with other groups for both the acute injury phase and the chronic progressor phase.

Figure 8 shows the temporal relationship between nephrin and podocin mRNA excretion in urine for the progressor group in relation to proteinuria. Figure 8, top, shows the biphasic peak of proteinuria seen after DT injection, which is followed by long-term sustained proteinuria in the progressor group. The nephrin: AQP2 ratio peak corresponds closely to the first proteinuria peak seen in the acute phase. The second proteinuria peak corresponds to the podocin:AQP2 peak of mRNA in urine. The high level of sustained proteinuria in progressors correlates with continued podocin:AQP2 mRNA in urine.

The seeming discrepancy between nephrin and podocin mRNA profiles in urine was confirmed by time-course correlation analysis (Table 1). There was no temporal correlation between nephrin mRNA excretion and podocin mRNA excretion in urine (r² = 0.17), thereby suggesting that these podocyte markers are not identifying the same populations of cells in urine. In addition, nephrin profiles obtained using a second independent set of rat nephrin primers were consistent (r² = 0.74; n = 35). The podocin mRNA profile correlated closely with the podocalyxin mRNA profile (r² = 0.61; n = 70) but not the nephrin mRNA profile (r² = 0.26; n = 70), thereby linking these podocin data to previous reports using podocalyxin as a marker for urine podocytes.

We conclude that two podocyte phenotypes are identifiable in the urine of progressor rats. These include a nephrin-posi-
tive (and podocin-positive) phenotype present in the acute phase immediately after DT injection and associated with the first proteinuria peak. A nephrin-negative, podocin/podocalyxin/GLEPP1-positive podocyte phenotype was present in association with the second wave of proteinuria and persisted for several months during the progression process in rats that progressed to ESKD but not in those that did not progress. Nephrin-negative but podocin-positive detached podocytes were also identified in ectatic tubules of progressor rats (Figure 4). These data are consistent with the concept that detached podocytes lose their nephrin expression at both the mRNA and protein levels during the progression phase of glomerular injury.

Quantitative Analysis Based on Podocin mRNA Excretion

Table 2 shows that podocin mRNA loss in urine during the chronic progression phase was on average 230-fold higher than in normal rats (ranging from 40-fold in slow progressors to 350-fold in rapid progressors). This is in contrast to nonproteinuric and nonprogressor rats, in which the rate of urine podocin mRNA loss during the chronic phase was not significantly different from control. On the basis of assumptions outlined in the Concise Methods section, we estimate that progressor rats were losing on average 130,000 podocyte-equivalents in their urine per day compared with approximately 500 to 600 podocyte-equivalents per day found in normal and nonprogressor rat urine.

Correlation between Proteinuria and Urine Podocyte mRNAs

If urine podocyte markers reflect the degree of podocyte loss, then they should correlate with the degree of proteinuria. As shown in Figure 9, this was indeed the case. The level of proteinuria in the acute phase correlated closely with the nephrin mRNA excretion ($r^2 = 0.84$) but less well with the podocin mRNA excretion ($r^2 = 0.29$). Proteinuria in the chronic phase correlated with both the low-level nephrin ($r^2 = 0.80$) and high-level podocin ($r^2 = 0.69$) mRNA excretion.
Systolic BP

Systolic BP (SBP) of rats that progressed to ESKD was significantly higher than controls as assessed by the “last measured” SBP ($P < 0.05$; progressors 194 ± 6 mmHg; limited proteinuria 162 ± 8; nonproteinuric 140 ± 7; controls 153 ± 10). The last measuring days of the progressor rats were approximately 1 wk before being killed and at 5 mo after DT injection for the other groups.

