Activation of NFAT Signaling in Podocytes Causes Glomerulosclerosis

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ABSTRACT

Mutant forms of TRPC6 can activate NFAT-dependent transcription in vitro via calcium influx and activation of calcineurin. The same TRPC6 mutants can cause FSGS, but whether this involves an NFAT-dependent mechanism is unknown. Here, we generated mice that allow conditional induction of NFATc1. Mice with NFAT activation in nascent podocytes in utero developed proteinuria and glomerulosclerosis postnatally, resembling FSGS. NFAT activation in adult mice also caused progressive proteinuria and FSGS. Ultrastructural studies revealed podocyte foot process effacement and deposition of extracellular matrix. NFAT activation did not initially affect expression of podocin, synaptopodin, and nephrin but reduced their expression as glomerular injury progressed. In contrast, we observed upregulation of Wnt6 and Fzd9 in the mutant glomeruli before the onset of significant proteinuria, suggesting a potential role for Wnt signaling in the pathogenesis of NFAT-induced podocyte injury and FSGS. These results provide in vivo evidence for the involvement of NFAT signaling in podocytes, proteinuria, and glomerulosclerosis. Furthermore, this study suggests that NFAT activation may be a key intermediate step in the pathogenesis of mutant TRPC6-mediated FSGS and that suppression of NFAT activity may contribute to the antiproteinuric effects of calcineurin inhibitors.


Glomerulosclerosis is a common cause of chronic kidney disease. The glomerulus contains a number of unique cell types and structures to ensure the efficiency and selectivity of blood filtration. The glomerular filtration barrier consists of podocytes, fenestrated endothelium, and the intervening glomerular basement membrane.1,2 Podocytes play such a key role in maintaining the structural and functional integrity of the filtration barrier that the progression of glomerular disease almost always has podocyte involvement.3 Primary insults to podocytes tend to change the cytoskeleton eventually, leading to podocyte foot process effacement and proteinuria.4,5 A common consequence of podocyte injury is the development of glomerulosclerosis characterized by the accumulation of extracellular matrix and progressive loss of filtration function.3,6–8 When glomerulosclerosis involves glomeruli focally (<50%) and involves limited segments of the affected glomerulus (<50%), it is described as FSGS.9,10 FSGS can progress to global glomerulosclerosis and renal failure.

Calcineurin is a Ca2+-dependent serine/threonine phosphatase composed of a catalytic subunit, CnA, and a regulatory subunit, CnB.11,12 Calcineurin has a wide range of roles in organ development and cellular functions,13,14 including the reg-
ulation of transcription in various renal cells. The NFATc transcription factors are the most extensively studied calcineurin substrates and the major regulators of transcription in response to Ca\(^{2+}\)/calcineurin signals. Upon activation by increased intracellular Ca\(^{2+}\), calcineurin dephosphorylates the NFATc proteins that reside in the cytoplasm in resting cells. This dephosphorylation exposes the concealed nuclear localization signals of the NFATc proteins, leading to the cytoplasm to nucleus translocation of these proteins. In the nucleus, the NFATc proteins form NFAT transcription complexes with their nuclear partners to control the transcription of target genes. Nuclear import and activation of NFATc are opposed by phosphorylation of NFATc by GSK3 and other NFAT kinases; however, when the serines targeted for phosphorylation by NFATkinesases and for dephosphorylation by calcineurin are changed to alanines, the modified NFATc1 protein becomes constitutively nuclear and insensitive to either the control of NFAT kinases or calcineurin.

Calcineurin inhibitor cyclosporine (CsA) is a potent immunosuppressive drug, owing to its inhibitory effect on NFAT activation in T cells. CsA has also been used to treat nephrotic syndrome and FSGS, especially those resistant to steroid treatment. Although the efficacy of CsA was thought to derive from its suppression of NFAT activation in T cells through the inhibition of calcineurin, not all drugs suppressing T cell activation have protective effects on glomeruli. A recent study showed that calcineurin dephosphorylates synaptopodin, a podocyte foot process cytoskeletal component, facilitating its degradation by Cathespin L. This study thus identified a new calcineurin substrate in podocytes, and the authors attributed the antiproteinuric effect of CsA to its inhibition of calcineurin-mediated degradation of synaptopodin. Although arguing against an antiproteinuric role of CsA through the suppression of T cells, this study did not rule out the involvement of NFATc proteins downstream of calcineurin in podocytes. Because NFATc proteins are important substrates of calcineurin, it is possible that, in parallel to synaptopodin regulation, there is an additional pathway from calcineurin to glomerulosclerosis that involves NFATc-mediated regulation of known and novel factors important for podocyte function.

