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Nephrin is involved in podocyte maturation but not survival during glomerular development

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Nephrin, a major component of the glomerular slit diaphragm (SD), is both a structural protein as well as a signaling molecule influencing foot process (FP) formation and maintenance of podocyte integrity. Analyses of near-term embryonic kidneys showed normal cellular viability and no apoptosis in glomeruli from nephrin knockout mice. Moreover, expression and location of other SD or glomerular basement membrane components were similar in wild-type and mutant mice as was the location and levels of most podocyte-specific proteins. Transcriptional profiling showed that the lack of nephrin had minor impact on the expression of genes for FPs and SD proteins. Claudin 3, a tight-junction protein normally absent in glomeruli, was upregulated threefold in the knockout mice, suggesting a role of nephrin in claudin 3 gene expression within the glomeruli. Our results suggest that nephrin is expressed late in the process of podocyte differentiation and is a locus for the formation of SD and FP maintenance and physical integrity *in vivo*. Nephrin does not seem to have a primary role in cell survival but has a small impact on gene regulation during glomerular development.

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The glomerular filtration barrier constitutes a fenestrated capillary endothelium, a glomerular basement membrane (GBM), and the slit diaphragm (SD) localized between foot processes (FPs) of adjacent podocytes. The porous SD resembles a zipper-like filter[1,2] and is structurally related to classical junction complexes.

The molecular composition of the SD started to be unveiled through the discovery of nephrin,[3] the first SD constituent to be identified.[4] Nephrin, a 180-kDa transmembrane protein expressed exclusively at the podocyte SD and its gene are mutated in congenital nephrotic syndrome of the Finnish type (CNF), characterized by aberrant podocyte FP formation, lack of SD, and massive proteinuria.[3] Over 60 different mutations have been identified in both Finnish and non-Finnish patients with CNF,[5,6] some mutations giving rise to a milder focal segmental glomerulosclerosis-like phenotype.[7]

The discovery of nephrin was soon followed up by identification of mutations in several other genes encoding SD and SD-associated proteins including *NPHS2*,[8,9] *NEPH-1*,[10,11] *CD2AP*,[12,13] and *FAT-1*,[14,15] all giving rise to nephrotic syndrome phenotypes including podocyte effacement and proteinuria. Moreover, mutations found in *ACTN4*,[16,17] *WT-1*,[18] *LAMB2*,[19] *TRPC6*,[20] and *PLCE1*[21] were shown to lead to a similar phenotype.

Several SD and SD-associated proteins are thought to interact into an intricate network with both structural and signaling functions, important for podocyte viability, cell polarity, cell differentiation, regulation of cytoskeleton rearrangements, and maintenance of podocyte integrity.[22–24]

To further elucidate the involvement of nephrin in glomerular development, assembly, and organization of FP and SD *in vivo*, we investigated the impact of nephrin on these processes in nephrin-deficient embryonic kidneys, using fluorescence and electron microscopy imaging techniques, and microarray transcriptome analyses.

RESULTS

Glomerular phenotype in nephrin null mice

Nephrin-deficient mice present massive neonatal proteinuria and death within 24 h after birth.[25] Here, periodic acid-Schiff staining of E18.5-day mouse kidneys revealed characteristic dilatations of Bowman's spaces and tubuli, and massive accumulation of proteins in many tubules already *in utero* in

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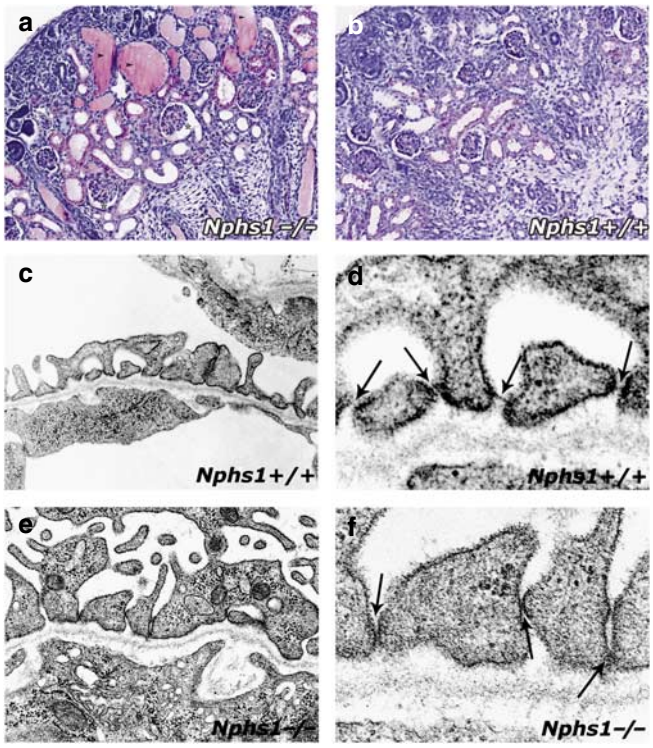

Figure 1 | Histological analysis of kidney cortex from *Nphs1*$^{+/+}$ and *Nphs1*$^{-/-}$ mice. (**a**) Periodic acid-Schiff staining reveals characteristic dilated Bowman's space (*) and proximal tubuli with accumulation of tubular protein precipitates (arrowheads) in *Nphs1*$^{-/-}$ kidneys compared with a control littermate (**b**). Transmission electron microscopy reveals a thin glomerular basement membrane (GBM) with numerous foot processes (FPs) toward the Bowman's space in normal mice (**c**). At higher magnification, the presence of slit diaphragm (SD; arrows) between FPs is observed (**d**). In *Nphs1*$^{-/-}$ kidneys, electron microscopy reveals thicker GBM with broader-looking FPs (**e**). At higher magnification (**f**), no SD is observed between the FPs in *Nphs1*$^{-/-}$ kidney, and the distance between two adjacent podocytes is narrower, the FPs being connected with each other via an undefined adherence junction-like structure (arrow).