Human Urine mRNA

To confirm that the urine mRNA system developed for the rat can be applied to the clinic, we examined urine from patients with biopsy-proven glomerular disease as a result of SLE. Nephrin, podocin, and AQP2 mRNAs were detectable in normal human urine. The measured amounts of each mRNA decreased in urine samples stored at both 4°C (30 to 40% by 6 h) and room temperature (80% by 6 h), but the ratio of nephrin:AQP2 or podocin:AQP2 mRNA did not change significantly up to 48 h of storage at 4°C (Figure 10). Data are therefore presented as the nephrin:AQP2 or podocin:AQP2 molar ratios (MRs). The upper limit of the normal MR ranges (mean ± 2 SD) were established from eight normal humans under conditions of fasting, nonfasting, time of day, storage, and exercise ($n = 100$; Table 3). None of these physiologic stimuli changed the MRs.

Urine from four patients with SLE-associated glomerular disease was evaluated at renal biopsy (Table 3). Three patients with diffuse proliferative glomerulonephritis or membranous glomerulonephropathy had elevated MRs for both nephrin:AQP2 MR (1.3- to five-fold) and podocin:AQP2 MR (five- to 17-fold). One patient with longstanding membranous glomerulonephropathy with proteinuria and stable normal renal function for >11 yr had MR values in the normal range. This

![Figure 5. RNA parameters for the progressor group: Excretion of total RNA (A), urine AQP2 mRNA (B), urine nephrin mRNA (C), and urine podocin mRNA (D) for the acute (left) and chronic (right) progressor phases of injury. *$P < 0.05$ and **$P < 0.01$ as assessed by Kruskal-Wallis test and then Scheffe test. Unless otherwise specified, units are arbitrary, based on standard curves from wild-type animals as described in the Concise Methods section.](image)

![Figure 6. Comparison of urine nephrin and podocin mRNA excretion between groups through day 56: The progressor group excreted high-level urine nephrin mRNA (red) as an initial peak followed by low-level mRNA excretion. Podocin mRNA (blue) was excreted persistently throughout the time course. A similar pattern but reduced in quantity was seen for the limited proteinuric group. Unless otherwise specified, units are arbitrary, based on standard curves from wild-type animals as described in the Concise Methods section.](image)
result confirms that podocyte mRNA products are detectable in normal human urine, that a kidney reference gene can be a useful method for adjusting data for RNA stability and other confounding variables, that proteinuria per se was not associated with elevated MRs, and that podocyte MRs are increased in association with active glomerular injury such as occurs in lupus nephritis.

**DISCUSSION**

The podocyte hypothesis predicts that progression will be associated with continued podocyte loss from glomeruli. Consistent with this hypothesis, we observed persistent podocyte mRNAs in urine for months as progression occurs. Quantification (making the critical assumption that the amount of podocin mRNA per podocyte is a constant) revealed that normal and nonproteinuric rats excreted approximately 500 to 600 podocyte-equivalents (0.006%) of podocin mRNA per day. In contrast, progressor rats excreted a 230-fold higher rate of podocyte-equivalents of podocin mRNA in urine (mean 130,000 podocyte-equivalents per day). Progressor rats lost from 24,000 podocyte-equivalents (0.2%) per day (slow progressor) up to 333,000 podocyte-equivalents (3%) per day (rapid progressor). This result therefore directly supports a key role for podocyte loss in the progression process. Furthermore, glomeruli became substantially depleted of podocytes (>90%) as demonstrated by both podocyte counting using the WT1 podocyte nuclear marker and immunofluorescence for the podocyte markers nephrin, podocin, podocalyxin, and gcpp1. Global depletion of podocytes was therefore associated with reaching ESKD, further linking the progression process to podocyte depletion and compatible with the concept that intact podocytes are necessary for glomerular maintenance.

A single limited episode of podocyte injury and loss triggered subsequent prolonged podocyte loss leading to ESKD. The implication is that whatever the initial mechanism of podocyte injury, an autonomous process that can lead to progression through further podocyte loss can supervene. This process is likely analogous to both the "hyperfiltration hypoth-
Podocyte detachment may therefore require a phenotypic switch that downregulates nephrin expression, thereby allowing a detaching podocyte to disengage from its neighbors. This result also suggests that the biology of the glomerular podocyte may be monitored by urine mRNA analysis and that clinically useful diagnostic and therapeutic parameters may be available through this approach.

