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p35, the non-cyclin activator of Cdk5, protects podocytes against apoptosis *in vitro* and *in vivo*

Paul T. Brinkkoetter[1,2], Jimmy S. Wu[1], Takamoto Ohse[1], Ronald D. Krofft[1], Bernhard Schermer[2], Thomas Benzing[2], Jeffrey W. Pippin[1] and Stuart J. Shankland[1]

[1]Division of Nephrology, Department of Medicine, University of Washington, Seattle, Washington, USA and [2]Department of Medicine and Centre for Molecular Medicine and Cologne Excellence Cluster on Cellular Stress Responses in Aging-Associated Diseases, University of Cologne, Cologne, Germany

Cyclin-dependent kinase-5 is widely expressed and predominantly regulated by the non-cyclin activator p35. Since we recently showed that expression of p35 in the kidney is restricted to podocytes, we examined here its function in mice in which p35 was genetically deleted. The mice did not exhibit kidney abnormalities during glomerular development or during adult life. Conditionally immortalized cultured podocytes, derived from these null mice, did not have any change in their morphology, differentiation, or proliferation. However, when these cultured podocytes were exposed to UV-C irradiation, serum depletion, puromycin aminonucleoside, or transforming growth factor-β-1, they showed increased apoptosis compared to those from wild-type mice. Levels of Bcl-2 were decreased in these null podocytes but increased after transduction with human p35. Restoration of p35 or the ectopic expression of Bcl-2 reduced the susceptibility of p35-null podocytes to apoptosis. Experimental glomerulonephritis, characterized by podocyte apoptosis and subsequent crescent formation, was utilized to test these findings *in vivo*. Podocyte apoptosis was significantly increased in diseased p35-null compared with wild-type mice, accompanied by increased glomerulosclerosis and decreased renal function. Our study shows that p35 does not affect glomerulogenesis but controls podocyte survival following injury, in part, by regulating Bcl-2 expression.

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Correspondence: *Stuart J. Shankland, Division of Nephrology, Department of Medicine, University of Washington, Box 356521, BB-1265 HSB, 1959 NE Pacific Street, Seattle, Washington 98195-6521, USA. E-mail: stuartjs@uw.edu*

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Glomerular diseases are a leading cause of chronic and end-stage kidney failure. The terminally differentiated and highly specialized glomerular epithelial cell, the podocyte, is a critical component of the glomerular filtration barrier, and is required for normal glomerular integrity. A major mechanism underlying the development of glomerulosclerosis and proteinuria in disease is reduced podocyte number resulting from apoptosis, detachment, and the inability to adequately proliferate. Podocyte apoptosis has been reported in diabetic kidney disease,[1] membranous nephropathy,[2] HIV-associated nephropathy, and focal segmental glomerulosclerosis.[3,4]

Contrary to earlier belief that cell-cycle protein function was limited to the regulation of proliferation, recent studies have shown that specific cell-cycle proteins also have biological roles governing apoptosis.[5–8] Among these is cyclin-dependent kinase (Cdk)-5, which unlike other Cdks, is not involved in the regulation of cell-cycle progression and proliferation. Cdk5 modulates cell maturation, differentiation, migration, and apoptosis in various tissues.[9–11] Within the kidney, glomerular expression of Cdk5 is limited to podocytes. In contrast to other Cdks that are activated by partner cyclins, published data show that Cdk5 is activated by non-cyclin proteins p35 or p39.[12] p35, but not p39, is constitutively expressed in podocytes and forms an active complex with Cdk5.[13] However, the role of p35 in podocytes is poorly understood.

In this study we delineated one function of p35 in podocytes and glomerular biology by using p35-null mice and cultured immortalized p35-null podocytes.

RESULTS

Characterization of conditionally immortalized p35-null podocytes

To generate cultured immortalized podocytes, wild-type and p35-null mice were crossed with H-2Kb-tsA58 mice (ImmortoMice; Jackson Laboratory, Bar Harbor, ME, USA[14]), and podocytes from the resultant wild-type and p35-null mice were isolated as previously reported (for review see reference Shankland *et al.*[15]). Quiescence and differentiation were induced by culturing cells at 37 °C for 13–15 days in the absence of interferon-γ. Under these

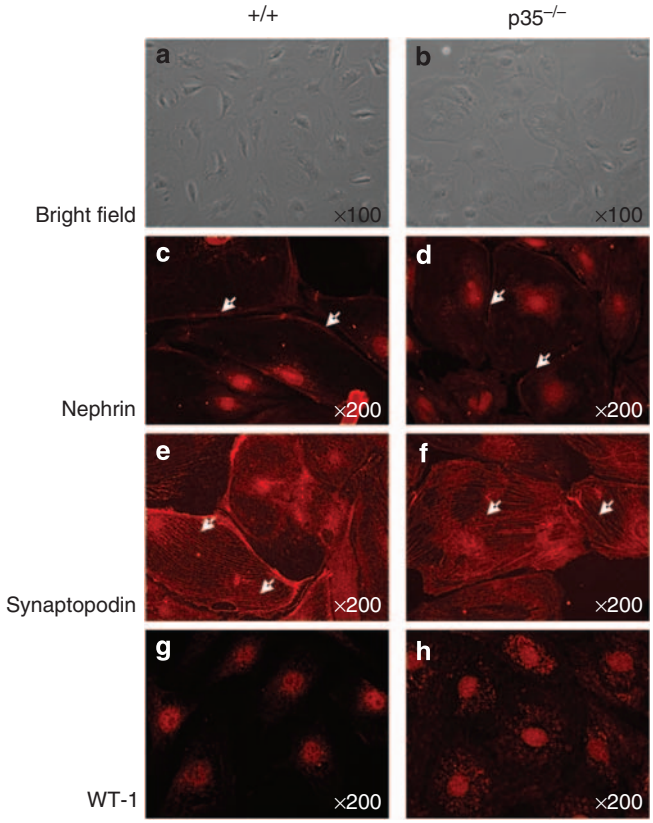

Figure 1 | Characterization of conditionally immortalized p35-null podocytes. Representative images of wild-type (left column) and p35-null (right column) podocytes at day 14: bright field (**a**, **b**), nephrin (**c**, **d**) synaptopodin (**e**, **f**), and WT-1 (**g**, **h**) staining. There were no differences in intensity or subcellular localization between p35-null and wild-type cells.

conditions, absence of p35 did not affect podocyte proliferation (data not shown) nor morphology (Figure 1). To assess the effect of p35 on podocyte differentiation, the expression of podocyte-specific marker proteins WT-1, nephrin, and synaptopodin was measured by indirect immunofluorescence (Figure 1). Both wild-type and p35-null cells showed nuclear staining for WT-1, characteristic radial distribution of synaptopodin, as well as cortical localization of nephrin. Thus, lack of p35 had no effect on podocyte differentiation in culture as judged by expression of these proteins. This observation was similar to *in vivo* results, where p35-null mice had normal kidney histology when bred under physiological conditions (see below).