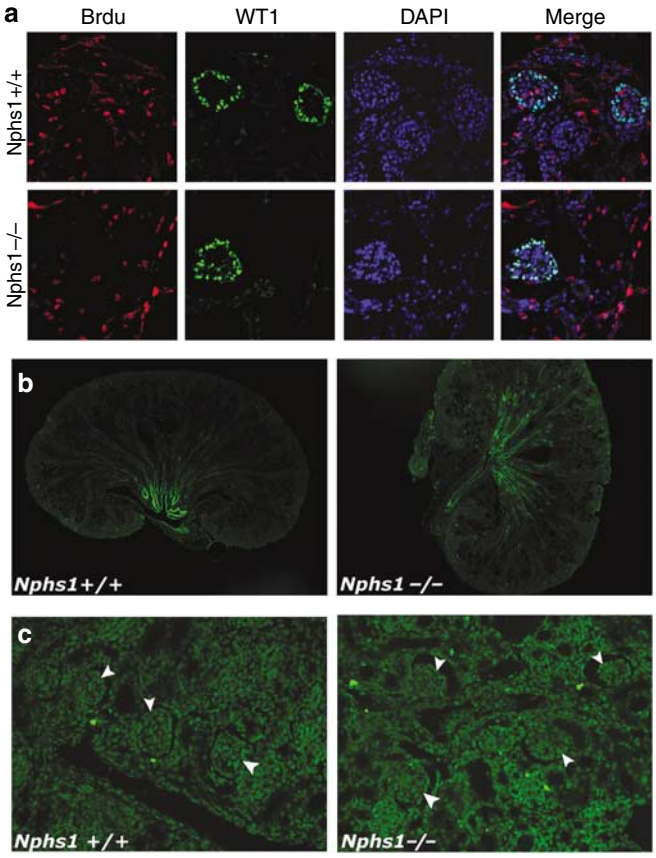

Figure 2 | Effects of nephrin absence on cell proliferation and apoptosis in the kidney cortex. (**a**) Bromodeoxyuridine (BrdU) incorporation analysis shows that the absence of nephrin does not cause impairment in cell proliferation. (**b** and **c**) Transferase dUTP Nick End Labeling staining analyses reveal no clear differences in apoptosis of the kidney of nephrin knockout mice. Apoptosis rate is not increased in the glomeruli in *Nphs1*$^{-/-}$ mice (**c**, white arrowheads), but counting of apoptotic nuclei show significant difference between *Nphs1*$^{-/-}$ and *Nphs1*$^{+/+}$ in the tubules.

Nphs1$^{-/-}$ kidneys (Figure 1a), but not in littermate controls (Figure 1b). By light microscopy, the appearance of glomeruli was similar in both genotypes, but electron microscopy revealed, as previously reported,[25] complete loss of the SD in *Nphs1*$^{-/-}$ (Figure 1c–f). However, podocyte FPs appeared to be formed, but they were broader in *Nphs1*$^{-/-}$ than in *Nphs1*$^{+/+}$. The width of the slit between two adjacent podocytes is clearly narrower in *Nphs1*$^{-/-}$, and the FP connect with each other through some other, as yet undefined, junction-like structure (Figure 1f). Endothelial and mesangial cells did not seem to be affected in nephrin null mice (data not shown).

Nephrin loss does not affect podocyte viability

Since nephrin expression has been associated with podocyte survival,[26] we studied the relationship of nephrin with cell proliferation and cell survival of E18.5 stage glomeruli *in vivo*. As shown in Figure 2, the absence of nephrin neither interfered with cell proliferation nor cell survival at E18.5. Thus, bromodeoxyuridine (BrdU) staining (Figure 2a) revealed that the proliferation rate in *Nphs1*$^{-/-}$ is not notably different from that in *Nphs1*$^{+/+}$. Moreover, WT-1 staining confirmed that the proliferation events are occurring in other glomerular cells types, but not in podocytes.

Transferase dUTP Nick End Labeling (TUNEL) staining of kidneys of E18.5 stage (Figure 2b) and newborn mice revealed that in *Nphs1*$^{-/-}$ mice, the number of apoptotic nuclei in the kidney was somewhat higher than in *Nphs1*$^{+/+}$, but these events were concentrated to the tubules, in the medulla of the kidney. In contrast, in the glomeruli (Figure 2b, white arrowheads), the number of apoptotic nuclei was similar in both *Nphs1*$^{-/-}$ and *Nphs1*$^{+/+}$ kidneys. Again, the podocytes did not present any sign of apoptosis events, indicating that absence of nephrin does not lead to podocyte death, at this embryonic stage.

Absence of nephrin does not affect the expression of other SD proteins

Some intracellular FP proteins and SD components interact directly and/or indirectly, and disruption of this structure can

either directly or indirectly cause collapse of the SD and proteinuria. We, therefore, investigated the impact of nephrin absence on expression and organization of other FP and SD components. Figure 3 shows that the absence of nephrin did not affect the expression of any of the other components studied. Proteins, such as podocin, synaptopodin, CD2AP, podocalyxin, and WT-1, were expressed in *Nphs1*$^{-/-}$ glomeruli at levels and with distribution comparable to those in *Nphs1*$^{+/+}$, and similar findings were observed for dendrin and ZO-1 (data not shown). Immunoelectron microscopy revealed ZO-1 to be located at FP-like locations in both wild-type and null nephrin mice (Figure S1) Furthermore, no differences were observed in the levels of GBM proteins, such as the collagen IV α1–6 chains; laminin α1, α2, α5, and β2 chains; nidogen; perlecan; integrin α3 chain; or fibronectin between *Nphs1*$^{-/-}$ and *Nphs1*$^{+/+}$ glomeruli (data not shown). Taken together, these data indicate that the absence of nephrin causes minimal disturbances in the expression and location of the other FP/SD proteins or GBM components during glomerular development.

Nephrin regulates a small set of genes during glomerular development

The glomerular protein expression profile suggested that loss of nephrin does not interfere with the expression or gross location of other FP or SD components. Therefore, the transcriptional profiles were analyzed by microarray expression assay from E18.5 stage kidneys of *Nphs1*$^{+/+}$ and *Nphs1*$^{-/-}$ mice. Glomerular RNA was isolated from four *Nphs1*$^{+/+}$ and five *Nphs1*$^{-/-}$ mice, and hybridized in nine individual arrays. Table 1 shows that only seven genes were downregulated more than twofold and 11 genes were upregulated more than twofold in *Nphs1*$^{-/-}$ mice. None of these genes have previously been associated with the structure or turnover of the glomerular filter. The 11 upregulated genes included Smoc2 (fourfold upregulation), the expression of which has been shown to potentiate angiogenic effects of vascular growth factors,[27] and claudin 3 (threefold upregulation), a tight-junction (TJ) protein. Table S1 shows that the expression levels of known FP/SD proteins, as well as members of the claudin gene family (with the exception of claudin 3), do not differ between wild-type and nephrin null mice. Thus, nephrin has little impact on podocyte gene regulation during late-stage development of glomeruli.