Urine podocyte mRNAs can be semi-quantified and used for analysis of the events taking place in the kidney. We previously reported detection of mRNA for nephrin in rat urine after PAN injury. Szeto and colleagues have since reported detecting podocyte mRNAs in human urine and have linked this to progression in patients with glomerular diseases including diabetes and SLE. An innovation reported here is the use of a kidney-specific reference gene mRNA that is robustly expressed even in the end-stage kidney. This approach has several advantages: It allows for spot urine sample analysis, variations in proportion of RNA recovered from kidney as opposed to the numerous other sources of RNA that could end up in the urine, variations in RNA quality and degradation between samples, and variations in efficiency of the reverse transcriptase–PCR (RT-PCR) step. We elected to use AQP2 mRNAs as our reference gene in this report, but other kidney-specific candidates (RT-PCR) step. We elected to use AQP2 mRNAs as our reference gene in this report, but other kidney-specific candidates may turn out to be preferable.

Finally, we confirmed that the observations made in rat urine could potentially be extended to patients with glomerular diseases. We found that podocyte mRNA products were detectable in normal human urine and that differences in mRNA stability during urine storage could be accounted for by mRNA stability during urine storage could be accounted for by mRNA stability during urine storage could be accounted for by mRNA stability during urine storage could be accounted for by using the reference kidney gene (AQP2). Three patients with active glomerular disease as a result of SLE had significantly increased urine podocin:AQP2 MRs (five- to 17-fold). A patient with longstanding membranous lupus nephropathy and proteinuria with stable normal renal function did not have elevated MRs. The podocin mRNA excretion (top; $r^2 = 0.84$) but not with urine podocin mRNA excretion (bottom; $r^2 = 0.29$). In the chronic phase (right), both nephrin and podocin mRNA correlated with proteinuria ($r^2 = 0.80$ and 0.69, respectively). Unless otherwise specified, units are arbitrary, based on standard curves from wild-type animals as described in the Concise Methods section.

Table 2. Chronic phase urine podocin mRNA and podocyte-equivalents per day

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Urine Podocin mRNA (ng/d)</th>
<th>Urine Podocyte-Equivalents per Day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>4</td>
<td>0.06 ± 0.10</td>
<td>552 ± 935</td>
</tr>
<tr>
<td>Nonproteinurics</td>
<td>5</td>
<td>0.06 ± 0.03</td>
<td>561 ± 316</td>
</tr>
<tr>
<td>Limited proteinurics 4</td>
<td>1.27 ± 0.76</td>
<td>11,782 ± 7056</td>
<td>4259 to 19814</td>
</tr>
<tr>
<td>Progressors</td>
<td>8</td>
<td>14.04 ± 11.22b</td>
<td>129,977 ± 103,916b</td>
</tr>
</tbody>
</table>

Values for podocyte-equivalents per day were calculated using a recovery rate of 0.03% (see the Concise Methods section).

Different urine podocyte phenotypes were identifiable through urine mRNA monitoring. We identified an initial nephrin-positive (and podocin-positive) podocyte phenotype in urine during the acute injury phase analogous to the podocyte phenotype found in the normal glomerulus. This podocyte phenotype was present in urine coincident with the first peak of proteinuria after acute DT-induced podocyte injury; however, the second peak of proteinuria, which we hypothesize is caused by the “podocyte damage damages podocytes” phenomenon described by Ichikawa et al., associated with podocyte mRNAs that were podocin and podocalxin positive but nephrin negative. This conclusion was further supported by identifying podocylxin- and podocin-positive but nephrin-negative podocytes in ectatic tubules of progressor rats. This would also be compatible with previous reports in which nephrin was not found to be a good marker of urine podocytes in contrast to podocalyxin and podocin.

