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Supplemental Information

Organoids from Nephrotic Disease-Derived iPSCs Identify Impaired NEPHRIN Localization and Slit Diaphragm Formation in Kidney Podocytes

Shunsuke Tanigawa, Mazharul Islam, Sazia Sharmin, Hidekazu Naganuma, Yasuhiro Yoshimura, Fahim Haque, Takumi Era, Hitoshi Nakazato, Koichi Nakanishi, Tetsushi Sakuma, Takashi Yamamoto, Hidetake Kurihara, Atsuhiro Taguchi, and Ryuichi Nishinakamura

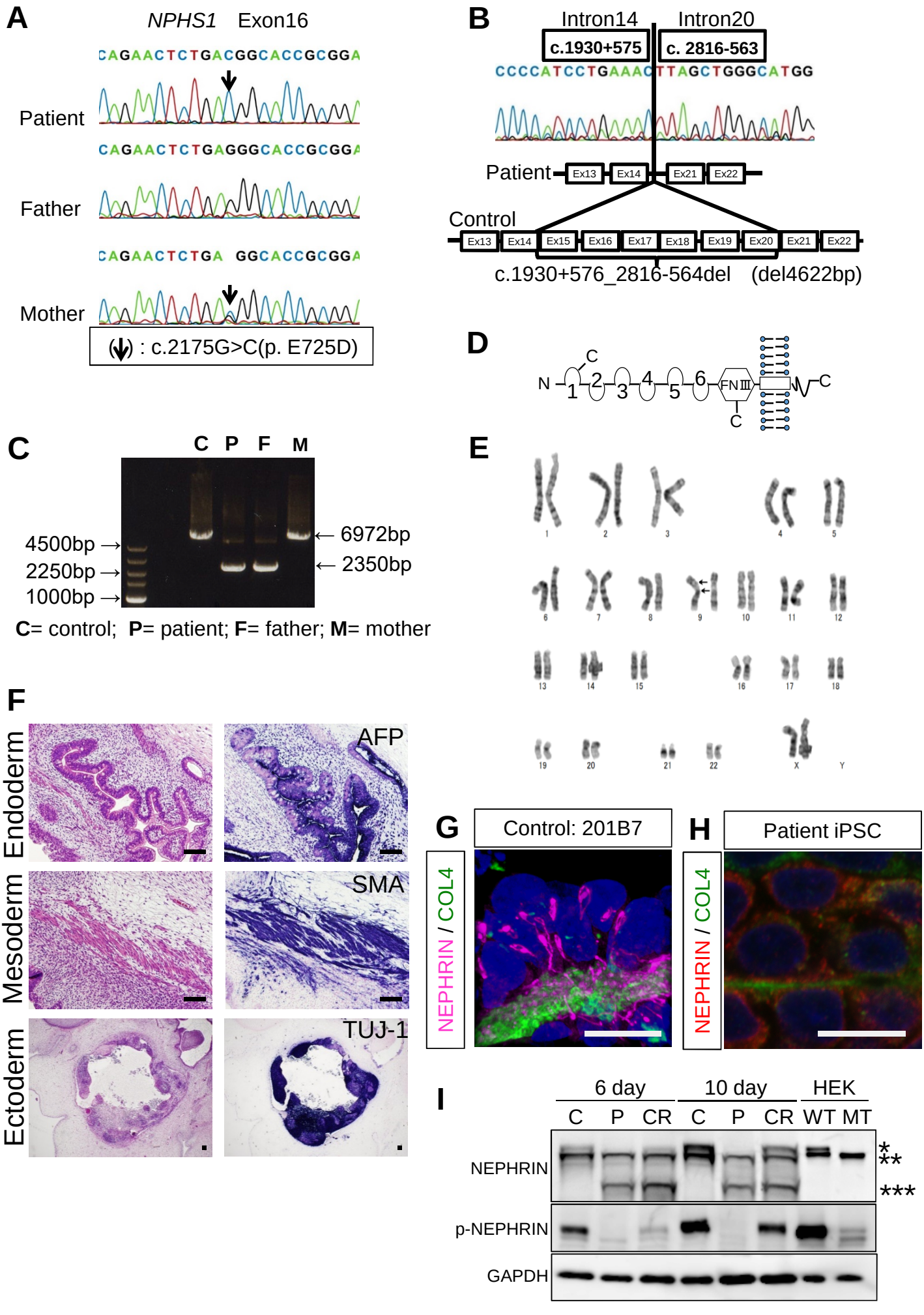
A
NPHS1 Exon16
CAGAACTCTGACGGCACCGCGGA
Patient
CAGAACTCTGAGGGCACCGCGGA
Father
CAGAACTCTGA GGCACCGCGGA
Mother
(↓) : c.2175G>C(p. E725D)
B
Intron14
Intron20
c.1930+575
c. 2816-563
CCCCATCCTGAAACTTAGCTGGGCATGG
Patient
Ex13 Ex14 Ex21 Ex22
Control
Ex13 Ex14 Ex15 Ex16 Ex17 Ex18 Ex19 Ex20 Ex21 Ex22
c.1930+576_2816-564del (del4622bp)
C
C P F M
4500bp →
2250bp →
1000bp →
← 6972bp
← 2350bp
C= control; P= patient; F= father; M= mother
D
N
C
1 2 3 4 5 6
FN III
C
E
F
Endoderm
Mesoderm
Ectoderm
AFP
SMA
TUJ-1
G
Control: 201B7
NEPHRIN / COL4
H
Patient iPSC
NEPHRIN / COL4
I
6 day
10 day
HEK
C P CR C P CR WT MT
NEPHRIN
p-NEPHRIN
GAPDH
*
**
***