Taken together, these results confirmed that both wild-type and p35-null podocytes generated for this study were indeed fully differentiated, quiescent podocytes, and were thus suitable for the proposed studies. These results also show that p35 was not essential for normal podocyte differentiation.

p35-null mice have normal renal function under physiological conditions *in vivo*

To test the role of p35 in renal development *in vivo*, wild-type Balb/C and p35-null Balb/C mice were used. Renal histology and function were evaluated in 12-week-old, male, wild-type and p35-null mice housed under non-stressed conditions. There were no significant differences in podocyte number or urinary protein excretion. Likewise, p35-null mice had no kidney histological abnormalities (by light microscopy) when bred under physiological (non-stressed) conditions. To assess podocyte differentiation, the expression of several podocyte-specific proteins was determined by immunohistochemistry (Figure 2). Absence of p35 did not affect immunostaining for WT-1, podocin, nephrin, and synaptopodin. Thus, these results were similar to that observed in cultured podocytes. Taken together, p35 alone is not required for normal glomerular development and differentiation under physiological conditions, and its absence does not affect renal function.

Cultured p35-null podocytes are more susceptible to apoptosis

To test the hypothesis that p35 governs podocyte survival and thereby sets a threshold for injury-induced apoptosis, the following forms of injury were used to induce apoptosis as we have previously reported:[16–18] (i) UV-C initiates both intrinsic and extrinsic caspase-dependent pathways of apoptosis;[19] (ii) puromycin aminonucleoside (PAN) primarily activates the intrinsic pathway independent of caspase-3 activation;[17,20] (iii) Transforming Growth Factor-β-1 (TGFβ1) induces caspase-3-dependent podocyte cell death;[18,21] and (iv) serum starvation causes caspase-3-dependent death.[22] The anti-glomerular antibody causes apoptosis that is associated with increased cleaved caspase-3 immunostaining.[23]

Apoptosis was determined by two methods: DNA condensation measured using Hoechst-33342 and caspase-3 cleavage. In response to each form of injury described above, the onset of apoptosis occurred significantly earlier in p35-null podocytes ($P<0.05$ versus wild-type cells) (Figure 3). Moreover, with each form of injury, the magnitude of apoptosis was also increased in p35-null podocytes ($P<0.05$ versus wild-type cells) (Figure 3a–f). Importantly, increased apoptosis was confirmed in p35-null podocytes by a second method, showing caspase-3 cleavage products by Western blot analysis (Figure 3g). Taken together, these results show that although the absence of p35 alone is not sufficient to induce apoptosis, but that p35 is required to prevent apoptosis induced by four different forms of injury in cultured podocytes.

Lowering p35 by siRNA in wild-type podocytes increases apoptosis

A second approach was used to confirm a role for p35 in enhancing podocyte survival and thus limiting apoptosis by transfecting wild-type podocytes with small interfering RNA (siRNA) to p35, to transiently lower p35 levels. Non-transfected podocytes and podocytes transfected with scrambled siRNA served as controls. Successful p35 gene silencing was confirmed by real-time quantitative PCR (Figure 4a). There was a 68% ± 19% reduction in p35 mRNA