Figure 9. Relationship between proteinuria and mRNA excretion of urine nephrin and podocin: In the acute phase (left), proteinuria correlated highly with urine nephrin mRNA excretion (top; $r^2 = 0.84$) but not with urine podocin mRNA excretion (bottom; $r^2 = 0.29$). In the chronic phase (right), both nephrin and podocin mRNA correlated with proteinuria ($r^2 = 0.80$ and 0.69, respectively). Unless otherwise specified, units are arbitrary, based on standard curves from wild-type animals as described in the Concise Methods section.
cyte mRNA:AQP2 ratios therefore seemed to be a potentially clinically useful indicator of podocyte injury as predicted from the rat studies. Szeto and colleagues also found urine podocin mRNA to correlate with progression in human diabetic glomerulosclerosis. Other investigators have demonstrated the presence of podocytes in urine using antibodies to podocalyxin and podocin and have correlated podocyturia with outcome in a range of human glomerular diseases. Fragments of podocytes have also been demonstrated in human urine. We initially identified microvesicles in urine and demonstrated their apparent release from podocytes during injury in an anti-glomerular basement membrane model in the rabbit using a procoagulant assay system to quantify and identify these structures. We also demonstrated the presence of microvesicles in normal human urine (termed "urosomes"). Knepper and colleagues characterized urine exosomes and defined a urine exosome proteome that includes podocin and podocalyxin. For the studies described, the centrifugation step would predominantly pellet whole cells; however, it is possible that podocyte mRNAs could be packaged and protected from degradation within lipid membranes as microvesicles and that their association with larger structures in urine (e.g., polymerized Tamm-Horsfall protein) might allow them to be pelletted under the conditions used for the assay. Both RNA and micro-RNAs have been shown to travel via microvesicles. Further studies will be required to evaluate the possibility that downstream nephron signaling can occur via this mechanism.

CONCISE METHODS

Experimental Design

All animal studies were approved by the University of Michigan Committee on Use and Care of Animals. Heterozygous hDTR Tg rats received an injection of DT in normal saline containing 0.1 mg/ml rat albumin as a carrier (n = 17; 11 male, six female) or normal saline containing rat albumin at 0.1 mg/ml as controls (n = 4; two male, two female). Rats were injected intraperitoneally when they reached approximately 100 g of weight. Then they were kept on an ad libitum rat food and water diet until they were killed under anesthesia for analysis up to 175 d. All studied rats were kept in metabolic cages. We performed daily urine collection for 4 wk, followed by 4 d/wk urine collection for 8 wk and subsequent 1-wk urine collections at monthly intervals. All rats were inspected daily and evaluated for lethargy, ruffled fur, and failure to eat or drink as assessed by reduced urine volume and feces production as evidence for uremia. Eight progressor rats were killed before the 6-mo time point as noted in the Results section. In these experiments, a variable portion of the injected DT in 10 ml of saline leaked out of the peritoneal cavity through the injection site. Some rats therefore received a lower dosage of DT than others, resulting in a variable level of podocyte injury (see below for data on uniformity of transgene expression and intrinsic model reproducibility in response to DT injection).

RNA from Urine Sediments

To maximize RNA harvest, we washed metabolic cage collection surfaces thoroughly and rinsed with alcohol every afternoon, urine collection tubes were set up, and the urine was harvested the next morning. Average collected time was approximately 14 h/d. The urine samples were centrifuged at 3200 g for 15 min at 4°C. Urine was removed and the remaining pellet was suspended in 1.5 ml of DEPC-treated PBS then centrifuged 13,000 g for 5 min at 4°C. The pellet was suspended in 1.0 ml of Trizol (cat. no. 15596-026; Invitrogen, Carlsbad, CA) and stored at −80°C until use. Total RNA was isolated using the protocol of the Trizol Reagent and RNeasy Mini Kit (cat. no. 74106; Qiagen, Valencia, CA). The contaminating chromosomal DNA was digested with RNase-Free DNase (cat. no. 79254; Qiagen). The coefficient of reproducibility of RNA recovery was 28% (n = 6).
The 300 ng of RNA was reverse-transcribed to cDNA using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA) and stored at −20°C until use.

RNA Quality Analysis
RNA was analyzed by the University of Michigan Affymetrix Core using Agilent Technologies and both the Nano and Pico systems (Agilent Technologies, Santa Clara, CA). RINs are used as a measure of RNA quality as defined (http://www.agilent.com/chem/labonachip).