Figure S1. related to Figure 1, 3, 5.

Identification of *NPHS1* mutations in the patient and generation of patient-derived iPSCs

A. *NPHS1* sequences showing the mutated nucleotide (black arrows) in blood samples from the patient and her parents.

B. Paternal *NPHS1* sequence at the junction caused by the exon 15–20 deletion. A total of 4622 base pairs were deleted.

C. Deletion of the paternal allele, as confirmed by genomic PCR. The primers shown in Figure 1A were used. The patient shows a 2350-bp band corresponding to the paternal allele.

D. The putative NEPHRIN protein produced from the paternal allele. The spacer region and the 7th and 8th Ig-like domains are deleted.

E. Normal karyotype of the patient-derived iPSC clone. Arrows: pericentric inversion within chromosome 9.

F. Teratomas formed by the patient-derived iPSCs. AFP: α-fetoprotein (endoderm marker); α-SMA: α-smooth muscle actin (mesoderm marker); TUJ-1: neuron-specific class III α-tubulin (ectoderm marker). Scale bars: 100 μm.

G. Z-stack visualization of NEPHRIN and COL4 in control iPSC-derived podocytes at day 20. Scale bar: 10 μm.

H. Impaired localization of NEPHRIN in podocytes of another patient-derived clone (#3-3) at day 20. Clone #1-5 was used for Figure 3. Scale bar: 10 μm.

I. Western blots of NEPHRIN in the podocytes at earlier differentiation time points. *: the upper band detected in the control and corrected clones; **: lower bands detected both in the controls and the mutants; ***: the additional bands presumably representing the paternal truncated protein. NEPHRIN is phosphorylated in the control and corrected clones, but not in the mutant clones. Wild-type NEPHRIN over-expressed in HEK 293 cells is also phosphorylated. Slower kinetics are also noted for the upper NEPHRIN band and NEPHRIN phosphorylation in the corrected podocytes. C: control; P: patient; CR: genetically corrected; HEK: HEK293 cells; WT: wild-type NEPHRIN; MT: mutant NEPHRIN.