Upregulation of claudin 3 in Nphs1$^{-/-}$ glomeruli

Claudin 3 belongs to a family of 24 proteins that are major components of TJ, each claudin being expressed in a tissue-specific manner.[28] Although the SD has been described as a modified TJ and/or adherence junction (AJ),[29] these structures have only been reported in immature glomeruli,[30] prior to the formation of podocytes. In contrast, mature podocytes are totally devoid of TJ or AJ. Since claudin 3 was upregulated in E18.5 day glomeruli ($2^{1.6}$, $P = 0.0267$), we studied expression of the protein in *Nphs1*$^{-/-}$ kidneys. When

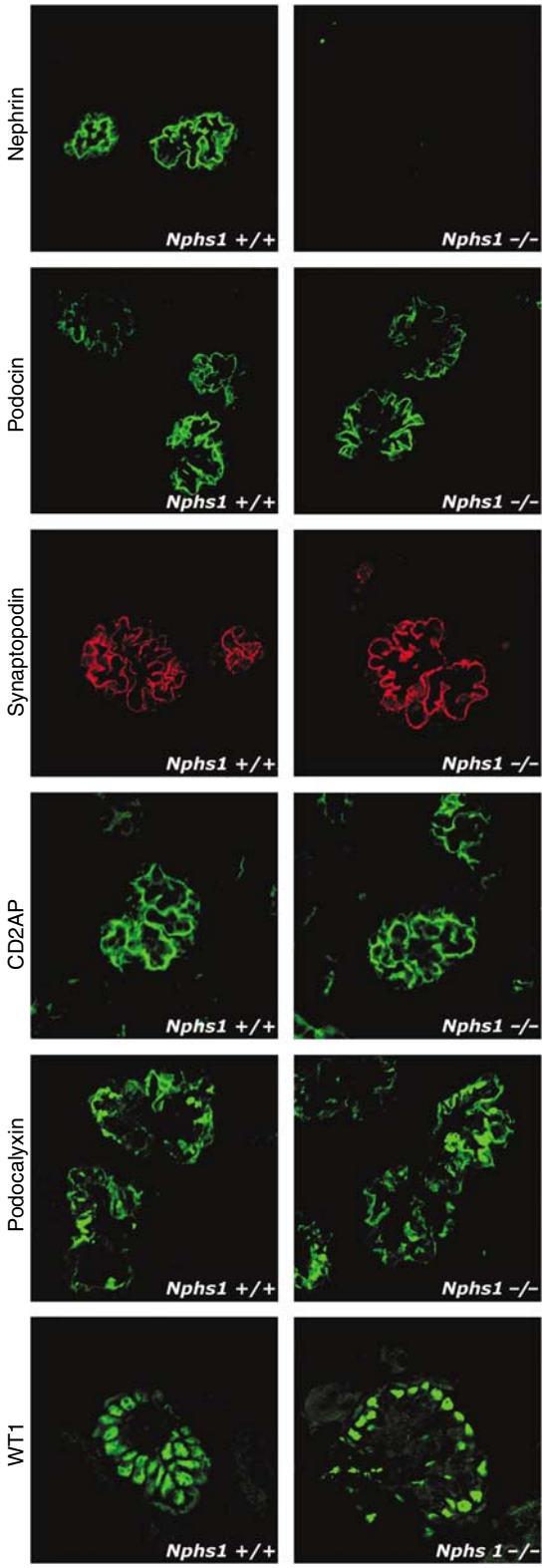

Figure 3 | Absence of nephrin does not affect the expression of the other foot process and slit diaphragm components. Comparison of the expression of podocyte-specific proteins podocin, synaptopodin, CD2AP, podocalyxin, and WT-1 by fluorescence immunohistochemistry in *Nphs1*$^{-/-}$ and *Nphs1*$^{+/+}$ mice reveals no differences in expression levels or location of the proteins.

Table 1 | List of genes up- or downregulated at least twofold in *Nphs1*$^{-/-}$ glomeruli when compared with those from *Nphs1*$^{+/+}$ mice

Probe set ID	Gene title	Gene symbol	Log 2 (KO/WT)	*P*-value
Downregulated genes				
1. 1422142_at	Nephrosis 1 homolog, nephrin (human)	Nphs1	−5.22	0.00215
2. 1452135_at	Glutathione peroxidase 6	Gpx6	−2.50	0.03097
3. 1449883_at	FXYD domain-containing ion transport regulator 2	Fxyd2	−2.10	0.02962
4. 1450344_a_at	Prostaglandin E receptor 3 (subtype EP3)	Ptger3	−1.85	0.03946
5. 1421286_a_at	ATPase, H+/K+ transporting, α polypeptide	Atp4a	−1.69	0.03974
6. 1425138_at	Guanylate cyclase activator 1B	Guca1b	−1.34	0.04590
7. 1423407_a_at	Fibulin 2	Fbln2	−1.31	0.01354
Upregulated genes				
1. 1415935_at	SPARC-related modular calcium binding 2	Smoc2	1.91	0.01271
2. 1445626_at	Lectin, galactose binding, soluble 3 (Lgals3), mRNA	Lgals3	1.65	0.03965
3. 1434651_a_at	Claudin 3	Cldn3	1.60	0.02670
4. 1449195_s_at	Chemokine (C-X-C motif) ligand 16	Cxcl16	1.55	0.02731
6. 1428664_at	Vasoactive intestinal polypeptide	Vip	1.31	0.00531
7. 1417156_at	Keratin complex 1, acidic, gene 19	Krt1-19	1.26	0.00892
8. 1418203_at	Phorbol-12-myristate-13-acetate-induced protein 1	Pmaip1	1.22	0.01715
10. 1437213_at	Nudix (nucleoside diphosphate-linked moiety X)-type motif 21	Nudt21	1.16	0.04729
11. 1451021_a_at	Kruppel-like factor 5	Klf5	1.16	0.01668

KO, knockout; WT, wild type.
Ratio refers to the log 2 ratio between glomeruli from *Nphs1*$^{-/-}$ and *Nphs1*$^{+/+}$ mice.
Probe ID is listed according to Affymetrix annotation.
The analysis was carried on using gcrma normalization. *Nphs1*$^{-/-}$, n=5 and *Nphs1*$^{+/+}$, n=4, $P<0.05$.

compared with expression of the podocyte-specific synaptopodin, immunofluorescence revealed claudin 3 expression mainly in non-podocyte regions (Figure 4a) in *Nphs1*$^{+/+}$, while the glomerular capillaries of *Nphs1*$^{-/-}$ were strongly positive, with clear podocyte staining overlapping with that of synaptopodin.