Quantitative Real-Time PCR
Quantification of the absolute nephrin, podocin, AQP2, podocalyxin, uromodulin, GLEPP1, WT1, and glyceraldehyde-3-phosphate dehydrogenase mRNA abundance was performed using the 7900 HT Fast Real-Time PCR System (Applied Biosystems) using TaqMan Fast Universal PCR Master Mix, with sample cDNA in a final volume of 10 μl per reaction. TaqMan Probes (Applied Biosystems) were used as follows: Rat homologue for NPHS1 (nephrin) spanned exons 20 to 21 (cat. no. Rn00575235_m1, for regular use); rat homologue for NPHS1 spanned exons 6 to 7 (cat. no. Rn01457444_g1, alternative nephrin primer/probe set), rat homologue for NPHS2 (podocin) spanned exons 3 to 4 (cat. no. Rn00709834_m1); rat AQP2 spanned exons 1 to 2 (cat. no. Rn00563755_m1), rat Podxl (podocalyxin) spanned exons 4 to 5 (cat. no. Rn00593804_m1), rat Umod (uromodulin or Tamm-Horsfall protein) spanned exons 9 to 10 (cat. no. Rn00567180_m1), rat GLEPP1 (Ptpro) spanned exons 11 to 12 (cat. no. Rn01462616_m1), rat WT1 (Wilms' tumor 1) spanned exons 7 to 8 (cat. no. Rn00580566_m1), and rat GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was within exon 3 (cat. no. Rn99999916_s1). All data were from 100 ng of sample cDNA. Standards were obtained from glomerular or cortical RNA prepared from a wild-type rat. For these experiments, glomeruli (or cortex) were isolated by sieving and perfused and fixed kidney tissue followed by paraffin embedding for sectioning as described previously.

Histologic Analysis
All histology was performed on parafomaldehyde/lipidine/periodate-perfused and fixed kidney tissue followed by paraffin embedding for sectioning as described previously.

Quantification of Glomerular Segmental/Global Lesions
A murine anti-rat GLEPP1 mAb was used as a podocyte marker as described previously.

Quantification of GLEPP1-Positive and Periodic Acid-Schiff–Positive Glomerular Areas
Staining was done on 4-μm sections as outlined in the previous section. Podocytes were identified using peroxidase/DAB immunohistochemistry using the monoclonal anti-rat GLEPP1 as primary antibody.

Table 3. Human SLE urine podocyte mRNA study

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age/ Gender</th>
<th>Duration of SLE</th>
<th>SCr (mg/dl)</th>
<th>Up:Cr</th>
<th>Renal Biopsy Findings</th>
<th>Unep:AQP2 MR (ULNR = 2.1)</th>
<th>Upod:AQP2 MR (ULNR = 0.26)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>21/F</td>
<td>2 yr</td>
<td>1.0</td>
<td>5.9</td>
<td>DPGN (WHO IV), 7/12 crescentic glomeruli, focal mild interstitial scarring</td>
<td>3.0</td>
<td>1.6</td>
</tr>
<tr>
<td>2</td>
<td>34/F</td>
<td>7 yr</td>
<td>0.9</td>
<td>2.9</td>
<td>Severe DPGN (WHO IV), 3/13 crescentic glomeruli, no interstitial changes</td>
<td>10.9</td>
<td>4.4</td>
</tr>
<tr>
<td>3</td>
<td>19/M</td>
<td>3 mo</td>
<td>0.8</td>
<td>1.6</td>
<td>Membranous GN (WHO V), no global glomerulosclerosis, diffuse mild interstitial fibrosis</td>
<td>3.1</td>
<td>1.4</td>
</tr>
<tr>
<td>4</td>
<td>41/F</td>
<td>&gt;11 yr</td>
<td>0.7</td>
<td>4.3</td>
<td>Membranous GN (WHO V), mild focal proliferation, no glomerulosclerosis, focal mild interstitial fibrosis</td>
<td>0.2</td>
<td>0.1</td>
</tr>
</tbody>
</table>

*DPGN, diffuse proliferative glomerulonephritis; SCr, serum creatinine; ULNR, upper limit of normal range; Unep, urine nephrin; Upod, urine podocin; Up:Cr, urine protein-creatinine ratio; WHO, World Health Organization.
Podocyte Counts per Glomerulus
Podocyte counts per glomerulus were performed as described previously using the two-section thickness method. In parallel studies, this method correlated with the GLEPP1-positive area method outlined in the previous section ($r^2 = 0.88$).