TRPC6 is a member of the large transient receptor potential superfamily of nonselective cation channels. After Winn et al., discovered a mutation in TRPC6 in familial autosomal dominant FSGS, many more TRPC6 mutations have been found in patients with FSGS. Interestingly, TRPC6 mutations associated with FSGS are dominant mutations representing potential gain-of-function alleles. Because activation of TRPC6 is thought to result in a rise of intracellular calcium level, many calcium-dependent factors and pathways, including calcineurin, may be potential targets of TRPC6 activation in podocytes. In fact, a recent study showed that TRPC6 mutants associated with FSGS cause activation of NFAT-depentent transcription in cultured cells. Is it possible that the pathogenic effect of TRPC6 mutations is mainly or partially delivered through NFAT activation? To investigate the possibility that NFAT activation itself is sufficient to cause glomerulosclerosis in vivo, we generated a murine model in which podocyte-specific expression of an active form of NFATc1 is induced by the administration of doxycycline. Activation of NFAT signaling in podocytes resulted in proteinuria and glomerulosclerosis, suggesting a role for NFAT signaling in podocyte function and in the pathogenesis of glomerulosclerosis.

RESULTS

Generation of a Murine Model in Which NFAT Signaling Can Be Conditionally Activated in Podocytes

To study the role of NFAT signaling in podocytes and glomerular disease, we created a system for inducible activation of NFAT signaling in podocytes (Figure 1, A and B). In this system, a Podocin-Cre transgene was used to induce the removal of the transcriptional stop cassette in a ROSA26-rtTA allele only in podocytes. Once this cassette is deleted, the ROSA26 promoter drives the production of reverse tetracycline-controlled transactivator (rtTA) in podocytes. In the presence of doxycycline, the doxycycline–rtTA complex binds to the TetO sequence of the TetO-NFATc1Nuc transgene (to induce the transcription of NFATc1Nuc, an activated form of NFATc1). We refer to mice carrying all three alleles (Podocin-Cre, ROSA26-rtTA, and TetO-NFATc1Nuc) as mutants. Their littermates without the full set of three alleles cannot express NFATc1Nuc, even in the presence of doxycycline, and thus are regarded as controls. Indeed, NFATc1Nuc transcripts were detected in glomeruli isolated from mutants but not from controls after doxycycline treatment (Figure 1C). Besides weak cytoplasmic signal, we did not observe significant nuclear presence of NFATc1 in control podocytes (WT1) with or without doxycycline treatment (Figure 1, D and E). In mutants treated with doxycycline, 100% of the glomeruli contained podocytes showing nuclear staining that represented the transgene-produced NFATc1Nuc. Nuclear presence of NFATc1Nuc at various levels was detected in approximately half of the WT1 podocytes (51%, n = 798) in these mutants (Figure 1, F and G, and data not shown). We cannot rule out the possibility that NFATc1Nuc was present in the nucleus and functional in the remaining WT1 podocytes because it is not unusual for proteins to have measurable functions at levels below detection limits of immunostaining.