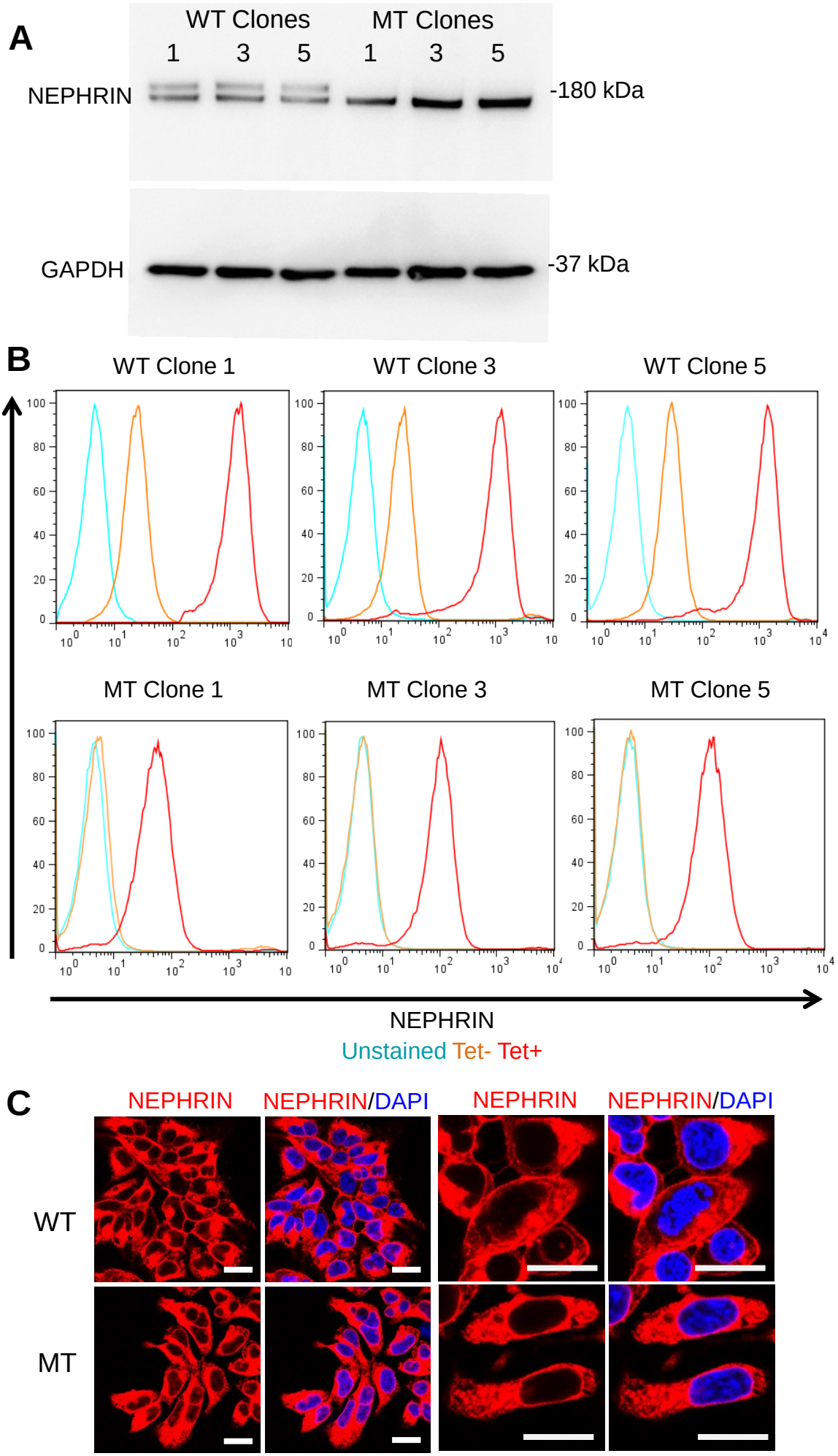
A
WT Clones
MT Clones
1
3
5
1
3
5
NEPHRIN
-180 kDa
GAPDH
-37 kDa
B
WT Clone 1
WT Clone 3
WT Clone 5
MT Clone 1
MT Clone 3
MT Clone 5
Percent of MAX
NEPHRIN
Unstained Tet- Tet+
C
NEPHRIN
NEPHRIN/DAPI
NEPHRIN
NEPHRIN/DAPI
WT
MT

Figure S2. Generation of HEK293 cell lines expressing wild-type and mutant NEPHRIN proteins. Related to Figure 2.

A. Minimal variations in expression levels of wild-type (WT) or mutant (MT) NEPHRIN proteins across multiple HEK293 clones. The upper band is absent in the mutant clones. Note the absence of the shorter extra band (Figure 1E), suggesting the presence of the paternally-derived truncated protein only in the podocytes.

B. Flow cytometry analysis showing reduced mutant NEPHRIN expression on the surface of HEK293 cells. Tet: tetracycline treatment for 48 hr. The Y-axis shows the cell counts normalized to 100% of the total cells.

C. Immunostaining of HEK293 cells overexpressing WT or MT NEPHRIN using an antibody against the intracellular domain of NEPHRIN (Progen; GP-N2) in the presence of detergent (0.1% Triton X-100). Note the comparable signals of the WT and MT NEPHRIN proteins. Scale bars: 20μm.

