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.11,12,15–17 The NFATc transcription factors are the most extensively studied calcineurin substrates and the major regulators of transcription in response to Ca\(^{2+}\)/calcineurin signals.11,13,18,19 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 NFATc kinases 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.20–22

The calcineurin inhibitor cyclosporine (CsA) is a potent immunosuppressive drug, owing to its inhibitory effect on NFAT activation in T cells.11,12 CsA has also been used to treat nephrotic syndrome and FSGS, especially those resistant to steroid treatment.23–25 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.23 A recent study showed that calcineurin dephosphorylates synaptopodin, a podocyte foot process cytoskeletal component, facilitating its degradation by Cathespin L.26 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 degradation of synaptopodin. We refer to mice carrying all three alleles (Podocin-Cre, ROSA26-rtTA, and Tet-O-NFATc1Nuc) as mutants. Their littermates without the full set of three alleles cannot express NFATc1, 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.

**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* transgene31 was used to induce the removal of the transcriptional stop cassette in a ROSA26-rtTA allele32 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 Tet-O-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.
NFATc1 activation caused by germline mutations, whereas Doxycycline-Induced NFATc1 Activation in the Podocytes

NFATc1 activation in vivo

Podocytes (WT1) show significant nuclear presence of NFATc1 in the doxycycline-treated mutant. RT-PCR detects NFATc1 transgene transcripts only in the mutants. (D through G) Immunostaining with NFATc1 porter. (B) Strategy for doxycycline-induced NFATc1 activation in podocytes. (C) RT-PCR shows podocyte-specific expression as revealed by the ROSA-lacZ reporter. (A) Podocin-Cre shows podocyte-specific activation 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 J) 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 littermates 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).
Proliferation, Apoptosis, and Potential Molecular Changes in Glomeruli after Podocyte-Specific Activation of NFAT Signaling

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

Figure 2. NFATc1 activation in utero results in postnatal glomerulosclerosis. (A through I) Glomeruli from control (A and G) and mutant (B through F, H, and I) mice treated with doxycycline since conception. Kidneys are collected at approximately 3 months after birth. (A through F) Periodic acid–Schiff staining. (G through I) Silver staining. Black arrows point to thickened basement membrane of Bowman's capsule and proximal tubules (B, C, E, and H) or proximal tubule (D). White arrows in C, E, and F point to sclerotic areas. Whereas sclerosis is segmental in C and E, it is global in F. Asterisks in E and I mark proteinaceous materials in tubules.

Figure 3. Postdevelopmental activation of NFAT signaling in podocytes causes progressive proteinuria. Total protein-to-creatinine ratio in urine revealed a relatively rapid increase in the level of proteinuria in mutants treated with doxycycline to induce NFATc1 activation at weaning. Total protein-to-creatinine ratio on day 0 for control is 22.75 ± 3.89 (n = 11) and for mutant is 22.36 ± 6.13 (n = 8; P = 0.873). On day 2, control is 20.54 ± 3.92 (n = 8) and mutant is 27.81 ± 7.80 (n = 8; P = 0.045). On day 7, control is 21.82 ± 3.52 (n = 7) and mutant is 79.60 ± 40.97 (n = 6; P = 0.006). On day 14, control is 22.75 ± 7.18 (n = 6) and mutant is 122.63 ± 80.21 (n = 6; P = 0.012).
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 ± 5.4 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).

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-
was also upregulated (Figure 7B). Similar to Rcan1, both Wnt6 and Fz9 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. The expression of Snail, a gene reported to be affected by Wnt activation in podocytes, was also 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
NFAT.4 0–4 3

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also, that the suppression of NFAT signaling in podocytes
dephosphorylation of synaptopodin by calcineurin. It ispossi-

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NFAT signaling,

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attributed to its immunosuppressive effects, but the study of

angiotensin receptor blockers seem to go beyond their sys-

teinuric effects of angiotensin-converting enzyme inhibitors and

angiotensin II blockers deserves further investigation. Further-

more, NFATc1 activation can occur as a result of genetic mu-
tations and environmental factors affecting the phosphoryla-
ton 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 cal-
cineurin), mutations in other genes or chemicals that could
increase the intracellular calcium level and thereby activate cal-
cineurin, or mutations and factors inhibiting the kinases that
usually phosphorylate NFATc proteins to prevent their nuclear
entry.13,14,51,52 Results from this study suggest that all of these
genetic and environmental alterations have the potential to
cause proteinuria and glomerulosclerosis through the activa-
tion 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 glomer-
ulii. 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.20–22 Rcan1, Wnt6, and Fzd9 were
found to be upregulated in mutant glomeruli before significant
pathophysiologic changes, making them potentially direct tar-
gets of NFATc1. Rcan1 is a known target of NFAT and can
either inhibit or facilitate calcineurin-NFAT signaling under
different circumstances.36–38,53 The upregulation of Rcan1 here
may simply reflect a built-in regulation loop encompassing
calcineurin, NFAT, and Rcan1. Moreover, upregulation of
Wnt signaling, by adriamycin and other means, was found to
be detrimental to podocytes.39 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
seen 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 un-
derstanding of the pathogenesis of glomerulosclerosis, reveal-