Immunofluorescence Studies
Paraformaldehyde/lysine/periodate-fixed paraffin-embedded 4-µm sections of progressor rat kidney and normal kidney were deparaffinized and incubated for 4 h at 92°C with Retrieve-All (Signet Laboratories, Dedham, MA) for antigen unmasking. They were developed using rabbit anti-murine nephrin and rabbit anti-podocin antibodies (supplied by Dr. Larry Holzman, University of Michigan, Ann Arbor, MI). The murine monoclonal anti-podocalyxin antibody 2A4 was produced as previously reported. FITC- and Cy3-labeled secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA). Sections were mounted in DAPI-containing mounting fluid (SlowFade; Invitrogen, Eugene, OR) and photographed using a Leica DM inverted microscope (Bannockburn, IL) and a SPOT camera system (Diagnostic Instruments, Sterling Heights, MI).

Quantification of Podocin mRNA Excretion
The standard curves used for mRNA quantification were derived from known numbers of isolated glomeruli. A total of 95% of podocytes were lost from progressor rat glomeruli by the time they reached ESKD (Figure 3A). We can therefore estimate the proportion of total glomerular podocin mRNA that was recovered in the urine of progressing rats by the time they reached ESKD. This value was 0.03 ± 0.02% (1 SD; $n = 8$). This calculation assumes that the relationship between podocyte podocin mRNA in urine and in the normal glomerulus remained constant during the disease process. The data in Table 2 compare the excretion rates of podocin mRNA in different groups. Assuming there are approximately 76,000 glomeruli per rat and each rat glomerulus contains 128 podocytes, there are $9.7 \times 10^6$ podocytes per rat. These values were used to calculate the excretion of podocyte-equivalents shown in Table 2.

Urine Protein and Creatinine Assays
Urine and serum creatinine measurements were performed using the Sigma Creatinine kit (cat. no. 553-A; St. Louis, MO) or the Teco Diagnostics Creatinine kit (cat. no. C513-480; Anaheim, CA). For protein measurements, urine samples were precipitated with an equal volume of 30% TCA, dissolved in 1 M NaOH, then assayed using the Bio-Rad Protein Assay (cat. no. 500-0006; Hercules, CA) as described previously. Urine protein was expressed as the protein:creatinine ratio.

BP
BP was measured by the tail-cuff technique at approximately monthly intervals during the study period. Before measurement, the rats were anesthetized using isoflurane (4% induction and 1.5% maintenance) and placed on a warming pad to maintain their temperature. Eight tail-cuff BP measurements were taken for each rat at each time point (ITTC Life Science, Woodland Hills, CA) as previously reported. The tail-cuff method of measuring BP is less precise than direct intra-arterial monitoring and gave higher values for SBP than expected for controls. Nevertheless, this method is adequate for comparisons between groups.