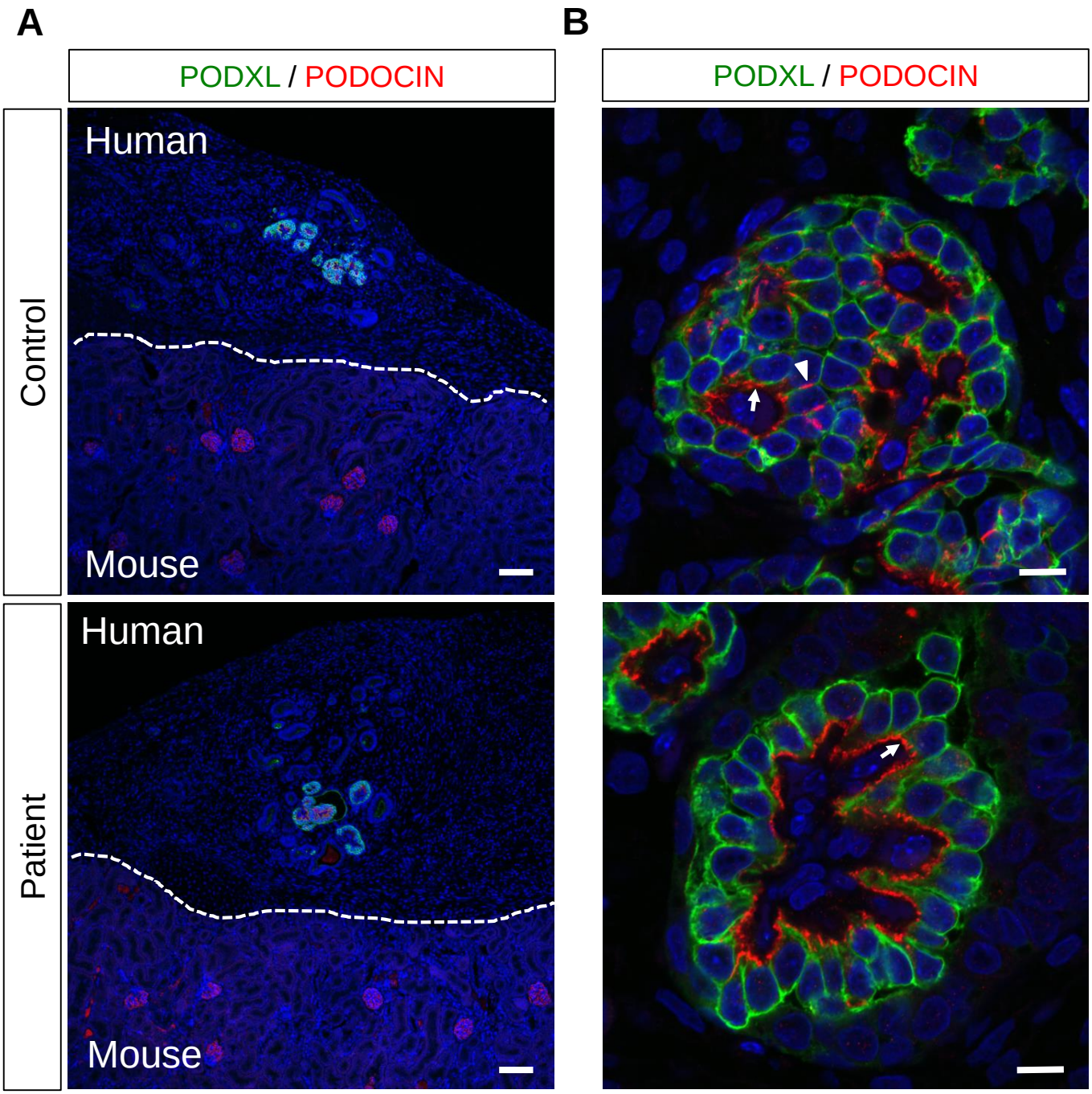

Figure S3. Analysis of human iPSC-derived kidney tissues after transplantation. Related to Figure 4.

A. Human iPSC-derived kidney tissues are clearly separated from the kidney parenchyma of the host mice at day 20 after transplantation. Staining with an anti-human PODXL-specific antibody confirms the human origin of the transplanted tissues. PODOCIN is detected in both the human and mouse glomeruli. Dashed lines: boundaries between the transplanted tissue and the host kidney. Scale bars: 100 µm.

B. Apical PODXL and basal PODOCIN expression in the control and mutant podocytes after transplantation. While the expression patterns are not markedly different, small numbers of PODOCIN+ pre-SD domains are detected in the control podocytes. Arrows: basal SD domains; arrowheads: lateral pre-SD domains. Scale bars: 10 µm.

