Supplemental Information

Nephrin-binding ephrin-B1 at slit diaphragm controls podocyte functions through JNK pathway

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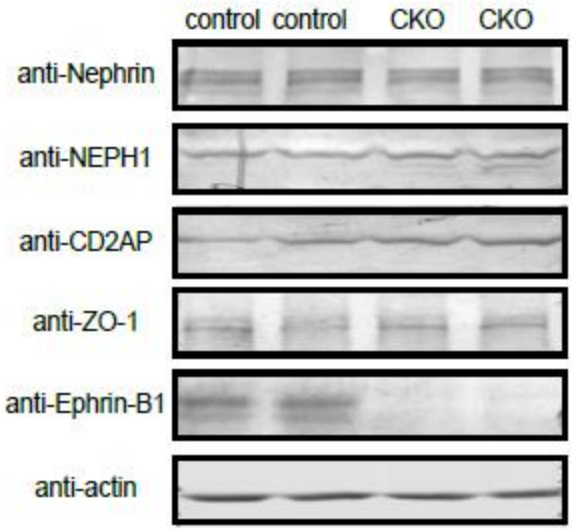
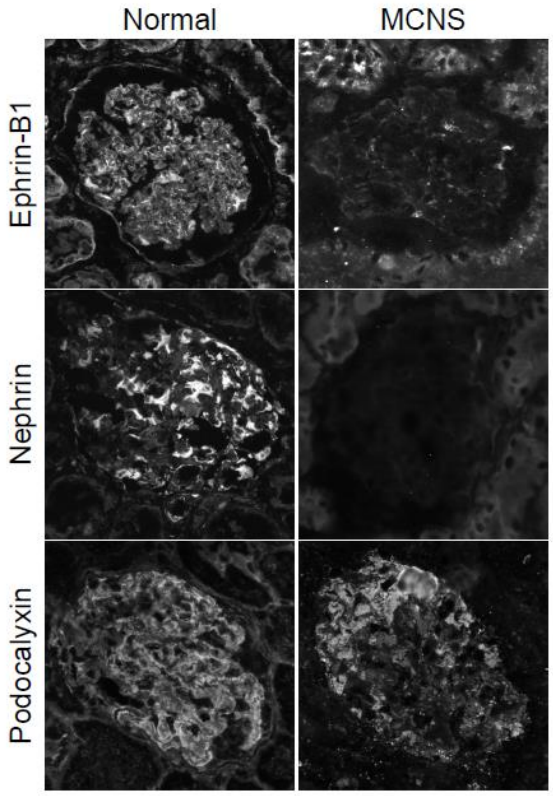


Supplemental Figure 1. Western blot findings of the slit diaphragm associated molecules of the glomerular lysates of the Ephrin-B1 CKO mice. No evident differences in the amounts of the expression of nephrin, NEPH1, CD2AP or ZO-1 were detected.



Supplemental Figure 2. Glomerular expression of ephrin-B1 in human nephrotic syndrome. The expression of ephrin-B1 as well as nephrin was clearly reduced in glomeruli of the case with active MCNS, whereas podocalyxin staining was reserved.

Supplemental Table 1. Antibodies in this study.

Antibodies	Reference or suppliers
Rabbit anti-intracellular site of nephrin antibody	Ref. 34
Rabbit anti-extracellular site of nephrin antibody	* supplemental text
Mouse anti-nephrin antibody	Ref. 26
Rabbit anti-podocin antibody	Ref. 35
Rabbit anti-CD2AP antibody	Ref. 37
Rabbit anti-NEPH1 antibody	Ref. 38
Rabbit anti-ephrin-B1 antibody	Santa Cruz Biotechnology, Dallas, TX
Goat anti-ephrin-B1 antibody	Ref. 7
Rabbit anti-ephrin-B2 antibody	Atlas Antibodies, Stockholm, Sweden
Rabbit anti-synaptopodin antibody	Atlas Antibodies, Stockholm, Sweden
Rabbit anti-phospho nephrin antibody	Abcam, Cambridge, UK
Rabbit anti-phospho ephrin-B antibody	Cell Signaling, Danvers, MA
Rabbit anti-ZO-1 antibody	Invitrogen, Camarillo, CA
Mouse anti-RECA1 antibody	Ref. 7
Mouse anti-Thy1.1 antibody	Ref. 7
Rabbit anti-phospho JNK antibody	Cell Signaling, Danvers, MA
Rabbit anti-JNK antibody	Cell Signaling, Danvers, MA
Rabbit anti-FLAG antibody	Sigma-Aldrich, St Louis, MO
Mouse anti-HA antibody	Santa Cruz Biotechnology, Dallas, TX
EphB2-Fc probe	R&D systems, Minnerapolis, MN
FITC-conjugated swine anti-rabbit IgG	DAKO, Glostrup, Denmark
TRITC-conjugated rabbit anti-mouse IgG	DAKO, Glostrup, Denmark
FITC-conjugated rabbit anti-human IgG	Southern Biotech, Birmingham, AL

(*, information on rabbit anti-extracellular site of nephrin antibody is provided in the Materials and Methods.)