To further localize claudin 3 in glomeruli, we performed immunoelectron microscopy in kidneys from both *Nphs1*$^{-/-}$ and *Nphs1*$^{+/+}$. As shown in Figure 4b, in *Nphs1*$^{-/-}$ glomeruli, the location of claudin 3 is very distinct as almost all gold particles are found at junction-like structures between adjacent podocytes (Table S2). On the other hand, only a few scattered particles were observed in the glomeruli of *Nphs1*$^{+/+}$ mice, and it is not sure if those were signals above background (Figure 4b; Table S2).

Thus far, the presence of claudin 3 has been reported only in tubules, more specifically, in thin ascending limb, thick ascending limb, distal tubule, and collecting duct.[31,32] The present data clearly showed expression of claudin 3 in the Bowman's capsule in both *Nphs1*$^{-/-}$ and, to a lesser extent, in *Nphs1*$^{+/+}$ glomeruli (Figure 4a). Importantly, the expression of claudin 3 in *Nphs1*$^{-/-}$ glomeruli extended to the podocytes (Figure 4b).

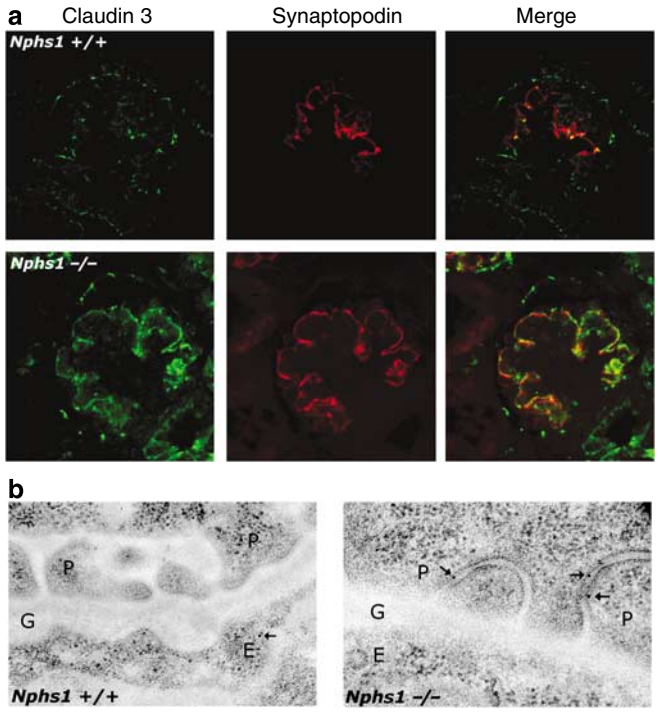

Figure 4 | Absence of nephrin leads to upregulation of claudin 3 in glomeruli. The expression of claudin 3 was examined by fluorescence immunohistochemistry and immunoelectron microscopy. (**a**) Compared with *Nphs1*$^{+/+}$ kidneys, the expression of claudin 3 was significantly increased in *Nphs1*$^{-/-}$ kidneys. It is to be noted that claudin 3 is partially colocated with synaptopodin, a podocyte-specific protein. (**b**) Expression of claudin 3 is increased in *Nphs1*$^{-/-}$ podocytes as compared with *Nphs1*$^{+/+}$ (arrows). The claudin 3 expression is primarily localized to the junctions of adjacent podocyte. P, podocyte foot processes; E, endotheluim; G, glomerular basement membrane.

DISCUSSION

Analyses of embryonic E18.5 stage kidneys of *Nphs1*$^{-/-}$ and transcriptional profiling of isolated glomeruli demonstrated that absence of nephrin does not significantly affect the glomerular morphogenesis, podocyte viability, or expression of other SD protein complex genes *in vivo*, although the embryonic glomeruli do not have fully developed FPs but loss of SD, tubular dilatations, and massive protein leakage.

Podocyte proteins, such as podocin, synaptopodin, CD2AP, podocalyxin, and WT-1 were expressed at similar levels as in wild-type glomeruli from the same embryonic stage. Also, the expression levels of only a few genes were significantly changed as observed by microarray transcriptome analyses. One of the upregulated genes in embryonic *Nphs1*$^{-/-}$ mouse glomeruli was claudin 3, a TJ protein not normally expressed in glomeruli. Collectively, the results suggest that nephrin is expressed quite terminally in podocytes, is not essential for glomerular morphogenesis, and that it does not largely affect regulation of other SD protein genes or podocyte viability.

On the basis of transmission electron microscopy results, Rodewald and Karnovsky[1] proposed that the SD is an isoporous zipper-like structure, the nature of which remained unclear for about three decades. The discovery of nephrin in 1998 and demonstration of its role in the organization in the SD,[2–4] followed by the discovery of other novel SD components and SD related proteins,[33–36] have given new insight into the structure and biology of the glomerular filter, and mechanisms of inherited and acquired glomerular disease. Thus, defects in the genes for several highly FP-associated proteins have revealed crucial interactions of many of these proteins and interplay in biology and disease. With reference to that, the present results showing remarkably little effects of nephrin absence on the overall expression profile in embryonic glomeruli were, therefore, surprising.

