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A missense mutation in podocin leads to early and severe renal disease in mice

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Mutations in the *NPHS2* gene, encoding podocin, are responsible for familial autosomal recessive and sporadic cases of steroid-resistant nephrotic syndrome. We have successfully generated a mouse model in which the common p.R138Q mutation found in nephrotic patients is expressed in the kidney. Homozygous mice express the mutant protein, which is mislocated to the cytoplasm, along with a portion of the nephrin pool. These mice die within the first month of life, but their survival depends on the genetic background. Albuminuria manifests early and leads to progressive renal insufficiency, characterized histologically by diffuse mesangiolysis and mesangial sclerosis, endothelial lesions along with podocyte abnormalities such as widespread foot process effacement. Gene expression profiling revealed marked differences between these and the podocin-null mice, including significant perturbations of podocyte-expressed genes such as *Cd2ap*, *Vegfa* and the transcription factors *Lmx1b* and *Zhx2*. Upregulation of *Serpine1* and *Tgfb1* implicates these as potential mediators of disease progression in these mice. This mouse model of nephrotic syndrome may serve as a valuable tool in studies of *in vivo* intracellular protein trafficking of podocyte proteins, as well as testing therapeutic modalities aimed at correcting the targeting of mutant proteins.

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Efforts over the past decade aimed at unravelling the genetic basis of nephrotic syndrome have led to significant advances in our understanding of the molecular basis of glomerular function and the pathophysiological mechanisms leading to proteinuric renal diseases. The *NPHS2* gene, encoding the slit diaphragm protein podocin, has been shown to be mutated in familial forms of autosomal recessive steroid-resistant nephrotic syndrome (SRNS),[1] and in sporadic cases.[2,3] Although early[4] and adult-onset[5] forms have been described, SRNS most commonly manifests between 3 and 6 years of age,[1] rapidly progresses to end-stage renal disease, and does not recur after transplantation. Mutations may involve both N- and C-terminal intracytoplasmic regions of the protein, and include both loss-of-function mutations as well as missense changes.[2] Indeed, the most commonly found variant is the p.R138Q mutation, observed in 32% of all affected alleles,[2] and leads to a severe, early-onset form.[4] Missense variants of podocin have been studied in cell culture models and some, including the p.R138Q mutation, have been shown to result in intracellular trafficking defects of podocin, whereas some are able to maintain plasma membrane targeting.[6] Moreover, podocin has been shown to function not only as a scaffold necessary for slit diaphragm assembly via its interactions with CD2AP and nephrin,[7] but also in nephrin targeting to lipid rafts, where signal transduction events occur.[8] Missense podocin variants have been shown to differ in their ability to affect nephrin trafficking to lipid rafts and plasma membrane.[9,10] More recently, podocin has been shown to bind to cholesterol regulating the activity of the transient receptor potential channel 6.[11]

Murine models have proven to be invaluable tools in elucidating the pathophysiological mechanisms leading to renal disease. Indeed, we have previously inactivated the murine *Nphs2* gene and showed that these mice develop early, and severe terminal renal disease, which differs phenotypically from disease seen in humans.[12] We have, therefore, developed a mouse model expressing the murine equivalent of the missense variant p.R138Q to better understand the

pathophysiological mechanisms involved in renal disease caused by mutant forms of podocin. These mice developed renal disease similar to that of the *Nphs2*-null model, due to failure of the mutant protein to target properly in the podocyte. We further demonstrated that survival can be modulated by genetic background and that the renal disease results in significant perturbations of gene expression in the kidney.

RESULTS

Successful targeting leads to expression of the R140Q podocin variant

The *Nphs2* gene in mice encodes a protein with 385 amino acids, two amino acids more than the human homolog. To generate a mouse model bearing a mutant R140Q variant of the podocin gene, corresponding to the p.R138Q mutation found in humans, a 6.6-kb targeting construct was generated, in which the c.505G > A, c.506A > G mutations were achieved by site-directed mutagenesis of exon 3 of the *Nphs2* gene, and a floxed *phosphoglycerate kinase–hygromycin* cassette was inserted into intron 3 for positive selection (Figure 1a). Successful homologous recombination in 2/299 embryonic stem cells was verified by Southern blot hybridization (Figure S1) and two embryonic stem cell clones were expanded and subsequently injected into C57BL/6 blastocysts. Germline transmission of the mutant allele was achieved by mating of >80% chimeric mice from one clone and verified by polymerase chain reaction (PCR) genotyping. Heterozygous $Nphs2^{R140Q/+}$ mice were crossed with Meu-Cre40 mice constitutively expressing Cre recombinase,[13] leading to excision of the floxed hygromycin cassette. Finally, the *Cre* allele was selected against by breeding heterozygotes with wild-type mice.