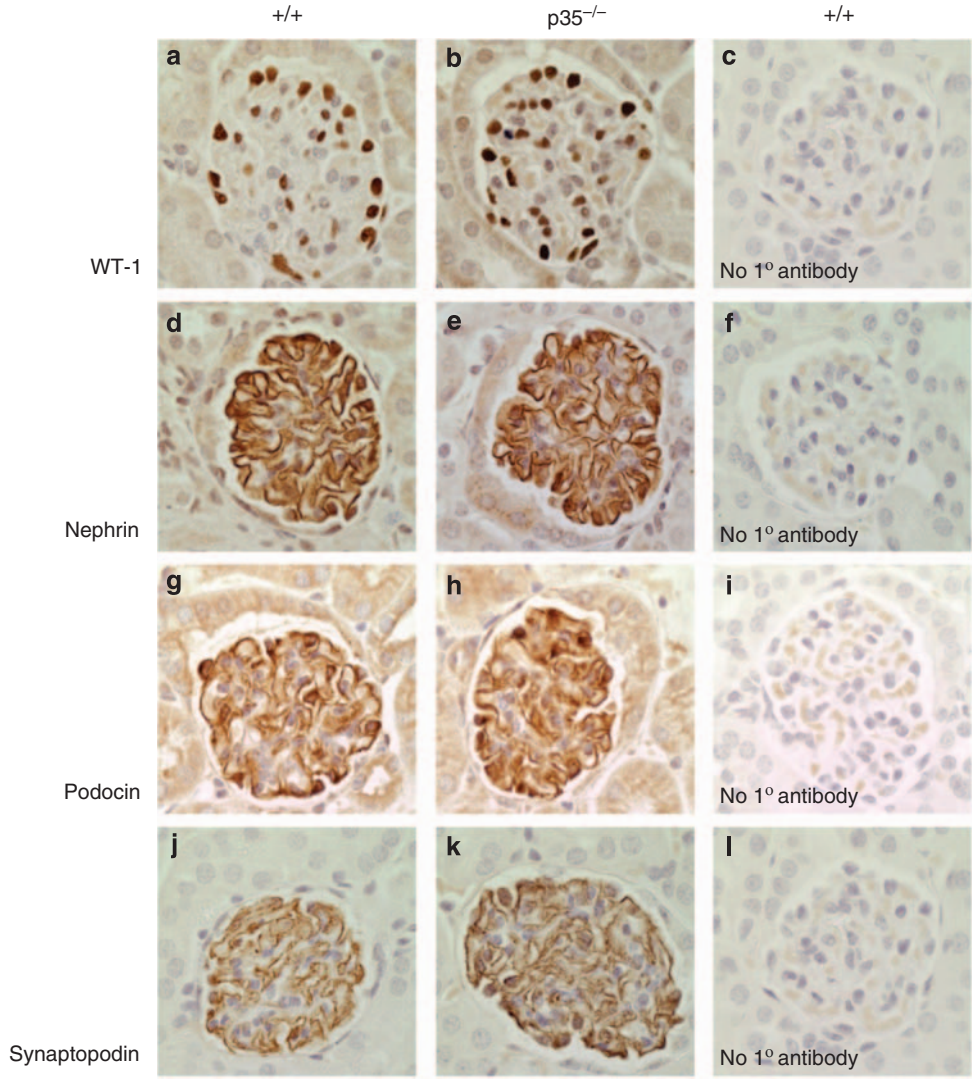

Figure 2 | p35-null mice exhibit normal glomerular and podocyte differentiation. Representative images of wild-type (left column) and p35-null (middle column) glomeruli, and immunostaining controls showing there were no differences in staining for WT-1 (**a**, **b**), nephrin (**d**, **e**), podocin (**g**, **h**) or synaptopodin (**j**, **k**) between wild-type and p35-null mice. As expected, staining was not detected when the primary antibody was omitted (**c**, **f**, **i**, **l**).

levels 48 h post transfection as compared with the house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Cells were exposed to UV-C irradiation (25 J/m^2) to induce caspase-dependent apoptosis. Caspase-3 cleavage was substantially increased after lowering p35 levels with p35 siRNA as compared with that in control siRNA-transfected wild-type podocytes (Figure 4b). These results confirm that specific depletion of p35 increases podocyte susceptibility to apoptosis following injury.

Increasing levels of p35 in p35-null podocytes decreases apoptosis

To further confirm a role for p35 in podocyte survival, p35 was re-expressed in p35-null podocytes by lentiviral gene transfer. First, p35 expression was confirmed by Western blotting. p35-null podocytes transduced with human p35.V5 revealed a strong band at approximately 37 kDa (Figure 4c). A green fluorescent protein (GFP) construct (28 kDa) served as control. Restoration of p35 protein in p35-null podocytes partially decreased UV-C-, and PAN-induced apoptosis (Hoechst-33342 staining; Figure 4d). Similar results were obtained by Western blot for caspase-3 cleavage products following UV-C (data not shown). These results further confirm a role for p35 in podocyte susceptibility to apoptosis following injury.

Restoring p35 or Bcl-2 protein expression rescues p35-null podocytes from apoptosis

To determine how p35 might regulate podocyte apoptosis, we focused on the pro-survival protein Bcl-2 as our group has previously shown that changes in Bcl-2 are involved in podocyte apoptosis.[17,18] Figure 5a shows that Bcl-2 expression is constitutive and abundant in cultured wild-type podocytes. In contrast, Bcl-2 protein levels were barely