Nephrin has been shown to be a signaling molecule involved in development of the FPs and SD during development and regeneration after injury.[37,38] The SD and its associated proteins are a complex and highly dynamic structure formed by different classes of proteins that interact with each other and with the podocyte cytoskeleton. Evidence has been presented showing that SD acts not only as a physical filtration barrier, but also participates in common signaling pathways necessary to maintain the functional integrity of podocytes. For example, Huber *et al.*[26] proposed that nephrin, together with podocin and CD2AP, induced AKT activation, which is associated with a strong inhibition of detachment-induced apoptosis in cultured podocytes. The damage followed by loss of podocytes from glomeruli has been proposed as one of the hallmarks of both primary and secondary forms of glomerulosclerosis.[39]

The present histological analyses of E18.5 stage kidneys in *Nphs1*$^{-/-}$ mice show that the overall size and appearance of the glomerular tuft are not grossly affected by the absence of nephrin, but that the Bowman's space was frequently dilated and multiple tubuli were dilated with massive protein accumulation. These results agree well with previous descriptions of embryonic glomeruli of patients with CNF. Moreover, as observed in human CNF patients, transmission and scanning electron microscopy have demonstrated the presence of interdigitating, abnormally broad cellular protrusions rather than authentic FPs and complete absence of an SD.[40] The slit was collapsed, and the FPs seemed to be attached to each other through some undefined junction structures. On the basis of these observations, it was to be expected that extensive morphological and functional changes in the glomeruli caused by nephrin absence could generate major alterations in glomerular gene expression.

The results of this study, however, showing no significant effects of nephrin absence on early glomerular development are in line with its late appearance during normal kidney development. In the early stages of kidney development, the podocytes are polygonal cells, which divide rapidly. When entering a later developmental stage, the capillary loop stage, specific markers start to be expressed, and the podocytes start to develop their characteristic cellular architecture and the FP are formed. At this stage, the podocytes exit from the cell cycle and they loose their ability to divide. This process is accompanied by the expression of cell cycle inhibitors such as p27Kip1 and p57Kip2.[41–44] Expression of nephrin, which also starts in the late S-shaped body or capillary loop stage, coincides with ceasing of cell proliferation and could have been thought to affect that process.

However, as shown in Figure 2, we could not detect any significant differences in the rate of glomerular cell apoptosis or expression of cell cycle or apoptosis-related proteins such as cleaved caspase-3, Bad, and AKT (data not shown) between *Nphs1*$^{-/-}$ and *Nphs1*$^{+/+}$ glomeruli. This agrees with previous observations showing no differences in TUNEL-positive cells between CNF glomeruli and controls in humans.[45] Apoptosis events could be observed in the tubuli, located in the medulla of the kidney. These events may be due to excessive amount of protein accumulated in the tubuli. Reports in the literature appoint albumin as being responsible for endoplasmic reticulum stress and further development of apoptosis in renal proximal tubular cells.[46] Furthermore, there was no difference in the rate of cell proliferation between Nphs1$^{-/-}$ and Nphs1$^{+/+}$. These data indicate that the lack of nephrin alone has no influence either on podocyte apoptosis or proliferation at least during development. We also counted the number of WT-1-positive cells, which are thought to represent podocytes on the tissue sections, but we could not detect any significant difference in the number of WT-1-positive cells between Nphs1$^{-/-}$ and Nphs1$^{+/+}$, either (data not shown). Taken together, it seems that nephrin alone plays no role in maintaining podocyte viability during development. However, we cannot exclude the likelihood of nephrin participating in podocyte survival signaling in adulthood, when nephrin expression changes due to acquired disease conditions. Therefore, further experiments are necessary to evaluate the potential involvement of nephrin in podocyte survival in steady-state glomeruli.

Since nephrin is a signaling molecule and a critical component of the SD, we examined the expression of other FP/SD components and GBM components. These analyses revealed no differences in the expression of specific podocyte proteins between *Nphs1*$^{-/-}$ and *Nphs1*$^{+/+}$. It has been reported that the Neph1–nephrin interaction is important for maintaining SD integrity, since disruption of the Neph1–nephrin interaction *in vivo* results in complement- and

leukocyte-independent proteinuria with preserved FP.[11] This disruption modestly reduces Neph1 and nephrin protein expression in podocytes and dramatically reduces ZO-1 protein expression, independent of changes in mRNA expression of all three genes. We have not analyzed the expression of Neph1 protein in the lack of nephrin *in vivo*. However, we could not detect any difference in the expression of ZO-1 (data not shown). Our data, together with others, suggest that nephrin plays an important role in keeping the structure of the SD together without, however, interfering with the levels of protein expression in the SD.

The results of transcriptional profiles of isolated glomeruli showing only minor effects of nephrin on the overall glomerular gene expression were surprising, as nephrin has been shown to regulate FP formation and thus podocyte maturation. For example, tyrosine phosphorylation of the intracellular tail of nephrin can recruit docking proteins and trigger intracellular signaling cascades leading to FP and SD formation,[38,47] and treatment of differentiated podocytes puromycin aminonucleoside, an agent that causes FP effacement *in vivo*, disrupts actin and nephrin simultaneously.[48] The present results clearly indicate that such signaling events have small influence on the overall glomerular gene regulation even though proper FPs do not develop. It is possible that the absence of nephrin causes severe structural changes in the podocyte architecture through destabilization of the protein–protein interactions in the FP/SD area, as well as altering the links between FP/SD proteins and the podocyte cytoskeleton.

Therefore, a future challenge is to carry out proteomic comparison of *Nphs1*$^{-/-}$ and *Nphs1*$^{+/+}$ glomeruli, to analyze the impact of nephrin absence on the expression and modifications of the glomerular proteome.

The results of this study indicate that the absence of nephrin from the SD does not affect podocyte viability or expression of other FP/SD and GBM proteins, and it has little impact on glomerular gene expression, in general. However, there was a significant upregulation of the TJ family member claudin 3 in *Nphs1*$^{-/-}$ glomeruli when compared with those in *Nphs1*$^{+/+}$. Claudins are integral proteins with four transmembrane regions, two extracellular and one intracellular loops, and both N- and C-terminal cytoplasmic domains. To date, 24 members of this multigene family have been identified in mouse and man. Claudins are major components of TJ, each of the claudins being expressed in a tissue-specific manner, such that every cellular sheet has its own unique set of claudins.[15,49–51] This arrangement is responsible for the different barrier properties among the TJs. Thus far, nine different claudins have been found to be expressed in different segments of the nephron, and mutation in some of them causes kidney failure. However, only claudins 1 and 2 have been observed in the glomerulus, at the Bowman's capsule.[31,32,52,53] Among all the claudins, only claudin 3 was upregulated in nephrin null mice. It is possible that claudin 3 compensates for the absence of nephrin by providing a TJ type of link between the podocytes that do not develop proper FPs.