Reproducibility of the hDTR Model
One potentially important reason for nonreproducibility of the model would be mosaicism of transgene expression. We therefore formally addressed this question as follows: Transgene (human HB EGF) expression was compared in homozygous, heterozygous, and wild-type Fischer 344 rat glomeruli at both the mRNA and protein levels. Rat glomerular RNA prepared as outlined already was used to measure the relative amount of transgene mRNA in relation to the podocyte marker nephrin mRNA using quantitative RT-PCR (human HB-EGF TaqMan probe spanning exons 4 to 5; cat. no. Hs00181813_m1) expressed as a ratio to nephrin mRNA (TaqMan probe spanning exons 20 to 21; cat. no. Rn00575235_m1). The homozygous rat glomerular transgene:nephrin mRNA ratio was normalized to 100%. The values obtained (mean ± 1 SD) were as follows: Homozygous 100 ± 13% ($n = 10$), heterozygous 55 ± 11% ($n = 10$), and wild-type undetectable ($n = 6$). A similar approach was used for glomerular protein expression. Isolated glomerular transgene protein expression measured by Western blot (using goat polyclonal antibody AF-259-NA; R&D Systems, Minneapolis, MN) was expressed as a ratio with the podocyte marker GLEPP1 (using monoclonal murine antibody 1B4). The homozygous rat glomerular transgene:GLEPP1 ratio was normalized to 100%. The values obtained were as follows: Homozygous 100 ± 17% ($n = 6$), heterozygous 53 ± 17% ($n = 9$), and wild-type undetectable ($n = 5$). Therefore, both transgene mRNA and protein were expressed in glomeruli at approximately 50% in heterozygotes compared with homozygotes and were undetectable in wild-type glomeruli.

To examine the relative distribution of the transgene within and between glomeruli, we used double-label immunofluorescence comparing transgene expression (using goat polyclonal AF-259-NA) with GLEPP1 expression (using monoclonal mouse antibody 1B4) in DAPI-stained sections of homozygous ($n = 8$), heterozygous ($n = 16$), and wild-type rats ($n = 5$). We found no significant discrepancies in signal expression between transgene and GLEPP1 protein expression within or between glomeruli in either homozygous or heterozygous glomeruli. No detectable signal was seen in wild-type rat glomeruli.

As a further test of reproducibility of the model to DT, we measured the effect of low-dose intravenous diphtheria toxin injections (12.5 ng/kg) in heterozygous rats. The values for urine protein:creatinine ratio at 7 d were as follows: Mean 8.4 ± 2.2 (1 SD), range 5.9 to 13.7 ($n = 13$). For comparison, the urine protein:creatinine ratio at high-dosage DT is in the 60 to 80 range. This result therefore confirms that the model was very reproducible in response to intravenous DT (in contrast with intraperitoneal injection of DT used in these studies), even at a low dosage in the steep part of the DT dose-response curve; therefore, the variability of response seen in these experiments was not due to intrinsic variability of response to DT between individual animals.

Human Urine mRNA Analysis
Urine collection was approved by the University of Michigan human studies committee (IRB HUM2468 and 4729). Random urine samples
were collected from four patients with SLE at the time of renal biopsy (Table 3). Urine samples were collected from eight normal individuals (ages 30 to 63, six men, two women) to evaluate impact of time of day, hydration, exercise, and storage of urine at 4°C and at room temperature (n = 100 samples). These urine samples were collectively used to establish a normal range. RNA from human urine was processed as described for rat urine. For quantitative RT-PCR assay, standard curves were constructed for nephrin (using a full-length 6.2-kb cDNA construct provided by Dr. Larry Holzman), podocin (using a full-length cDNA construct provided by Dr. Geraldine Mollet, Hospital Necker-Enfants Malade, Paris, France), and for AQP2 (using a 7.1-kb construct provided by Peter Deen, Nijmegen, Netherlands). TaqMan Probes (Applied Biosystems) were as follows: Nephrin Hs 00190446–m1, podocin Hs 00922492–m1, and AQP Hs 00166640–m1. Data were corrected according to the differences in molecular weight of the cDNAs used for the standard curves so as to be able to express ratios in molar terms (i.e., the MR of nephrin:AQP2 or podocin:AQP2).

Statistical Analysis
Statistical analysis was performed using StatView 5.0 for Windows (SAS Institute, Cary, NC). All results were presented as means ± SE unless otherwise stated. Significance of difference among groups was tested by Kruskal-Wallis test. When the Kruskal-Wallis test was significant, a Scheffe test was carried out for post hoc analysis. P < 0.05 was considered statistically significant. Correlations between several parameters were compared by single regression analysis.

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DISCLOSURES
None.

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