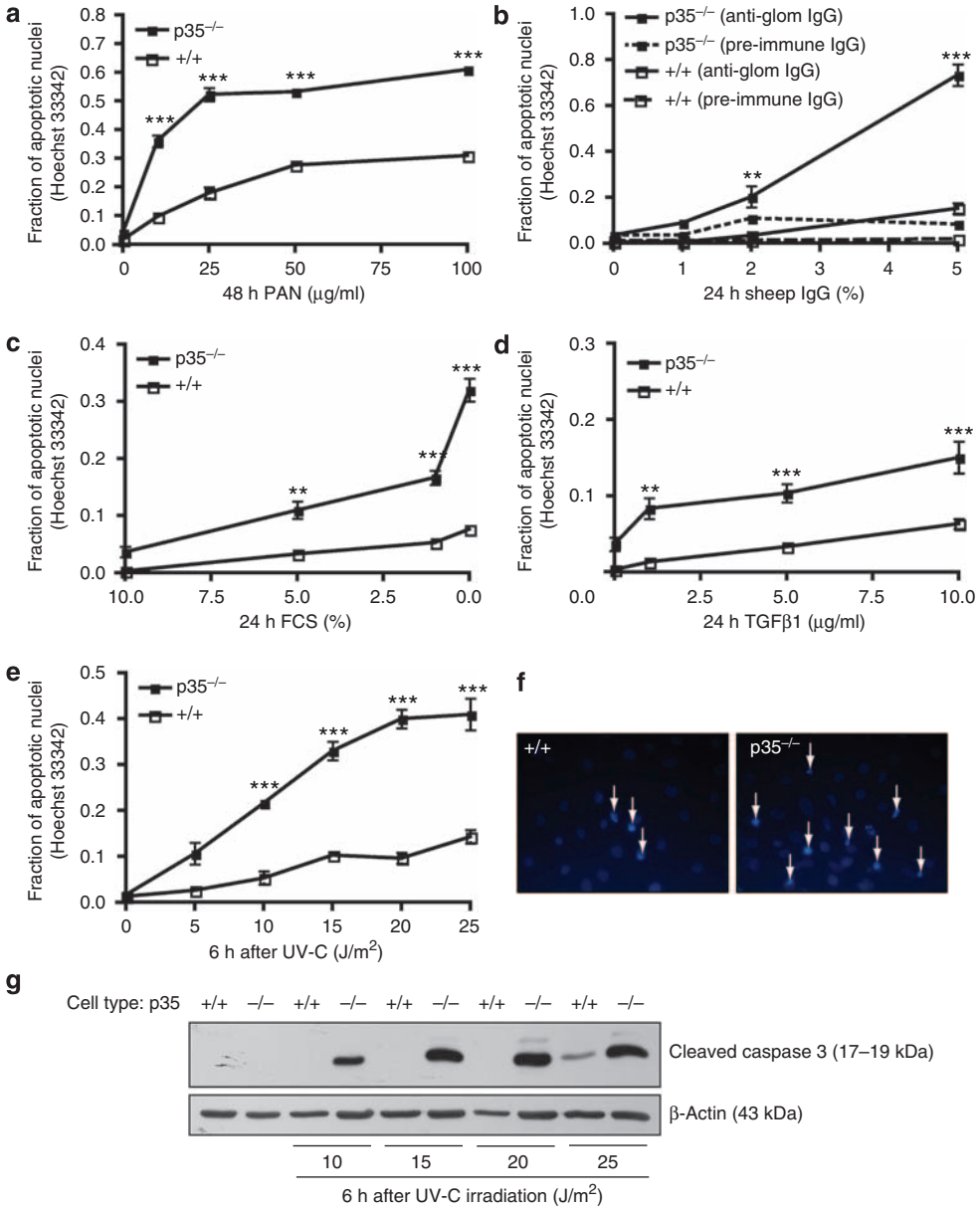

Figure 3 | Cultured p35-null podocytes are more susceptible to apoptosis. Apoptosis was quantified in non-fixed cells by nuclear Hoechst-33342 staining in wild-type (open square) and p35-null (filled square) cells under non-stressed conditions (reflected as zero in each experiment) (**a**) 48 h following exposure to PAN (0–100 μg/ml), (**b**) 24 h after exposure to anti-glomerular antibody (0–5%, pre-immune IgG served as control IgG), (**c**) 24 h after serum depletion (0–10%), (**d**) 24 h following TGFβ1 incubation (0–10 μg/ml), and (**e**) 6 h after UV-C irradiation (0–25 J/m^2). Experiments were performed in triplicates and a minimum of 300 cells were counted per experiment. For statistical analysis, analysis of variance for multiple testing was applied. *$P<0.05$; **$P<0.01$; ***$P<0.001$. (**f**) Representative photographs of Hoechst-33342 stainings following UV-C irradiation (25 J/m^2). (**g**) Increase in apoptosis in p35-null podocytes was confirmed by measuring cleaved caspase-3 by Western blot analysis 6 h following UV-C irradiation (0–25 J/m^2). β-Actin served as loading control.

detected in p35-null podocytes. To test the hypothesis that Bcl-2 protein levels underlie the pro-survival effect of p35, p35-null podocytes were stably infected with a retroviral construct (cMMP-IRES-GFP) containing human Bcl-2. As overexpression of Bcl-2 could potentially override any apoptotic phenotype, due to its important role in governing mitochondrial permeability and release of pro-apoptotic factors, the levels of ectopically expressed human Bcl-2 in p35-null podocytes were compared with those in wild-type cells. Ectopic expression of human Bcl-2 normalized Bcl-2 protein to levels comparable to endogenous levels in wild-type cells (Figure 5a). An empty plasmid served as control. Cells were then subjected to increasing doses of UV-C and cleaved caspase-3 was measured by Western blot analysis as a measure of apoptosis (Figure 5b). The results show that normalizing Bcl-2 protein levels reversed the apoptotic susceptibility of p35-null podocytes and that Bcl-2 underlies survival in podocytes.

Next, to prove that p35 indeed regulates Bcl-2 levels, we analyzed Bcl-2 protein expression in p35-null podocytes

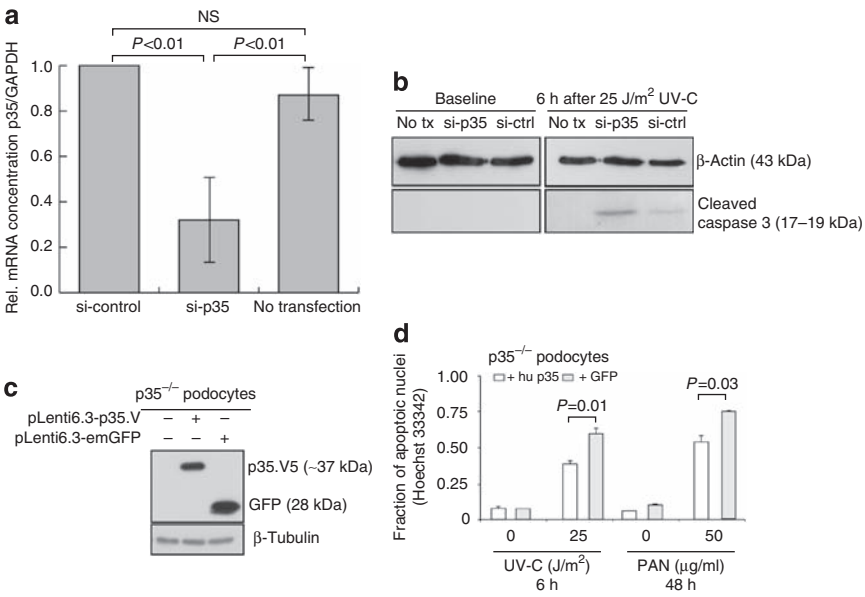

Figure 4 | p35 is a specific regulator of podocyte apoptosis. (**a**) Following p35 siRNA targeting (lane 2), p35 mRNA levels were significantly reduced as compared with control-transfected (lane 1) and non-transfected (lane 3) wild-type podocytes, ($P<0.01$, analysis of variance for multiple testing). The relative concentration of p35 is shown as a ratio of p35 normalized to the housekeeping control GAPDH. Samples were run in triplicate and mRNA from three independent experiments were included. Relative gene expression was analyzed using the 2-standard curve method. (**b**) Following p35 siRNA targeting and UV-C irradiation (25 J/m^2), caspase-3 cleavage was augmented (lane 2) in wild-type podocytes as compared with control (ctrl) siRNA-transfected cells (lane 3) or non-transfected cells (lane 1). No caspase-3 cleavage was detected in podocytes that were not exposed to UV-C. (**c**) p35 expression in p35-null podocytes was confirmed by Western blotting (lane 2). A GFP construct served as control (lane 3). β-Tubulin was included as loading control. (**d**) Apoptosis (Hoechst-33342 staining) was significantly reduced in p35-null podocytes expressing human p35 as compared with GFP-transduced cells 6 h after UV-C (25 J/m^2) irradiation and 48 h after PAN (50 μg/ml). hu, human; tx, transfection.

in which p35 expression was restored as described earlier. p35-null podocytes transduced with human p35 showed increase in Bcl-2 levels as compared with that in GFP-transduced cells (Figure 5c). These results show that the expression level of p35 influences the expression level of the pro-survival protein Bcl-2.

Podocyte apoptosis is increased in p35 null mice with experimental glomerulonephritis

Having shown decreased survival and increased susceptibility to apoptosis following injury in cultured p35-null podocytes, injury was induced *in vivo*. Experimental glomerulonephritis was induced in wild-type and p35-null mice with a sheep anti-rabbit glomeruli IgG, as previously described.[16,24] This animal model is characterized by podocyte apoptosis and subsequent glomerular crescent formation in the absence of any infiltrating leukocytes.

Comparable levels of sheep IgG deposition measured by immunofluorescence staining, was observed in wild-type and p35-null mice 7 days after injection of the anti-glomerular antibody, which induces disease (Figure 6a–c). Podocyte apoptosis was significantly increased in p35-null mice 3 days after injection (wild-type 0.04 ± 0.04 per glomerulus versus p35-null 0.08 ± 0.04 per glomerulus; $P<0.05$) as measured by terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining (Figure 6d and e). Increased apoptosis at day 3 was subsequently accompanied by decrease in podocyte number at day 7 (WT-1 staining, wild-type 3.03 ± 0.55 versus p35-null 2.15 ± 0.39; $P<0.001$; Figure 6f and g). The decrease in podocyte number at day 7 correlated with increased glomerulosclerosis in p35-null mice as compared with that in wild-type controls ($R^2 = 0.55$; Figure 7a).