Activation of NFAT Signaling in Podocytes Leads to Proteinuria

In this report, we primarily discuss two modes of doxycycline treatment to induce NFAT activation. In the first, mating pairs of appropriate genotypes were treated with doxycycline so that NFATc1 activation in the podocytes of their offspring began from the time when podocytes first appeared (Figure 1H, group 1). Mice born from such matings were monitored for proteinuria and killed at various points to study potential histopathologic and molecular changes. The second mode of treatment began at weaning after nephrogenesis is complete.
Doxycycline-induced NFATc1 activation in podocytes leads to proteinuria. (A) Podocin-Cre shows podocyte-specific expression as revealed by the ROSA-lacZ reporter. (B) Strategy for doxycycline-induced NFATc1 activation in podocytes. (C) RT-PCR detects NFATc1 transgene transcripts only in the mutants. (D through G) Immunostaining shows significant nuclear presence of NFATc1 in the doxycycline-treated mutant podocytes (WT1+) but not in similarly treated control podocytes. (H) Group 1 receives doxycycline from conception; group 2 receives it after weaning. (I through K) Urine samples (2 μl each lane on SDS-PAGE) from mice treated since conception (E0; I) and from postnatal day 24 (P24; J and K). Two and 8 weeks are the time from treatment to urine collection. CT, control; MT, mutant; Dox, doxycycline.

Treatment continued for variable periods of time (Figure 1H, group 2). We again monitored proteinuria and studied the pathologic changes in the kidney at various end points from 2 days to approximately 4 months after the start of the doxycycline treatment. Doxycycline treatment in group 1 mimics NFATc1 activation caused by germline mutations, whereas group 2 is associated with postdevelopmental/adult-onset NFATc1 activation. We detected albuminuria by SDS-PAGE in mutants from both groups (Figure 1, I through K), providing the first in vivo evidence that NFAT signaling in podocytes can have detrimental effects on podocytes and glomerular function.

**Doxycycline-Induced NFATc1 Activation In Utero Leads to Postnatal Glomerulosclerosis**

We harvested kidneys from mice treated with doxycycline since conception (group 1) at approximately 2 to 6 months of age. Periodic acid–Schiff staining and silver staining revealed abnormalities in mutant glomeruli (Figure 2). The control glomeruli had patent capillary loops, normal basement membrane in Bowman’s capsule and tubules, and no aberrant matrix deposits in the mesangium (Figure 2, A and G). All mutants examined showed focal segmental or global glomerulosclerosis (Figure 2, B through F, H, and I). Some glomeruli exhibited mild mesangial matrix expansion, adhesion of the capillary loops to Bowman’s capsule, and localized thickening of the Bowman’s capsule basement membrane (Figure 2, B through F, H, and I). The findings in these mutants resemble the pathologic findings of FSGS in humans. The percentage of affected glomeruli varied from <20 to >90% in different mice.

**Doxycycline-Induced NFATc1 Activation after Weaning also Leads to Glomerulosclerosis**

Next we wished to determine whether podocyte activation of NFATc1 at postdevelopmental stages, aiming to imitate glomerular injury in human adults, would also lead to glomerulosclerosis. We thus performed the second set of experiments in which mice were treated after weaning (group 2). A slight increase in urine protein-to-creatinine ratio was observed only in the mutants after 2 days of doxycycline treatment. Significant proteinuria was observed in the mutants 7 and 14 days after NFATc1 activation (Figure 3). Glomerulosclerosis was evident 2 to 8 weeks after initiation of doxycycline treatment (Figure 4). The degree of pathologic changes was very similar to that seen in group 1 samples. Segmental or global sclerosis was present in approximately 30 to 90% of mutant glomeruli in different animals. Although absent in the controls (Figure 4A), thickening of Bowman’s capsule basement membrane, expansion of the mesangial matrix, capillary loop adhesion to Bowman’s capsule, and glomerulosclerosis were present in the mutants (Figure 4, B through E). This provides a strong indication that postdevelopmental activation of NFAT signaling in podocytes is sufficient to cause glomerulosclerosis.

**Ultrastructural Changes in Mutant Glomeruli with NFATc1 Activation in the Podocytes**

We performed electron microscopy (EM) to identify ultrastructural changes in mutant glomeruli. In the example in Figure 5, the kidney from a control mouse treated from embryonic day 0 had normal glomerular structures postnatally (Figure 5A). The kidney from a similarly treated littermate mutant harvested at the same time showed podocyte foot process effacement (Figure 5B). We further examined control and mutant littersmates treated after weaning for 2 weeks (Figure 5, C and D). Foot process effacement was again observed only in the mutants (Figure 5D). These changes resemble those seen in human FSGS. The fenestrated endothelium did not show major differences between controls and mutants in any of the treatment groups (Figure 5).