In the mixed C57BL/6:129SvPas background, inheritance of the mutant allele deviated from expected Mendelian ratios, with 65/351 (18.5%) $Nphs2^{R140Q/R140Q}$ genotyped at birth ($\chi^2 = 8.11$, 2 df, $P = 0.0173$), suggesting intrauterine or perinatal mortality. Expression of the mutant *Nphs2* transcript in $Nphs2^{R140Q/+}$ and $Nphs2^{R140Q/R140Q}$ mice was verified by sequencing the PCR products obtained after reverse transcription of total RNA extracted from kidneys (Figure 1b). By northern blot hybridization, no alternative splice variants were seen in mutant *Nphs2* mice, but *Nphs2* transcriptional levels were higher than in wild-type mice (Figure 1c). Real-time PCR confirmed significant upregulation of the *Nphs2* transcript at postnatal (P) day 4 and at P12, although the latter did not achieve statistical significance (Figure 1d). These results confirmed successful targeting of the *Nphs2* gene leading to expression of a podocin variant at biologically relevant levels.

Mutant R140Q podocin is mislocalized, along with nephrin

Despite higher levels of *Nphs2* mRNA in targeted mice, western blotting revealed an overall decrease in the expression of podocin at the protein level as early as P1 (data not shown), which was sustained at P12 (Figure 2). Previous studies have revealed that the p.R138Q human podocin variant, when overexpressed in cell culture models, fails to localize to the plasma membrane and is retained in the endoplasmic reticulum.[6,10] We, therefore, investigated the localization of podocin in homozygous $Nphs2^{R140Q/140Q}$ mice by immunofluorescence. In wild-type mice, anti-podocin antibodies labelled podocytes in a pattern consistent with plasma membrane expression, in close juxtaposition to the glomerular basement membrane marked by nidogen (Figure 3a). In $Nphs2^{R140Q/R140Q}$ mice, however, podocin no longer colocalized with nidogen, instead displayed an intracellular pattern of expression with perinuclear staining, indicating retention in the endoplasmic reticulum (Figure 3a). Additionally, in mutant mice, partial colocalization of nephrin with mutant R140Q podocin suggests that the intracellular trafficking defect of podocin leads to mislocalization of a fraction of the nephrin in podocytes (Figure 3b).

Mutant mice develop proteinuria and early, terminal renal failure

Thereafter, cohorts of mutant R140Q mice were followed till death or were killed at the designated time points. Heterozygous $Nphs2^{R140Q/+}$ mice followed to 1 year of age did not demonstrate albuminuria and had no obvious renal histological abnormalities (data not shown). Homozygous mutant mice on the mixed genetic background died at a median age of 4 days (range 1–40 days), with additional mice likely dying either *in utero* or in the perinatal period (Figure 4a). Previously, we had demonstrated a modifying effect of genetic background on the survival of *Nphs2*-null mice.[12] We, therefore, backcrossed the mutant $Nphs2^{R140Q}$ allele, enriching for 129S2/SvPas alleles, and the mice died at a median age of 14 days, significantly later than mixed background mice (Figure 4a). Furthermore, we identified 37/133 (27.8%) newborn $Nphs2^{R140Q/R140Q}$ mice on the 129-enriched background, which suggests insignificant perinatal mortality and is consistent with Mendelian inheritance.

Although appearing normal at birth, $Nphs2^{R140Q/R140Q}$ newborn mice quickly developed albuminuria, which progressed with age (Figure 4b). Biochemical measures of renal function in 129 mutant mice demonstrated significant elevations in plasma urea at both P4 and P12 (Figure 4c). Similarly, plasma creatinine was elevated at P4 but not P12 (Figure 4d). This may potentially be accounted for by a trend toward decrease in body weight at P12 in mutant podocin (9.67 ± 0.78 mg in controls vs 6.92 ± 0.96 mg in mutants, $P = 0.06$), which may reflect loss of muscle mass. These data are consistent with early and significant impairment of renal function in $Nphs2^{R140Q/R140Q}$ mice, and a potential role for genetic modifiers in determining the length of survival.

Evolution of renal histological lesions in mutant mice

Thereafter, we characterized the evolution of renal histological lesions in mutant mice and compared these with littermate controls (Figures 5a and b). Despite the early onset of albuminuria, light microscopy revealed no glomerular or