The clinical consequences of increased podocyte loss and glomeruloscerosis in the absence of p35 were assessed by measuring kidney function. Both groups developed proteinuria following disease induction (Figure 7b). However, the levels of proteinuria in p35-null mice were significantly greater at day 7 (wild-type 624.15 ± 343.71 mg/dl versus p35-null $1{,}216.97 \pm 728.52$ mg/dl; $P<0.01$). Taken together these results confirm the *in vitro* findings and further emphasize the important role of p35 in podocyte survival following injury.

DISCUSSION

Podocytes lacking p35 differentiate normally

We have previously reported that p35 binds to and activates Cdk5 in glomerular protein lysates obtained from normal rats.[13] However, the role of p35–Cdk5 in podocyte biology has remained elusive. In contrast to other Cdks, Cdk5 is not directly involved in cell-cycle regulation.[9] It is expressed widely throughout the body and believed to be regulated by the presence of specific activator proteins.[25] There is a large body of literature showing that p35/39–Cdk5 is required for

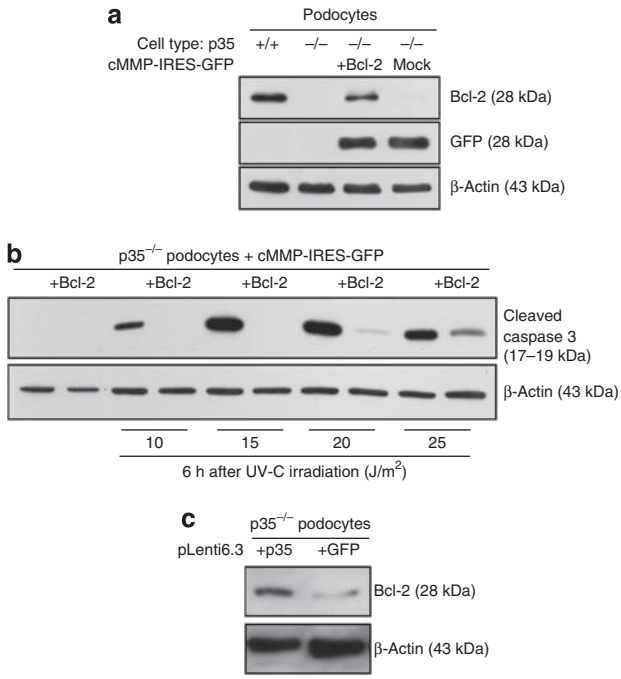

Figure 5 | Restoring Bcl-2 protein expression rescues p35-null podocytes from apoptosis. (**a**) Bcl-2 protein expression was significantly decreased in p35-null cells (lane 2) as compared with that in wild-type podocytes (lane 1). Retroviral infection with (cMMP-IRES-GFP) encoding human Bcl-2 or mock (empty) construct was confirmed by measuring GFP (lanes 3 and 4) and protein expression of Bcl-2 in p35-null podocytes (lane 3). Bcl-2 levels were comparable to endogenously expressed Bcl-2 levels in wild-type podocytes (lane 1). There was no effect of the mock (empty) vector on Bcl-2 protein levels. β-Actin served as loading control. (**b**) Apoptosis (caspase-3 cleavage) was markedly decreased in p35-null podocytes stably infected with Bcl-2 (lanes 2, 4, 6, 8, 10) as compared with that in mock (empty) vector (lanes 1, 3, 5, 7, 9)-infected p35-null podocytes 6 h after UV-C irradiation (0–25 J/m^2). (**c**) p35 expression in p35-null podocytes (lane 1) led to significant increase in Bcl-2 levels as compared with that in the GFP-transduced control (lane 2). β-Actin served as loading control.

cell maturation, differentiation, and control of apoptosis, especially in postmitotic neurons.[9] Podocytes share many similarities with neurons, as both cell types are considered to be terminally differentiated and highly specialized cells with complex architectures.[26]

The first finding of this study was that, despite being constitutively expressed in and restricted to podocytes in glomeruli, p35 is not involved in glomerulogenesis or podocyte differentiation. Moreover, mice lacking p35 have no ultrastructural kidney defects when grown under physiological conditions. Staining for the podocyte proteins nephrin, synaptopodin, and podocin were similar in p35-null and wild-type mice. Furthermore, neither podocyte number nor renal function was affected by absence of p35. Similar data were obtained *in vitro* using conditionally immortalized p35-null cell lines. Despite the lack of p35, there was no influence on cell proliferation or differentiation.

These data are in contrast to published data on neurons, where lack of p35 is associated with mislocalization of Purkinje neurons of the cerebellum.[27] Moreover, Griffin *et al.*[13] showed in cultured podocytes inhibition of Cdk5 protein expression by siRNA or Cdk5 activity by roscovitine-induced changes in cell morphology, with cellular elongation and loss of process formation. Additional activators of Cdk5 might account for these effects as the substrate specificity of Cdk5, like other Cdks, may depend in part on its particular activating subunit. A potential candidate could be p67, another known activator of Cdk5[28] that is also expressed in podocytes (data not shown). However, the role of p67–Cdk5 in podocytes is unknown.

p35 sets a threshold for podocyte apoptosis

Podocytes function as a major barrier in the kidney to proteinuria, and are required for normal glomerular architecture.[29] A decrease in podocyte number due to apoptosis and/or detachment causes proteinuria and glomerular fibrosis, events that lead to a decline in kidney function. Recent experimental and clinical data define the podocyte number 'threshold' at which proteinuria begins and renal function deteriorates as (i) a 0–20% decrease not associated with proteinuria or reduced glomerular filtration rate; (ii) a 20–40% decrease in podocyte number associated with microalbuminuria, but normal renal function and (iii) $>40\%$ decrease in podocyte number causing proteinuria (i.e. >300 mg/24 h) and reduced glomerular filtration rate.[30]

Another major finding in this study was that p35 sets the threshold for apoptosis in podocytes following cell injury, but not under non-stress states. Several lines of evidence support this observation. First, there was no increase in apoptosis in p35-null cells in the absence of cell injury. Second, similarly there was no apoptosis in podocytes (or any other kidney cell) in p35-null mice grown normally, without disease. Third, following four different forms of cell injury under culture conditions, onset of apoptosis occurred earlier in p35-null podocytes compared to wild-type cells. Fourth, the magnitude of apoptosis was always statistically significantly greater in injured cultured p35-null cells as compared with that in wild-type cells. Fifth, reducing p35 levels with siRNA in cultured wild-type podocytes increased the onset and magnitude of injury-induced apoptosis. Sixth, restoring p35 levels in p35-null cells by lentiviral gene transfer decreased apoptosis, at least in part. The increased apoptosis was independent of the triggers applied and the apoptotic pathways involved (intrinsic versus extrinsic apoptotic pathway or caspase-dependent versus independent).