**Figure 1.** Doxycycline-induced NFATc1 activation in podocytes leads to proteinuria. (A) Podocin-Cre shows podocyte-specific expression as revealed by the ROSA-lacZ reporter. (B) Strategy for doxycycline-induced NFATc1 activation in podocytes. (C) RT-PCR detects NFATc1 transgene transcripts only in the mutants. (D through G) Immunostaining shows significant nuclear presence of NFATc1 in the doxycycline-treated mutant podocytes (WT1+) but not in similarly treated control podocytes. (H) Group 1 receives doxycycline from conception; group 2 receives it after weaning. (I through K) Urine samples (2 μl each lane on SDS-PAGE) from mice treated since conception (E0; I) and from postnatal day 24 (P24; J and K). Two and 8 weeks are the time from treatment to urine collection. CT, control; MT, mutant; Dox, doxycycline.
To determine whether NFAT activation in podocytes affects proliferation or causes podocyte apoptosis, we examined tissue from mutants with proteinuria and glomerulosclerosis and tissue from mutants treated with doxycycline for only 2 days, before overt proteinuria had occurred (Figure 6, A through J). We failed to detect significant proliferation or apoptosis of podocytes or other glomerular cells. Absence of significant
proliferation in mature podocytes indicates that glomerular changes associated with NFAT activation are different from those seen in proliferative glomerular diseases. In fact, NFAT activation caused podocyte loss but not increase. For samples collected from mice treated for 2 days, the controls had 20.0/110065.4 podocytes per glomerulus, whereas the mutants had 19.2/110064.6 podocytes. There was no significant difference (P < 0.399). After 2 weeks of treatment, however, the mutants had significantly fewer podocytes (11.6/110065.6 per glomerulus) than controls (21.9/110065.9 per glomerulus; P < 0.001). The absence of detectable apoptosis of the podocytes in the mutants may be due to detection limitations, although podocyte loss by other means has not been ruled out.33–35 The expression of podocin, synaptopodin, and nephrin was not significantly changed in podocytes with NFAT activation for 2 days, before proteinuria occurs (Figure 6, K through V); however, by 2 weeks after NFAT activation, reduction of expression of all of these genes was apparent (Figure 6, K through V), consistent with the ongoing glomerulosclerosis. In particular, the reduction of Nephrin expression was seen even in the WT1+ podocytes remaining in the glomerulus (Figure 6, U and V). Desmin staining was not significantly changed at the 2-day time point but was upregulated in the glomerulus and interstitium at the 2-week time point, consistent with mesangial expansion and fibrosis observed in the mutant kidneys (Figure 6, K through N).

Because alteration in Wnt signaling as a result of NFAT activation was suspected in other systems,20,22 we screened all 19 Wnt and all 10 Fzd genes for expression changes in mutant glomeruli by reverse transcriptase–PCR (RT-PCR). Wnt6 and Fzd9 were selectively upregulated (Figure 7A). Rcan1, a known target for NFAT signaling in mul-

Figure 4. Postdevelopmental activation of NFATc1 in podocytes leads to glomerulosclerosis. (A through E) Glomeruli from control (A) and mutant (B through E) mice treated with doxycycline after weaning. (B and C) Kidneys are harvested approximately 2 weeks after the initiation of doxycycline induction. B shows segmental sclerosis (white arrow) and expansion of the parietal layer (white triangle). C shows extensive matrix expansion and capillary loop adhesion to the Bowman’s capsule (white arrows). (D and E) Kidneys are harvested 16 weeks after the initiation of doxycycline induction at weaning. Bowman’s capsule basement membrane thickening is apparent in D (black arrow). Sclerosis is seen in both D and E. Asterisks in C and E mark dilated tubules filled with proteinaceous materials.