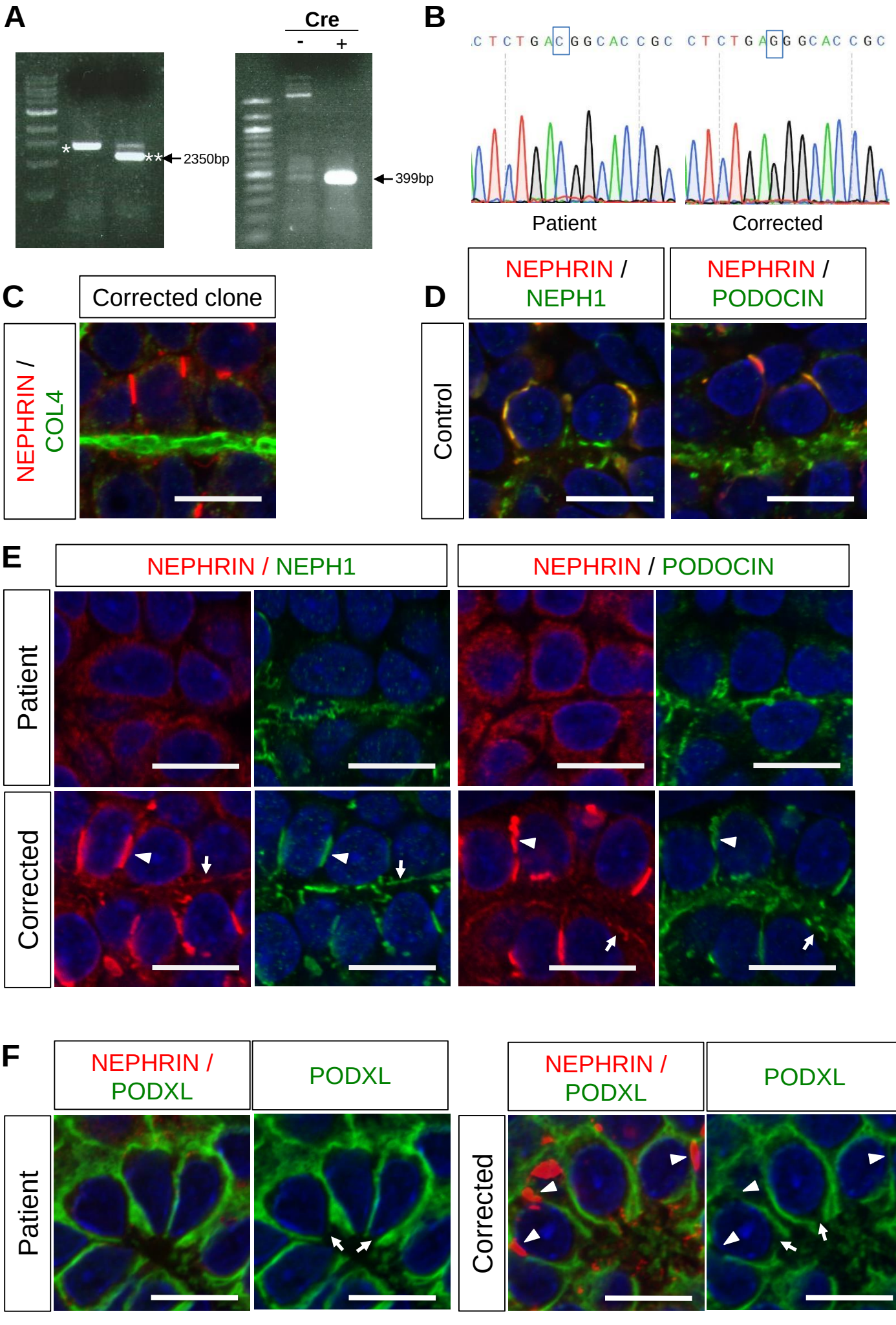
A
Cre
-
+
*
**
2350bp
399bp
B
Patient
Corrected
C
Corrected clone
NEPHRIN / COL4
D
NEPHRIN / NEPH1
NEPHRIN / PODOCIN
Control
E
NEPHRIN / NEPH1
NEPHRIN / PODOCIN
Patient
Corrected
F
NEPHRIN / PODXL
PODXL
Patient
Corrected

Figure S4. related to Figure 5.

Restored NEPHRIN expression by genetic correction

A. Genomic PCR showing the mutation correction. Left panel: clones before and after homologous recombination. *Nonspecific band. **Expected band spacing between the selection cassette and the patient's genomic DNA. Right panel: clones before and after removal of the selection cassette by Cre recombinase (Cre). The primer sequences are listed in Table S1.