Finally, the results of this study also showed that p35 limits podocyte apoptosis *in vivo* in experimental glomerular disease. Following injury p35-null mice showed significant increase in podocyte apoptosis, leading to reduced podocyte number, more severe glomerulosclerosis, and worsened proteinuria compared with wild-type controls.

The role for p35 in neuronal survival is controversial. Certain authors show that p35 protects neurons against apoptosis, especially during development.[9,31,32] However, others show hyperactive p35–Cdk5 in neurogenerative

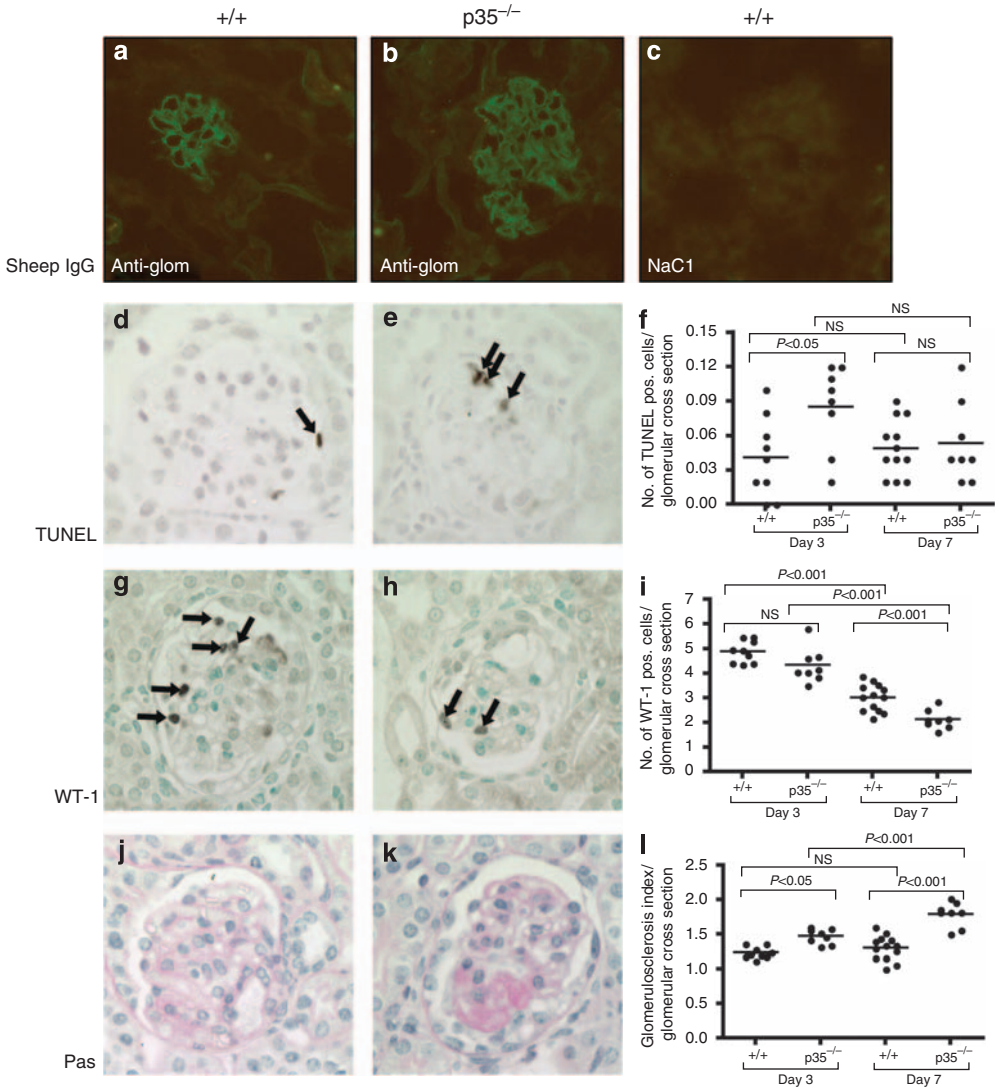

Figure 6 | Podocyte apoptosis is increased in p35-null mice with experimental glomerulonephritis. (**a**–**c**) There was comparable deposition of sheep-anti-glomerular IgG as measured by immunofluorescence at day 7 following administration of anti-glomerular antibody. (**d**–**f**) There was a significant increase in podocyte apoptosis (TUNEL staining) at day 3 of nephritis in p35-null mice ($P<0.05$, analysis of variance, ANOVA). (**g**–**i**) Podocyte number (WT-1 immunostaining) was decreased in both wild-type and p35-null mice following disease induction (day 7 $P<0.001$, ANOVA). However, there were significantly fewer podocytes in p35-null mice as compared with wild-type mice (day 7 $P<0.001$, ANOVA). (**j**–**l**) Glomerulosclerosis index (calculated based on the percentage of glomerular tuft area affected) increased by day 3 in mice lacking p35 as compared with wild-type mice ($P<0.05$, ANOVA), and was even more pronounced by day 7 ($P<0.001$, ANOVA). Representative images of glomeruli are shown.

diseases, where active p35–Cdk5 augments neuronal degeneration due to apoptosis. In the kidney, p35–Cdk5 levels and activity increase during glomerular development.[13] However, we have no evidence for any pathophysiological abnormalities in the kidney due to increased p35–Cdk5 activity.

p35 regulates the protein expression of Bcl-2 in podocytes

A major question is how p35 safeguards against podocyte apoptosis? We have shown that Bcl-2 safeguards podocytes from apoptosis.[17] To test the hypothesis that decreased Bcl-2 protein levels underlie the apoptotic threshold, p35-null podocytes were stably transduced with human Bcl-2. The third major finding of this study was that restoring Bcl-2 protein expression in p35-null podocytes rescued these cells from apoptosis following UV-C irradiation. In the absence of p35, Bcl-2 protein levels were substantially reduced in podocytes. Moreover, ecotopic expression of p35 in p35-null cells increased Bcl-2 protein expression. Taken together, these results indicate that p35 has pro-survival effects on podocytes, albeit modest. These effects are mediated by Bcl-2-dependent and Bcl-2-independent mechanisms. As rescue of p35-null cells was incomplete, it stands to reason that other mechanisms beyond p35 likely also contribute to the increased apoptosis rates observed in these cell lines. Thus, our findings are supported by recent reports in neurons showing that p35–Cdk5 induces Bcl-2

expression.[31] In addition, Cheung *et al.*[32] reported that human Bcl-2 is a direct phosphorylation substrate of active p35–Cdk5.