Figure 5. Ultrastructural changes in mutant glomeruli with NFATc1 activation in podocytes. (A) Normal foot processes (black arrows) and other structures in a control treated from conception (E0) until sample harvest at 5 months of age. (B) the glomerulus of a similarly treated littermate mutant shows podocyte foot process effacement (black arrows). Bars = 2 µm in A and B. (C) A control treated after weaning for 2 weeks shows normal podocyte foot processes (black arrows) and other structures. (D) A similarly treated mutant shows foot process effacement and irregularity (black arrows). Bars = 500 nm in C and D. WT1+ podocytes per glomerulus, whereas the mutants had 19.2 ± 4.6 podocytes. There was no significant difference (P = 0.399). After 2 weeks of treatment, however, the mutants had significantly fewer podocytes (11.6 ± 5.6 per glomerulus) than controls (21.9 ± 5.9 per glomerulus; P < 0.001). The absence of detectable apoptosis of the podocytes in the mutants may be due to detection limitations, although podocyte loss by other means has not been ruled out.33–35 The expression of podocin, synaptopodin, and nephrin was not significantly changed in podocytes with NFAT activation for 2 days, before proteinuria occurs (Figure 6, K through V); however, by 2 weeks after NFAT activation, reduction of expression of all of these genes was apparent (Figure 6, K through V), consistent with the ongoing glomerulosclerosis. In particular, the reduction of Nephrin expression was seen even in the WT1+ podocytes remaining in the glomerulus (Figure 6, U and V). Desmin staining was not significantly changed at the 2-day time point but was upregulated in the glomerulus and interstitium at the 2-week time point, consistent with mesangial expansion and fibrosis observed in the mutant kidneys (Figure 6, K through N).

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was also upregulated (Figure 7B). Similar to Rcan1, both Wnt6 and Frz9 have multiple potential NFAT binding sites within 2 kb of their transcriptional initiation sites, making them potentially direct transcriptional targets of NFATc1. By real-time PCR, we further confirmed that these genes were expressed at higher levels in the mutants (Wnt6 98.5 ± 45.9, P < 0.01; Fzd9 25.5 ± 4.1, P < 0.02; Rcan1 12.1 ± 1.1, P < 0.01; the numbers reflect fold changes in mutants); however, IL2, a well-established NFAT target in T cells, was not significantly expressed or changed (data not shown), consistent with previous studies in various settings that the effects of NFAT activation has cell type specificity.14 The expression of Snail, a gene reported to be affected by Wnt activation in podocytes,39 was not changed (Figure 7A), indicating a complex mechanism of Wnt action in podocytes with potentially different sets of players involved in different settings. In addition, we did not detect any significant changes in Podocin and Nephrin at this stage (Figure 7A), suggesting that they may not be the immediate and direct targets of NFAT activation.

**DISCUSSION**

In this report, we describe a model for conditional NFAT activation in podocytes and provide in vivo evidence that NFAT activation, either in utero or postdevelopmentally, can lead to multiple tissues.
proteinuria and glomerulosclerosis. These findings, combined with previous studies of TRPC6 mutations associated with glomerulosclerosis in human patients, suggest that activation of NFAT signaling may be a key pathogenic molecular change in at least a subset of glomerulosclerosis cases. The effectiveness of CsA for the treatment of nephrotic syndrome (especially the idiopathic and steroid-resistant cases) was initially attributed to its immunosuppressive effects, but the study of Faul et al. suggested a novel role for CsA in inhibiting the dephosphorylation of synaptopodin by calcineurin. It is possible, however, that the suppression of NFAT signaling in podocytes also plays a role in the antiproteinuric effect of CsA (Figure 8).

In addition to TRPC6 mutations, the renin-angiotensin system has been shown to be capable of activating calcineurin-NFAT. There is also growing evidence supporting a role of local renin-angiotensin action on podocytes. The antiproteinuric effects of angiotensin-converting enzyme inhibitors and angiotensin II blockers deserve further investigation. Furthermore, NFATc1 activation can occur as a result of genetic mutations and environmental factors affecting the phosphorylation of NFATc1 and/or its translocation to the nucleus. These include mutations destroying the kinase phosphorylation site on NFATc1, mutations or factors directly causing calcineurin activation (e.g., loss of the auto-inhibitory peptide of calcineurin), mutations in other genes or chemicals that could increase the intracellular calcium level and thereby activate calcineurin, or mutations and factors inhibiting the kinases that usually phosphorylate NFATc proteins to prevent their nuclear entry.