B. Confirmation of the mutation correction.

C. Restored localization of NEPHRIN in podocytes derived from a genetically corrected clone (#1-5-9-1) at day 20. Clone #1-5-9-10 was used for Figure 5. Scale bar: 10 μm.

D. Staining of NEPH1 and PODOCIN in the kidney organoids derived from the control iPSCs (201B7). Scale bar: 10 μm.

E. Single-colored images of NEPHRIN and SD-associated proteins shown in Figure 5C. Arrowheads: lateral pre-SD domains; arrows: basal pre-SD domains. Scale bars: 10 μm.

F. Staining of PODXL and NEPHRIN in *in vitro* kidney organoids (day 20) derived from patient and genetically corrected iPSCs. PODXL is excluded from the basal domains (arrows). It is also excluded from the lateral NEPHRIN+ pre-SD domains in the corrected podocytes (arrowheads). Scale bars: 10 μm.

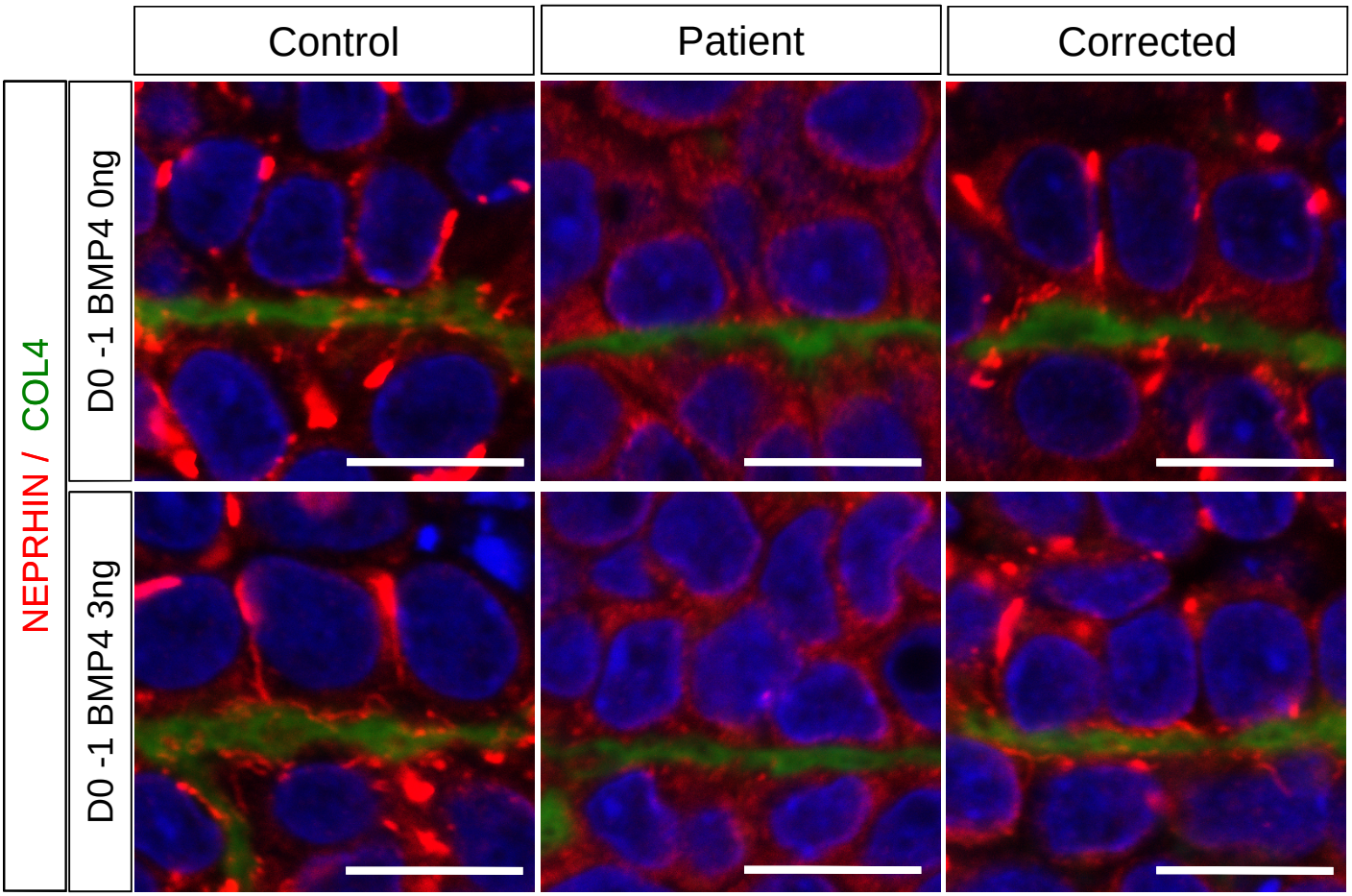

Figure S5. related to Figure 3, Experimental Procedures.