proteinuria and glomerulosclerosis. These findings, combined
with previous studies of TRPC628–30 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 effective-
ness of CsA for the treatment of nephrotic syndrome (espe-
cially the idiopathic and steroid-resistant cases) was initially
attributed to its immunosuppressive effects, but the study of
Faul et al.26 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 sys-
m has been shown to be capable of activating calcineurin-
NFAT.40–43 There is also growing evidence supporting a role of
local renin-angiotensin action on podocytes.44–48 The antipro-
teinuric effects of angiotensin-converting enzyme inhibitors or
angiotensin receptor blockers seem to go beyond their sys-
temic function in BP control to include direct effects on podo-
cytes.49,50 Thus, the potential involvement of NFAT signaling
in angiotensin-induced podocyte injury or in the antiprotein-

Figure 7. Increases in the transcription of selected Wnt signaling
components in the glomeruli of mice with podocyte-specific
NFAT activation. (A) RT-PCR using RNA purified from isolated
glomeruli of 4-week-old mice treated with doxycycline for 2 days.
All mutants (MT) express the NFATc1\textsuperscript{Nuc} transgene. No controls
(CT) show expression of NFATc1\textsuperscript{Nuc}. Among all 19 known Wnt
genes and all 10 known Fzd genes examined, only Wnt6 and Fzd9
show significant changes in mutants. Expression of Snail, Podocin,
and Nephrin remain unchanged. (B) A widely known target for
NFAT signaling, Rcan1, is upregulated in mutants with podo-
cyte-specific NFAT activation.

A

MT MT MT MT MT MT MT

GAPDH

NFATc1

Wnt6

Fzd9

Snail

Podocin

Nephrin

B

MT MT MT MT MT MT MT

GAPDH

RCAN1


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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.20,21,31,32 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.54 For transmission EM, tissues were fixed, embedded in plastic, sectioned, and stained as described previously.55

Reverse Transcriptase–PCR

RNA was purified from isolated glomeruli by using the Qiagen RNAeasy mini kit. Glomeruli were isolated as described previously.26 cDNA was prepared from RNA using Invitrogen ThermoScript RT-PCR System. PCR primer sequences were as follows: NFATc1–1 AAGAAGATGGTGCTGCTG and TN1 R1 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).
CAACTGCAACAACAGGAG and Wnt6-RT-R CAGAGGCAGCG -
GAACCCGAAA; Fzd9-RT-F ATCGGAGTCTTTTCCCATCTCTTAC
and Fzd9-RT-R CCGCCAGAAGTCCATGTTGAG; Snail-RT-F GC-
CTGGGGCTCCTGAAGATG and Snail-RT-R CAGTGGGGACGAC-
GAATGGGC; Podocin-RT-F AGGACAAAGACAGGCAAG
and Podocin-RT-R AATCTCAGGGCCATCTCTCA; Nephrin-RT-F
AAGAGGTGCTATGCGGCAAGC and Nephrin-RT-R GTGC-
CATTGTACTCTCATACAGG; and Rcan1-RT-F CTCCTC-
CCGTGGCTGGAAA and Rcan1-RT-R CTGGGATGTTGTCT-
CTGCGC. 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. Antibodies used were rabbit polyclonal anti-podocin
(P0372, 1:500; Sigma), mouse monoclonal anti-desmin (M0760,
1:200; DAKO), rabbit polyclonal and mouse monoclonal anti-WT1
(sc192 and sc7345, 1:50; Santa Cruz Biotechnology), goat polyclonal
anti-nephrin (AF3159, 1:100; R&D Systems), rabbit polyclonal anti-
caspase 3 (#9661, 1:100; Cell Signaling), mouse monoclonal anti-
anti-nephrin (AF3159, 1:100; R&D Systems), rabbit polyclonal anti-
Wt1 (P0372, 1:500; Sigma), mouse monoclonal anti-desmin (M0760,
1:200; DAKO), rabbit polyclonal and mouse monoclonal anti-WT1
(sc192 and sc7345, 1:50; Santa Cruz Biotechnology), goat polyclonal
anti-nephrin (AF3159, 1:100; R&D Systems), rabbit polyclonal anti-
caspase 3 (#9661, 1:100; Cell Signaling), mouse monoclonal anti-

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