Results from this study suggest that all of these genetic and environmental alterations have the potential to cause proteinuria and glomerulosclerosis through the activation of NFATc1. The focal segmental nature of the disease may be due to multiple reasons. Podocyte damage and glomerular diseases can start from subpopulations of podocytes before the gradual involvement of more and more podocytes and glomeruli. The variation in transgene expression, as shown in Figure 1, may cause focal and regional initiation and progression of the glomerulosclerosis. In addition, there are many examples that a uniform molecular change, such as a germline mutation in TRPC6, can cause focal segmental changes.

Previous studies have shown that, instead of causing non-specific transcriptional alterations, NFATc1 activation leads to distinctive changes in transcription and cellular behavior in different cell populations. The upregulation of NFATc1 here may simply reflect a built-in regulatory loop encompassing calcineurin, NFAT, and Rcan1. Moreover, upregulation of Wnt signaling, by adriamycin and other means, was found to be detrimental to podocytes. Although the mechanisms may be different in the NFAT-activation model with different Wnt components activated, the upregulation of Wnt signaling seems to be a specific response to NFAT activation and is likely linked to the pathogenesis of podocyte dysfunction and FSGS. More efforts are required to define further the role of Wnt signaling in NFAT-induced glomerulosclerosis and to identify other targets. Although a reduction of Nephrin expression was found in some WT1+ podocytes, the effect of NFATc1 activation on Nephrin may not be direct because the reduction of Nephrin expression was not found at the early time point (2 days after doxycycline treatment).

The model used in this study has a defined molecular change (in a single transcription factor, NFATc1) in a specific cell population (podocytes) that can be turned on and off as desired. It can be used to study further the molecular and pathogenic mechanisms leading from specific transcriptional changes to glomerulosclerosis (Figure 8). Besides a better understanding of the pathogenesis of glomerulosclerosis, reveal-
ing the molecular events responsible for the initiation and progression of glomerulosclerosis may provide a rich source of target factors for therapeutic intervention. In particular, treatments aimed at NFAT or NFAT transcriptional targets may prove to be more specific and with fewer adverse effects than those aimed at more upstream factors, such as calcineurin and TRPC6.

**CONCISE METHODS**

**Mouse (Mus musculus) Strains and Urinalysis**

All animal studies were approved by the Washington University Animal Studies Committee and were conducted according to relevant National Institutes of Health guidelines. The Podocin-Cre, ROSA26-rtTA, and TetO-NFATc1Nuc strains were described previously.\(^2\)\(^0\),\(^2\)\(^1\),\(^3\)\(^1\),\(^3\)\(^2\) The PCR genotyping methods are as follows: For Podocin-Cre, we used primers FCCreF1 TCGATGCAACGAGTGATGAG and FCCreR1 TCCATGAGTGAACGAACCTG to amplify a 420-bp product. PCR conditions were as follows: 95°C for 4 minutes and 35 cycles of 94°C for 35 seconds, 59°C for 35 seconds, and 72°C for 35 seconds. For ROSA26-rtTA, we used primers ROSA5 AGTTCTCTGCTGCCTCCTG, RTTA3 AAGACCGCGAAGAGTTTGTC, and ROSA3 CGAGGCGGATACAAGCAATA to amplify a wild-type band of 322 bp and a band of 215 bp corresponding to the ROSA26-rtTA allele. PCR conditions were as follows: 95°C for 4 minutes and 35 cycles of 94°C for 35 seconds, 59°C for 35 seconds, and 72°C for 35 seconds. For TetO-NFATc1Nuc, we used primers TnF1 AAGAAGATGGTCCTGTCTGG and TnR1 GTAGTCTGGTACGTCGTAC to amplify a 351-bp product. PCR conditions were as follows: 95°C for 4 minutes and 35 cycles of 94°C for 35 seconds, 56°C for 35 seconds, and 72°C for 35 seconds. Urine was collected by bladder massage of live mice or extraction of bladder content in dead or euthanized mice. Urine samples were subjected to electrophoresis on a 10% polyacrylamide gel. Proteins were visualized by BioSafe Coomassie stain (161-0786; Bio-Rad).