Initial BMP4 concentrations for nephron progenitor induction do not affect the resultant podocyte phenotypes

Immunostaining of *in vitro* kidney organoids derived from control, patient, and genetically corrected iPSCs. Different concentrations of BMP4 (0 and 3 ng/ml) were added at days 0–1 to induce nephron progenitors (13 days). Further culture for 20 days gives rise to nephrons containing glomeruli. Note that the BMP4 concentrations have no effect on the podocyte phenotypes. Scale bars: 10 μm.

Table S1. Sequences of the primers. Related to Figure 1, 2, 5, S1.

Purpose	Primer Name	5′ – Sequences – 3′
Construction of the E725D mutant vector for HEK293 cells	NEPHRIN mut-1	cggtatcgataagctCCAGGATCCCAGGCTTCCCG
	NEPHRIN mut-2	gcgcttccgcggtgcC**G**TCAGAGTTCTGGCAGTGC
	NEPHRIN mut-3	gcaccgcggaagcgcGGCTGCGGCTGGACGTGCAC
	NEPHRIN mut-4	tagaactagtggatcGGCGCCCCGTTGGTCCCCTG
To check mutagenic effect of TALEN	NEPHRIN rescue 13F	CTATCAGCTGCACTGCCAGA
	NEPHRIN rescue 13R	GAGCAGCTTCCGTGTCTAGG
To amplify 5′ arm of the correction vector	NEPHRIN rescue lt F1	actagtTGAAACTCCCGACCTTCATC
	NEPHRIN rescue rt R1	tgtacaGCTCCCACAATGAGGAGACT
To amplify 3′ arm of the correction vector	NEPHRIN rescue rt F2	gcggccgcGTCCCAGCCGCGGTGTAACC
	NEPHRIN rescue rt R2	gtcgacTTCATCCACCTGTTCATCCA
Screening 3′ recombination by PCR	NPR rt1F	GCTCAAAGAGCAGCGAGAAG
	NPR rt2R	CCATCTGTTCCTCCATCCAC
Screening 5′ recombination by sequencing	NPR lt2R	CGAGAAGCGTTCAGAGGAAA
	NPR seq F1	GTCCCTTATTCTGGCCTTCC
To amplify 3′ DIG probe for southern blot	NEPHRIN rescue 3F	CCTCTGTGGAGGGTGATTGT
	NEPHRIN rescue 3R	CAGAACTGG TGCTGTCTCCA
To confirm mutation correction	Ex 15-16F	CCTGATCTCCAATCTGTCCTTG
	Ex 15-16R	CCACAATGGGCAAGGTTCCTTG
To confirm a large deletion of multiple NPHS1 (exons 15-20)	NPHS1 Ex14-21 F	TGTTGGGTAAACACAGCAGAAATAGA
	NPHS1 Ex14-21 R	ACTCACAACCTTTAATCCTGATGGAG
To confirm excision of selection cassette after correction	NPR seq F1	GTCCCTTATTCTGGCCTTCC
	NPR right check (R)	GAGCAGCTTCCGTGTCTAGG

Supplemental Experimental Procedures

Immunohistochemical analysis

Samples were fixed in 10% formalin, embedded in paraffin, and cut into 6-μm sections. Antigen retrieval in citrate buffer was performed before staining. The following primary antibodies were used: rabbit anti-WT1 (Abcam; ab89901); goat anti-PODXL (R&D Systems; AF1658); guinea pig anti-NEPHRIN (Progen; GP-N2); rabbit anti-NEPHRIN (phospho Y1217) (Abcam; ab80298); rabbit anti-type IV collagen (Rockland; 600-401-106); rabbit anti-NEPH1 antibody (a kind gift from Dr. Y. Harita) (Harita et al., 2008); rabbit anti-PODOCIN (Immuno-Biological Laboratories; 29040); and rabbit anti-CD31 (Abcam; ab28364). Secondary antibodies were conjugated with Alexa 488 or 568 (Life Technologies). Immunofluorescence was visualized with an LSM780 confocal microscope (Zeiss) or a TCS SP8 confocal microscope (Leica). At least three samples from each of three independent induction experiments were serially sectioned and showed consistent results.