The SD is a special variant of tight or AJs, since the mature SD still retains some features of both. For instance, ZO-1, a typical TJ protein, has been localized to the SD region,[54] and some investigators have reported expression of AJ molecules such as cadherins at the SD.[14,29,30] In addition to being responsible for sealing the cell–cell contacts and regulating cell permeability, TJs also participate actively in orchestrating cell polarity, proliferation, and differentiation.[55] The present results strongly suggest that nephrin participates in such processes by regulating the expression of proteins, as claudin 3, within the glomerulus. It is tempting to speculate why claudin 3 is upregulated in the absence of nephrin. It might be due to a compensation to connect podocyte FPs to each other and prevent plasma protein leakage in the absence of the SD when nephrin is missing. Indeed, as shown in Figure 4b, the expression of claudin 3 is highest in areas where the SD should be located. This agrees with the hypothesis that junction proteins such as claudin 3 might work as a compensatory molecule for the SD protein complex when the SD structure is impaired. In accordance with this hypothesis, previous data have shown that coxsackievirus and adenovirus receptor is expressed in rat glomerular podocytes and its expression is upregulated in PAN-induced nephrosis.[56] Taken together, these data strongly support a model where, in the absence of nephrin, TJ proteins present in podocyte precursors fail to be downregulated, both as a counterbalance mechanism triggered to keep proper kidney function and as a result of impaired nephrin signaling. However, the precise mechanism involved in the regulation of claudin 3 within the podocytes and its biological role requires further studies.

In summary, the present data confirm that the absence of nephrin leads to loss of the SD, proteinuria, and dilation of Bowman's capsule and proximal tubuli *in utero*. Furthermore, despite being an essential component of the FP/SD, nephrin plays no direct role in regulating gene expression in the glomerulus. Upregulation of claudin 3 in the absence of nephrin is an interesting finding, which may, in part, explain the electron-dense junctions found in our electron microscope analysis. However, further experiments are necessary to elucidate the exact nature of FP contacts observed in the absence of nephrin.

MATERIALS AND METHODS

Antibodies

The following antibodies were used: anti-nephrin,[25] anti-podocin (kindly provided by Dr Corine Antignac, Hôpital Necker-Enfants Malades, Paris, France), anti-podocalyxin (kindly provided by Dr Hiroshi Kawashi, Niigata University School of Medicine, Niigata, Japan), anti-synaptopodin (PROGEN Biotechnik GmbH, Heidelberg, Germany), anti-CD2AP (kindly provided by Dr Eero Lehtonen, University of Helsinki, Helsinki, Finland), anti-WT-1 (Santa Cruz Technology, Santa Cruz, CA, USA), anti-BrdU (BD, Franklin Lakes, NJ, USA), and anti-claudin 3 (Abcam Plc, Cambridge, UK).

Mice

Nephrin knockout mice (*Nphs1*$^{-/-}$) developed previously[25] were used in this study. All mouse experiments were performed according to Swedish Animal Research Regulations and approved by the Local Ethical Committee.

Immunohistochemistry

Kidneys were excised from *Nphs1*$^{-/-}$ and *Nphs1*$^{+/+}$ littermate embryos at E18.5, fixed, and embedded accordingly. Sections (8 μm) were stained with primary antibodies overnight at 4°C. Bound antibodies were detected with fluorescent-labeled secondary antibody (Molecular Probes, Invitrogen, Carlsbad, CA, USA), using a Nikon E1000 laser microscope equipped with a digital camera (Nikon Coolpix 990) or by confocal laser scanning microscopy (Zeiss LSM 510; Carl Zeiss, Göttingen, Germany). Images were processed using Adobe Photoshop.

Transferase dUTP Nick End Labeling

Transferase dUTP Nick End Labeling assay was performed using TUNEL labelling mix (Roche Diagnostics, Rotkreuz, Switzerland), according to the manufacturer's instructions.

Bromodeoxyuridine staining

Pregnant female mice were treated with one single intraperitoneal injection of BrdU (50 mg kg^{-1}) (Sigma, Schnelldorf, Germany). After 2 h, mice were killed by cervical dislocation, kidneys were excised from the embryos, fixed with 4% paraformaldehyde, and embedded in 22-oxacalcitriol (Sakura Finetek Europe BV, Zoeterwoude, The Netherlands). Sections (8 μm) were stained with anti-BrdU antibody overnight, washed, and incubated with fluorescent-labeled secondary antibody. BrdU incorporation was analyzed under fluorescence microscope.

Microarrays

Glomeruli were isolated from E18.5 stage *Nphs1*$^{-/-}$ and *Nphs1*$^{+/+}$ embryos using a Dynabead (Dynal; Invitrogen) perfusion method as previously described.[57] Total RNA was extracted using Mini RNA Isolation kit (Zymo Research, Orange, CA, USA), labeled, and hybridized on Affymetrix Mouse Genome 430 2.0 chips (Affymetrix GeneChip Technology, Santa Clara, CA, USA), and the array data were analyzed using the gcrma package[58] and affy package in the Bioconductor project (http://www.bioconductor.org).

Periodic acid-Schiff staining

Periodic acid-Schiff staining was performed according to the manufacturer's instructions (Bio-Optica, Milan, Italy).

Electron microscopy

For transmission electron microscopy, fixed kidneys harvested from *Nphs1*$^{-/-}$ and *Nphs1*$^{+/+}$ littermate E18.5 embryos were dehydrated in graded ethanol and embedded in epoxy resin. Ultra-thin sections were stained with uranyl acetate and lead citrate, and analyzed under a Jeol 1200 EX electron microscope at 60 kV accelerating voltage. Immunoelectron microscopy was carried out as described previously.[59]

DISCLOSURE

Karl Tryggvason is the cofounder of Nephrogenex Inc.

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SUPPLEMENTARY MATERIAL

Table S1. Supplementary list of genes expressed in *Nphs1*$^{-/-}$ glomeruli compared to glomeruli from *Nphs1*$^{+/+}$ mice.
Table S2. Summary of claudin 3 labeling in immunoelectron microscopy of kidney glomeruli.