**Histology and EM**

Embryos or tissues were fixed with 4% paraformaldehyde and embedded in paraffin. Sections of 5 μm were collected and stained with periodic acid-Schiff and modified Gomori methenamine silver stain (HT100A; Sigma) following manufacturer’s instructions. X-gal staining on cryostat sections was performed as described previously.\(^5\)\(^4\) For transmission EM, tissues were fixed, embedded in plastic, sectioned, and stained as described previously.\(^5\)\(^5\)

**Reverse Transcriptase–PCR**

RNA was purified from isolated glomeruli by using the Qiagen RNAeasy mini kit. Glomeruli were isolated as described previously.\(^9\)\(^6\) cDNA was prepared from RNA using Invitrogen ThermoScript RT-PCR System. PCR primer sequences were as follows: NFATc1–1 AAGAAAGATGGTCCTGTCTGG and TnR1 GTAGTCTGGTACGTCGTAC to amplify a 351-bp product. PCR conditions were as follows: 95°C for 4 minutes and 35 cycles of 94°C for 35 seconds, 56°C for 35 seconds, and 72°C for 35 seconds. Urine was collected by bladder massage of live mice or extraction of bladder content in dead or euthanized mice. Urine samples were subjected to electrophoresis on a 10% polyacrylamide gel. Proteins were visualized by BioSafe Coomassie stain (161-0786; Bio-Rad).
CACTGCAACAACGGAGG and Wtnt6-RT-R CAGAGGGCAAG-
GAAACCGAAAA; Fzd9-RT-F ATCCAGGTCTTTTCCATTTTAC
and Fzd9-RT-R CCGCCGAAATGCATGGTGAAG; Snail-RT-F GC-
CTGGGCGCTCTGAAATG and Snail-RT-R CAGTTGGGACGAG-
GAATGGGC; Podocin-RT-F AGGACAAAGACAGGCAAG
and Podocin-RT-R AATCTCGAGCCATCTCC; Nephrin-RT-F
AAGAGGTCGATACCTGGCAAGC and Nephrin-RT-R GGTG-
CATTCTTGACCTCATGAGAGG; and Rcan1-RT-F CTCCTC-
CGTGGCTGGAAAGC and Rcan1-RT-R CTTGGAATGTTGCTC-
GTCG. PCR conditions were as follows: 95°C for 4 minutes and 35
cycles of 94°C for 35 seconds, 59°C for 35 seconds, and 72°C for 35
seconds). Real-time PCR was conducted in an ABI-7900HT system
using the following conditions: 95°C for 20 seconds and 40 cycles
of 95°C for 5 seconds and 60°C for 20 seconds.

**Immunostaining**

Immunostaining on cryostat sections was performed as described
previously.57 Antibodies used were rabbit polyclonal anti-podocin
(P0372, 1:500; Sigma), mouse monoclonal anti-desmin (M0760,
1:200; DAKO), rabbot polyclonal and mouse monoclonal anti-WT1
(sc192 and sc7345, 1:50; Santa Cruz Biotechnology), goat polyclonal
anti-neprhin (AF3159, 1:100; R&D Systems), rabbit polyclonal anti-
caspase 3 (#9661, 1:100; Cell Signaling), mouse monoclonal anti-
NFATc1 (556602, 1:100; PharMingen), and mouse monoclonal anti-
caspase 3 (#9661, 1:100; Cell Signaling), mouse monoclonal anti-
desmin (M0760, 1:200; DAKO), rabbit polyclonal and mouse monoclonal anti-WT1
(P0372, 1:500; Sigma), mouse monoclonal anti-desmin (M0760,
1:200; DAKO), rabbot polyclonal and mouse monoclonal anti-WT1
(sc192 and sc7345, 1:50; Santa Cruz Biotechnology), goat polyclonal
anti-neprhin (AF3159, 1:100; R&D Systems), rabbit polyclonal anti-
caspase 3 (#9661, 1:100; Cell Signaling), mouse monoclonal anti-
NFATc1 (556602, 1:100; PharMingen), and mouse monoclonal anti-
synaptotocin (1:300; gift from Dr. Kriz). AlexaFlour488-conjugated,
AlexaFlour555-conjugated, and NorthernLights-557–conjugated
secondary antibodies (1:500; Molecular Probes) were used for detec-

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**DISCLOSURES**

None.

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