For HEK293 cells, the staining procedure was carried out without Triton X-100 to detect the extracellular domain of NEPHRIN with mouse monoclonal antibody 50A9 (a kind gift from K. Tryggvason) (Ruotsalainen et al., 2004) in combination with wheat germ agglutinin (Invitrogen; W11261). iPSCs were fixed with 4% paraformaldehyde and treated with 0.1% Triton-X100 for detection of NANOG and OCT3/4. Cells were fixed with ice-cold methanol for TRA-1-60 staining. The following primary antibodies were used: rabbit anti-NANOG (Cell Signaling; 4903), mouse anti-OCT3/4 (Santa Cruz; sc5279), mouse anti-SEAA-4 (Millipore; MAB4304) and mouse anti-TRA-1-60 (Millipore; MAB4360).

Flow cytometry

Induced kidney tissues and HEK293 cells were dissociated by incubation with 0.25% trypsin/EDTA for 8 and 5 min, respectively. After blocking in normal mouse serum (Thermo Fisher Scientific), staining was carried out in a buffer comprising 1% bovine serum albumin, 1× Hank’s balanced saline solution, and 0.035% $NaHCO_3$. The antibodies used for staining of nephron progenitors were as follows: phycoerythrin-conjugated anti-PDGFRA (Biolegend; 323506); biotinylated anti-ITGA8 (R&D Systems; BAF4076), and allophycocyanin-conjugated streptavidin (Biolegend; 405207). The extracellular domain of NEPHRIN was stained with mouse monoclonal antibody 48E11 (a kind gift from K. Tryggvason) (Ruotsalainen et al., 2004) followed by phycoerythrin-conjugated anti-mouse IgG1 (eBioscience; 12-4015-82). PODXL was stained with a goat anti-PODXL antibody (R&D Systems; AF 1658) followed by an Alexa 633-conjugated donkey anti-goat secondary

antibody (Life Technologies; A21082). Data were obtained using a FACS SORPAria (BD Biosciences) or FACS CANTOII (BD Biosciences) and analyzed with FlowJo software (TOMY Digital Biology).

Western blot analysis

Ten iPSC spheres were lysed in 150 μl of lysis buffer containing 25 mM HEPES-KOH (pH 7.8), 150 mM KCl, 1 mM MgCl2, 1% Triton X-100, 1% sucrose, proteinase inhibitor cocktail (Roche; 04693159001), and phosphatase inhibitor cocktail (Roche; 4906845). Cell lysates were homogenized on ice three times for 15 s each using an ultrasonicator. The protein concentrations were determined with an Rc-protein assay kit and a BSA standard (Bio-Rad Laboratories). Protein samples (20 μg each) were denatured in 4× LDS sample buffer at 70°C for 10 min and resolved in 4–12% NuPAGE Bis-Tris gels with MOPS buffer (Life Technologies). Proteins were transferred to PVDF membranes (Millipore), blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Triton-X 100 (TBST), and incubated overnight at 4°C with primary antibodies. Membranes were washed with TBST, and incubated with HRP-conjugated secondary antibodies. Bound antibodies were visualized using the ECL Select Western Blotting Detection reagent (GE Healthcare) according to the manufacturer's instructions. The following primary antibodies were used: guinea pig anti-NEPHRIN (Progen; GP-N2), rabbit anti-NEPHRIN (Immuno-Biological Laboratories; 29070), mouse anti-NCK (BD Biosciences; 610099), mouse anti-GAPDH (Thermo; AM4300), and mouse anti-Transferrin receptor (Thermo Fisher Scientific; H68.4). The other antibodies used were described in the section for immunohistochemical analysis.

Biotin-mediated labeling of cell surface NEPHRIN proteins

HEK293 cells were treated with 1 μg/ml tetracycline for specified times. Cells were washed with ice-cold PBS containing 0.1 mM CaCl2 and 1 mM MgCl2, and subjected to cell surface biotinylation and affinity purification using a Cell Surface Protein Isolation Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. The purified protein samples (6 μg each) were analyzed by western blotting. Three independent experiments showed consistent results. Endo H treatment was performed as described (Drozdova et al., 2013).

References

Drozdova, T., Papillon, J., and Cybulsky, A. V (2013). Nephrin missense mutations: induction of endoplasmic reticulum stress and cell surface rescue by reduction in chaperone interactions. Physiol. Rep. *1*, e00086.

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