Figure S1. Expression of ZO-1 in podocytes of *Nphs1*$^{+/+}$ and *Nphs1*$^{-/-}$ glomeruli was examined by immunoelectron microscopy. ZO-1 is located in foot processes of *Nphs1*$^{+/+}$ and at corresponding podocyte junctions in *Nphs1*$^{-/-}$ glomeruli.

REFERENCES

1. Rodewald R, Karnovsky MJ. Porous substructure of the glomerular slit diaphragm in the rat and mouse. *J Cell Biol* 1974; **60**: 423–433.
2. Wartiovaara J, Ofverstedt LG, Khoshnoodi J *et al.* Nephrin strands contribute to a porous slit diaphragm scaffold as revealed by electron tomography. *J Clin Invest* 2004; **114**: 1475–1483.
3. Kestila M, Lenkkeri U, Mannikko M *et al.* Positionally cloned gene for a novel glomerular protein—nephrin—is mutated in congenital nephrotic syndrome. *Mol Cell* 1998; **1**: 575–582.
4. Ruotsalainen V, Ljungberg P, Wartiovaara J *et al.* Nephrin is specifically located at the slit diaphragm of glomerular podocytes. *Proc Natl Acad Sci USA* 1999; **96**: 7962–7967.
5. Beltcheva O, Kontusaari S, Fetissov S *et al.* Alternatively used promoters and distinct elements direct tissue-specific expression of nephrin. *J Am Soc Nephrol* 2003; **14**: 352–358.
6. Lenkkeri U, Mannikko M, McCready P *et al.* Structure of the gene for congenital nephrotic syndrome of the finnish type (NPHS1) and characterization of mutations. *Am J Hum Genet* 1999; **64**: 51–61.
7. Koziell A, Grech V, Hussain S *et al.* Genotype/phenotype correlations of NPHS1 and NPHS2 mutations in nephrotic syndrome advocate a functional inter-relationship in glomerular filtration. *Hum Mol Genet* 2002; **11**: 379–388.
8. Boute N, Gribouval O, Roselli S *et al.* NPHS2, encoding the glomerular protein podocin, is mutated in autosomal recessive steroid-resistant nephrotic syndrome. *Nat Genet* 2000; **24**: 349–354.
9. Tsukaguchi H, Sudhakar A, Le TC *et al.* NPHS2 mutations in late-onset focal segmental glomerulosclerosis: R229Q is a common disease-associated allele. *J Clin Invest* 2002; **110**: 1659–1666.
10. Donoviel DB, Freed DD, Vogel H *et al.* Proteinuria and perinatal lethality in mice lacking NEPH1, a novel protein with homology to NEPHRIN. *Mol Cell Biol* 2001; **21**: 4829–4836.
11. Liu G, Kaw B, Kurfis J *et al.* Neph1 and nephrin interaction in the slit diaphragm is an important determinant of glomerular permeability. *J Clin Invest* 2003; **112**: 209–221.
12. Shih NY, Li J, Cotran R *et al.* CD2AP localizes to the slit diaphragm and binds to nephrin via a novel C-terminal domain. *Am J Pathol* 2001; **159**: 2303–2308.
13. Kim JM, Wu H, Green G *et al.* CD2-associated protein haploinsufficiency is linked to glomerular disease susceptibility. *Science* 2003; **300**: 1298–1300.
14. Inoue T, Yaoita E, Kurihara H *et al.* FAT is a component of glomerular slit diaphragms. *Kidney Int* 2001; **59**: 1003–1012.
15. Ciani L, Patel A, Allen ND *et al.* Mice lacking the giant protocadherin mFAT1 exhibit renal slit junction abnormalities and a partially penetrant cyclopia and anophthalmia phenotype. *Mol Cell Biol* 2003; **23**: 3575–3582.
16. Kaplan JM, Kim SH, North KN *et al.* Mutations in ACTN4, encoding alpha-actinin-4, cause familial focal segmental glomerulosclerosis. *Nat Genet* 2000; **24**: 251–256.
17. Kos CH, Le TC, Sinha S *et al.* Mice deficient in alpha-actinin-4 have severe glomerular disease. *J Clin Invest* 2003; **111**: 1683–1690.
18. Mucha B, Ozaltin F, Hinkes BG *et al.* Mutations in the Wilms' tumor 1 gene cause isolated steroid resistant nephrotic syndrome and occur in exons 8 and 9. *Pediatr Res* 2006; **59**: 325–331.
19. Hasselbacher K, Wiggins RC, Matejas V *et al.* Recessive missense mutations in LAMB2 expand the clinical spectrum of LAMB2-associated disorders. *Kidney Int* 2006; **70**: 1008–1012.
20. Winn MP, Conlon PJ, Lynn KL *et al.* A mutation in the TRPC6 cation channel causes familial focal segmental glomerulosclerosis. *Science* 2005; **308**: 1801–1804.
21. Hinkes B, Wiggins RC, Gbadegesin R *et al.* Positional cloning uncovers mutations in PLCE1 responsible for a nephrotic syndrome variant that may be reversible. *Nat Genet* 2006; **38**: 1397–1405.
22. Lehtonen S, Ryan JJ, Kudlicka K *et al.* Cell junction-associated proteins IQGAP1, MAGI-2, CASK, spectrins, and alpha-actinin are components of the nephrin multiprotein complex. *Proc Natl Acad Sci USA* 2005; **102**: 9814–9819.
23. Huber TB, Benzing T. The slit diaphragm: a signaling platform to regulate podocyte function. *Curr Opin Nephrol Hypertens* 2005; **14**: 211–216.