Several studies have shown a role for cell-cycle proteins in governing podocyte apoptosis. Recently we showed that cyclin-I, a novel activator of Cdk5, also serves to minimize podocyte apoptosis.[23] Given that both cyclin-I and p35 activate Cdk5, a model is developing, showing that activation of Cdk5 is central to podocyte survival. By altering the levels of the activators of Cdk5 (cyclin-I and/or p35), podocytes are more vulnerable to injury-induced apoptosis. We speculate that activation of Cdk5 by several proteins allows for 'backup' processes in order to limit apoptosis.

In conclusion, the results reported here demonstrate that in contrast to neurons, p35 does not have a role in kidney development (glomerulogenesis) and does not affect podocyte differentiation. Rather, it sets a threshold for podocyte survival and apoptosis *in vitro* and *in vivo*, by maintaining Bcl-2 protein levels.

MATERIALS AND METHODS

Cell culture

Conditionally immortalized podocytes in culture were generated as previously described from wild-type and p35 null mice (for review see references Shankland *et al.*[15] and Griffin *et al.*[16]). Quiescence and differentiation were induced by culturing the cells at 37 °C for 13–15 days on Primaria plastic plates (BD Biosciences, San Jose, CA, USA) in the absence of interferon-γ.

Plasmid transfection

cMMP-IRES-GFP vectors encoding human Bcl-2[33] were transfected into Phoenix Eco packaging cells (ATCC, Manassas, VA, USA) to generate retrovirus. Retrovirus-containing media was harvested, filtered, and applied onto subconfluent, proliferating p35-null podocytes for infection. Infected cells were selected by fluorescence-activated cell sorting. Infected podocytes were further subcultured, passaged, and transferred to 37 °C to initiate growth arrest and differentiation.

Human p35 was cloned into a modified GATEWAY pENTR1A vector followed by recombination into pLenti6.3V5/Dest (Invitrogen, Carlsbad, CA, USA) as published previously.[34]

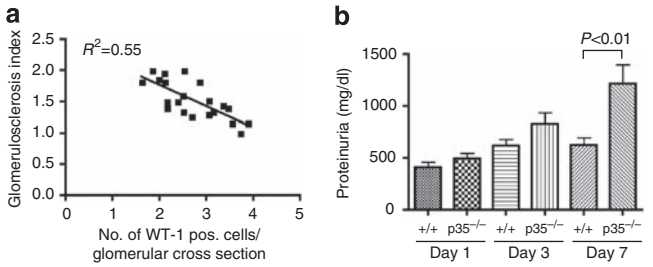

Figure 7 | Severity of glomerulonephritis is increased in p35-null mice. (**a**) There was significant correlation ($R^2 = 0.55$) between podocyte number (WT-1) and glomerulosclerosis for both groups of mice (glomerulosclerosis index, periodic acid Schiff staining). (**b**) Both groups developed proteinuria following anti-glomerular antibody administration. At day 7 the amount of proteinuria was significantly higher in p35-null mice as compared with that in WT controls ($P < 0.01$).

siRNA transfection

Wild-type podocytes were culture under growth-restrictive conditions. To reduce p35 levels in wild-type cells, growth-arrested podocytes at day 10 were transfected with 2 μg of pooled siRNA-targeting p35 by electroporation (175 volts, 200 μms, 5 pulses) according to the manufacturer's recommendation (Ambion, Austin, TX, USA). Non-specific siRNA (Ambion) was included as transfection control (Table 1).

Real-time quantitative PCR

To ensure the effectiveness of siRNA, RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's directions. 2-μg of RNA was used for cDNA synthesis using the Fermentas First Strand cDNA Synthesis kit (Fermentas, Glen Burnie, MD, USA). Quantitative real-time PCR was performed on a Rotor-Gene 6000 (Corbett Research, Sydney, Australia) using FAM-labeled primer probes (Table 2; all from Applied Biosciences, Austin, TX, USA) and the Quantace SensiMix dT reagent (Quantace, Norwood, MA, USA). The amount of product was determined at the end of each cycle using the Rotor-Gene software (Rotor-Gene 6000 series software 1.7). Relative gene expression was analysed with a normalizing gene using the 2-standard curve method.

Induction and detection of apoptosis *in vitro*

Apoptosis was induced in 80–90% confluent, fully differentiated, wild-type and p35-null podocytes. Each experimental condition was applied in triplicate. Apoptosis was induced by UV-C irradiation (0–25 J/m^2), serum depletion (0–10%), or incubation with PAN (0–100 μg/ml; Sigma-Aldrich, St Louis, MO, USA), sheep anti-rabbit glomerular antibody (0–5%), or TGFβ1 (0–10 μg/ml; R&D Systems, Minneapolis, MN, USA) and assessed by staining for nuclear condensation in non-fixed cells with Hoechst-33342 (10 mM; Sigma-Aldrich) and by detection of caspase-3 cleavage products by Western blot analysis.

Western blot analysis

Protein levels were measured by Western blot analysis as follows. Cells were washed twice with cold phosphate-buffered saline (PBS) and cells were harvested by scraping on ice. The cells were lysed in radioimmunoprecipitation assay lysis buffer (50 mM Tris–HCl (pH 8.0), 5 mM EDTA, 150 mM NaCl, 1% IP-40, 1% Trion X-100, 50 mM NaF, 1 mM Na-orthovanadate (all from Sigma-Aldrich)) in the presence of protease inhibitors (Roche, Indianapolis, IN, USA).

Table 1 | siRNAs

siRNA	Target mRNA	Strand sequence	Source
4390771/s63800	p35	5′-CAAGUGUUCUCUGACUUGAtt-3′	Ambion
4390771/s63801	p35	5′-GGACCAGGGUUUCAUCACAtt-3′	Ambion
4390771/s63802	p35	5′-GGUAGAGAGCUGUAAGGAAtt-3′	Ambion
AM4611	Non-targeting		Ambion

Abbreviation: siRNA, small interfering RNA.

Table 2 | qPCR primer probes (FAM reporter dye)

Primer probe	Target mRNA	Strand sequence	Source
Mm99999915_g1	GAPDH	TGAACGGATTTGGCCGTATTGGGCG	Applied Biosystems
Mm00438148_s1	p35	ACCTTGCGCGAACCCAAAAGATGCA	Applied Biosystems

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; siRNA, small interfering RNA.

Table 3 | Primary antibodies

Target protein	Antibody	Dilution (WB)	Dilution (IHC/IF)	Source
β-Actin	ab6276	1:100,000	—	Abcam
Bcl-2	CST-2876	1:1000	—	Cell Signaling
Cleaved caspase-3	CST-9664	1:1000	—	Cell Signaling
GFP	CST-2555	1:1000	—	Cell Signaling
Nephrin	RDI-PROGN2	—	1:1500/1:100	Fitzgerald
Podocin	Ab50339	—	1:4000	Abcam
Synaptopodin	RDI-PRO65194	—	1:20/1:1	Fitzgerald
β-Tubulin	sc-9104	1:200	—	Santa Cruz
V5 tag	MCA1360	1:5000	—	Serotec
WT-1	sc-192	—	1:2000/1:100	Santa Cruz

Abbreviations: GFP, green fluorescent protein; IF, immunofluorescence; IHC, immunohistochemistry; WB, Western blotting.

Protein concentrations were determined by BCA protein assay (Pierce, Rockford, IL, USA). The samples were separated on a 10% sodium dodecyl sulfate–polyacrylamide electrophoresis gel and blotted on a polyvinylidene difluoride membrane (Sigma-Aldrich). The membrane was incubated in 5% non-fat milk powder in TBS (10 mM Tris–HCl (pH 8.0), 150 mM NaCl) solution for 3 h at room temperature. Thereafter, the blot was incubated overnight at 4 °C with primary antibodies (Table 3) followed by incubation with appropriate horseradish peroxidase-conjugated secondary antibodies (Sigma-Aldrich). Proteins were visualized by enhanced chemiluminescence according to the manufacturer's instructions (GE Healthcare, Piscataway, NJ, USA). In some cases, membranes were incubated in stripping buffer (100 mM glycine, 1% SDS, pH 2.5) and re-stained as stated.

Animal models

Podocyte apoptosis was studied *in vivo* in wild-type and p35-null mice backcrossed onto the Balb/C background for at least 10 generations. Mice were housed according to the standardized specific pathogen-free conditions in the University of Washington animal facility. The Animal Care Committee of the University of Washington, Seattle, reviewed and approved the experimental protocol. Crescentic glomerulonephritis was induced in 8- to 12-week-old, male, wild-type and p35-null mice by intraperitoneal injection of sheep anti-rabbit glomerular antibody (20 mg/g body weight) on two consecutive days as described previously.[16,24] This model is characterized by podocyte injury, apoptosis, and development of proteinuria and glomerulosclerosis independent of complement activation and leukocyte infiltration of the glomeruli.

Mice were killed on days 3 and 7 after the second injection of anti-glomerular antibody ($n = 8$–13 per strain per time point). Urine was collected overnight from each animal before disease induction and prior to killing for quantification of proteinuria (sulfosalicyclin assay). Renal tissue was embedded in OCT compound (Miles, Elkart, IN, USA) and frozen at −70 °C or fixed in either 10% neutral buffered formalin or methyl-Carnoy's solution for immunostaining.

Immunostaining

Indirect immunoperoxidase staining was performed using methyl-Carnoy's solution- and formalin-fixed tissue from wild-type and p35-null mice. Briefly, 4-μm tissue sections were deparaffinized in Histo-Clear (National Diagnostics, Atlanta, GA, USA) and rehydrated in graded ethanol. Endogenous peroxidase activity was blocked with 3% hydrogen peroxidase. The sections were incubated overnight at 4 °C with primary antibodies (Table 3) diluted in 1% bovine serum albumin/PBS. The sections were washed repeatedly in PBS before incubation with biotinylated mouse anti-rabbit secondary antibody (Jackson Immunoresearch, West Grove, PA, USA) diluted in 1% bovine serum albumin/PBS for 1 h at room temperature. The ABC kit (Vector Laboratories, Burlingame, CA, USA) was used for signal amplification and 3,3′-diaminobenzamidine (Sigma) was used as chromogen. Slides were counterstained with methyl green (Vector Laboratories, Burlingame, CA, USA) or hematoxylin (Sigma-Aldrich), dehydrated, and covered with Histomount (National Diagnostics, Atlanta, GA, USA). Apoptosis was measured by the TUNEL assay as described previously. Periodic acid Schiff staining was performed for semi-quantitative assessment of glomerulosclerosis. Frozen sections were rehydrated in PBS and stained with fluorescein isothiocyanate-conjugated antibodies against sheep IgG (Cappel, Durham, NC, USA) to ensure comparable glomerular antibody deposition.

To perform immunostaining on cultured cells, podocytes in culture were fixed by 2% paraformaldehyde, 4% sucrose followed by 0.3% Triton X-100 for permeabilization. Staining was performed by primary antibodies diluted in PBS/bovine serum albumin (1%) overnight at 4 °C. After three washes with PBS, appropriate secondary antibodies, conjugated to Texas-Red (Invitrogen, Karlsruhe, Germany), were added for 30 min at room temperature. The coverslips were extensively washed and analyzed by fluorescence microscopy (Leica, Deerfield, IL, USA).

Assessment of glomerulosclerosis

Glomerulosclerosis was determined using periodic acid Schiff-stained sections for a minimum of 50 glomeruli in each animal and

was graded quantitatively based on the percentage of glomerular tuft area involvement as follows: grade-1, <25%; grade-2, <25–50%; grade-3, <50–75%; and grade-4, <75–100%.

Statistical analysis

All results are expressed as mean ± s.d. Statistical significance was evaluated using GraphPad Prism version 4.00c for Macintosh (GraphPad Software, San Diego, CA, USA). Analysis of variance (ANOVA) with Tukey–Kramer adjustment for multiple comparisons was applied. A *P*-value <0.05 was considered significant.

DISCLOSURE

All the authors declared no competing interests.

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