24. Benzing T. Signaling at the slit diaphragm. *J Am Soc Nephrol* 2004; **15**: 1382–1391.
25. Putaala H, Soininen R, Kilpelainen P *et al.* The murine nephrin gene is specifically expressed in kidney, brain and pancreas: inactivation of the gene leads to massive proteinuria and neonatal death. *Hum Mol Genet* 2001; **10**: 1–8.
26. Huber TB, Hartleben B, Kim J *et al.* Nephrin and CD2AP associate with phosphoinositide 3-OH kinase and stimulate AKT-dependent signaling. *Mol Cell Biol* 2003; **23**: 4917–4928.
27. Rocnik EF, Liu P, Sato K *et al.* The novel SPARC family member SMOC-2 potentiates angiogenic growth factor activity. *J Biol Chem* 2006; **281**: 22855–22864.
28. Van Itallie CM, Anderson JM. Claudins and epithelial paracellular transport. *Annu Rev Physiol* 2006; **68**: 403–429.
29. Reiser J, Kriz W, Kretzler M *et al.* The glomerular slit diaphragm is a modified adherens junction. *J Am Soc Nephrol* 2000; **11**: 1–8.
30. Tassin MT, Beziau A, Gubler MC *et al.* Spatiotemporal expression of molecules associated with junctional complexes during the *in vivo* maturation of renal podocytes. *Int J Dev Biol* 1994; **38**: 45–54.
31. Kiuchi-Saishin Y, Gotoh S, Furuse M *et al.* Differential expression patterns of claudins, tight junction membrane proteins, in mouse nephron segments. *J Am Soc Nephrol* 2002; **13**: 875–886.
32. Li WY, Huey CL, Yu AS. Expression of claudin-7 and -8 along the mouse nephron. *Am J Physiol Renal Physiol* 2004; **286**: F1063–F1071.
33. Tryggvason K, Patrakka J, Wartiovaara J. Hereditary proteinuria syndromes and mechanisms of proteinuria. *N Engl J Med* 2006; **354**: 1387–1401.
34. Kerjaschki D. Caught flat-footed: podocyte damage and the molecular bases of focal glomerulosclerosis. *J Clin Invest* 2001; **108**: 1583–1587.
35. Takemoto M, He L, Norlin J *et al.* Large-scale identification of genes implicated in kidney glomerulus development and function. *EMBO J* 2006; **25**: 1160–1174.
36. Somlo S, Mundel P. Getting a foothold in nephrotic syndrome. *Nat Genet* 2000; **24**: 333–335.
37. Reeves W, Caulfield JP, Farquhar MG. Differentiation of epithelial foot processes and filtration slits: sequential appearance of occluding junctions, epithelial polyanion, and slit membranes in developing glomeruli. *Lab Invest* 1978; **39**: 90–100.
38. Verma R, Kovari I, Soofi A *et al.* Nephrin ectodomain engagement results in Src kinase activation, nephrin phosphorylation, Nck recruitment, and actin polymerization. *J Clin Invest* 2006; **116**: 1346–1359.
39. Shankland SJ. The podocyte's response to injury: role in proteinuria and glomerulosclerosis. *Kidney Int* 2006; **69**: 2131–2147.
40. Autio-Harmainen H. Renal pathology of fetuses with congenital nephrotic syndrome of the Finnish type. 2. A qualitative and quantitative electron microscopic study. *Acta Pathol Microbiol Scand [A]* 1981; **89**: 215–222.
41. Nagata M, Nakayama K, Terada Y *et al.* Cell cycle regulation and differentiation in the human podocyte lineage. *Am J Pathol* 1998; **153**: 1511–1520.
42. Nagahama H, Hatakeyama S, Nakayama K *et al.* Spatial and temporal expression patterns of the cyclin-dependent kinase (CDK) inhibitors p27Kip1 and p57Kip2 during mouse development. *Anat Embryol (Berl)* 2001; **203**: 77–87.
43. Griffin SV, Petermann AT, Durvasula RV *et al.* Podocyte proliferation and differentiation in glomerular disease: role of cell-cycle regulatory proteins. *Nephrol Dial Transplant* 2003; **18**(Suppl 6): vi8–vi13.
44. Pavenstadt H, Kriz W, Kretzler M. Cell biology of the glomerular podocyte. *Physiol Rev* 2003; **83**: 253–307.
45. Kuusniemi AM, Merenmies J, Lahdenkari AT *et al.* Glomerular sclerosis in kidneys with congenital nephrotic syndrome (NPHS1). *Kidney Int* 2006; **70**: 1423–1431.
46. Ohse T, Inagi R, Tanaka T *et al.* Albumin induces endoplasmic reticulum stress and apoptosis in renal proximal tubular cells. *Kidney Int* 2006; **70**: 1447–1455.
47. Jones N, Blasutig IM, Eremina V *et al.* Nck adaptor proteins link nephrin to the actin cytoskeleton of kidney podocytes. *Nature* 2006; **440**: 818–823.
48. Saleem MA, Ni L, Witherden I *et al.* Co-localization of nephrin, podocin, and the actin cytoskeleton: evidence for a role in podocyte foot process formation. *Am J Pathol* 2002; **161**: 1459–1466.
49. Furuse M, Tsukita S. Claudins in occluding junctions of humans and flies. *Trends Cell Biol* 2006; **16**: 181–188.
50. Tsukita S, Furuse M, Itoh M. Multifunctional strands in tight junctions. *Nat Rev Mol Cell Biol* 2001; **2**: 285–293.
51. Van Itallie CM, Anderson JM. The molecular physiology of tight junction pores. *Physiology (Bethesda)* 2004; **19**: 331–338.
52. Hirano T, Kobayashi N, Itoh T *et al.* Null mutation of PCLN-1/Claudin-16 results in bovine chronic interstitial nephritis. *Genome Res* 2000; **10**: 659–663.
53. Reyes JL, Lamas M, Martin D *et al.* The renal segmental distribution of claudins changes with development. *Kidney Int* 2002; **62**: 476–487.
54. Schnabel E, Anderson JM, Farquhar MG. The tight junction protein ZO-1 is concentrated along slit diaphragms of the glomerular epithelium. *J Cell Biol* 1990; **111**: 1255–1263.
55. Bilder D. Epithelial polarity and proliferation control: links from the Drosophila neoplastic tumor suppressors. *Genes Dev* 2004; **18**: 1909–1925.
56. Nagai M, Yaoita E, Yoshida Y *et al.* Coxsackievirus and adenovirus receptor, a tight junction membrane protein, is expressed in glomerular podocytes in the kidney. *Lab Invest* 2003; **83**: 901–911.
57. Takemoto M, Asker N, Gerhardt H *et al.* A new method for large scale isolation of kidney glomeruli from mice. *Am J Pathol* 2002; **161**: 799–805.
58. Wu Z, Irizarry RA. Stochastic models inspired by hybridization theory for short oligonucleotide arrays. *J Comput Biol* 2005; **12**: 882–893.
59. Hultenby K, Reinholt FP, Oldberg A *et al.* Ultrastructural immunolocalization of osteopontin in metaphyseal and cortical bone. *Matrix* 1991; **11**: 206–213.