Supplemental Table 2. Characteristics of ephrin-B1 CKO mice.

	control	CKO	p value
Body weight (g)	17.0 ± 1.53	17.3 ± 1.68	0.723
Kidney weight (mg)	112.1 ± 13.36	113.6 ± 10.06	0.842
Kidney weight (mg) /Body weight (g)	6.60 ± 0.29	6.60± 0.33	0.881
Glomerulus diameter (mm)	68.1 ± 5.23	67.6 ± 4.93	0.890
Number of glomeruli (mm^2)	17.2 ± 2.09	17.1 ± 3.03	0.935
Creatinine clearance (mL/min/kg BW)	4.38 ± 1.31	3.83 ± 0.71	0.419

Materials and Methods

Generation of conditional knockout mice.

To generate tamoxifen-inducible Cre recombinase (CreERT2) transgenic mice under the control of podocyte-specific promoter, the 2.6-kb promoter of the human NPHS2 was PCR amplified using the following primers: 5'-ACCGAGCTCGCCCTCCTATTTAGTCTCTCTGCCACCTAC-3' and 5'-AATTTGAGCTCGGTCAGAGCTGCCGGGCGGCTGGAGCAGCAGCGC-3'. The PCR product was subcloned into the pCR-Blunt vector (Invitrogen). The plasmid pCAG-CreERT2 (Addgene, Cambridge, MA) was digested with *EcoRI* and *NotI*, and the 2.0-kb fragment of CreERT2 was subcloned into the *EcoRI-NotI* site of the pAcGFP1-1 vector (Clontech, Foster City, CA). A 2.6-kb *Sac*I fragment of the pCR-blunt-human NPHS2 promoter was inserted into the pAcGFP1-1-*Sac*I–CreERT2, and the nucleotide sequence was confirmed. The *ApaLI-Afl*II fragment of the pAcGFP1-1 NPHS2 promoter-CreERT2 vector was used to generate Jcl;B6C3F1 background Nphs2-CreERT2 transgenic mice. PCR was performed using tail tissue DNA with following primers specific for Cre recombinase; the forward primer (5'- GCATTTCTGGGGATTGCTTA -3') and a reverse primer (5'- CCCGGCAAAACAGGTAGTTA-3').

Ephrin-B1$^{loxP/loxP}$ mutant mice [1] were generated from frozen sperm from Jackson Laboratory (Stock No. 007664). A PCR for Ephrin-B1$^{lox/lox}$ genotyping was performed using the forward primer (5'- GGCCTTTGAAGGAATGTGAA-3') and a reverse primer (5'-TTGTCCTAATGGGGCATTTC-3') used at Jackson Laboratory. Ephrin-B1$^{loxP/loxP}$ female mice were bred with Nphs2-CreERT2-Ephrin-B1$^{lox/y}$ male mice. Nphs2-CreERT2-Ephrin-B1$^{loxP/loxP}$ mice at 9-11 week of age were injected intraperitoneally with 200 mg/kg body weight of tamoxifen (Sigma-Aldrich, St Louis, MO) in sunflower seed oil (Sigma-Aldrich) for 10 days and the phenotypes of Nphs2-CreERT2-Ephrin-B1$^{loxP/loxP}$ mice were analyzed at day 20 after the first injection. Age-matched Ephrin-B1$^{loxP/loxP}$ mice were used as a control.

RT-PCR

Semi-quantitative RT-PCR with isolated glomerular RNA was performed basically according to the method described previously [2-4]. Tissues were homogenized, and then, total RNA was extracted (n = 3). Primers for Ephrin-B1, the forward primer (5'-GGCAAGCATGAGACTGTGAA-3') and a reverse primer (5'-CCGTAAGGGGATGATGATGT-3'), Ephrin-B2, the forward primer (5'-

TCCAACAAGACGTCCAGAGC-3') and a reverse primer (5'-AGAACAAGGTGCGAGTTCCC-3'), and GAPDH, the forward primer (5'- CTCCACTCA CGGCAAATTCAA-3') and a reverse primer (5'-GGATGACCTTGCCCACAGC-3'), were used.

Immunofluorescence (IF) and Western blot analysis

The IF studies with the CKO mice and rats were performed basically according to the method previously reported [5-7]. The antibodies used in this study were summarized in Supplemental Table 1. To produce the rabbit anti-extracellular site of nephrin antibody, we chose a peptide of 400 amino acids, which located at the center area of the extracellular region of rat nephrin (amino acids 335 to 734 (accession No.AF161715.1)), as the immunogen. Rabbit anti-extracellular site of nephrin antibody was prepared in rabbits immunized according to the method described previously[5]. IF staining for CD2AP, nephrin, NEPH1, podocin, ZO-1, and synaptopodin was semiquantified basically according to the method described by Macconi et al. [8] (score 0, completely absent; 1, signal covering 0 to 25% of the glomerular tuft area; 2, 25 to 50%; 3, 50 to 75%; and 4, 75 to 100%). The stainings were evaluated in 30 glomeruli of each mouse in a blinded manner. The data are expressed as mean ± SD of five to seven mice of each genotype. For the developmental study, a pair of Wistar rats was purchased from Charles River Japan, Inc., and mated, and the kidneys were removed from 3 neonatal rats. The kidney sections from each rats were used for dual-labeling IF analysis.

Western blot analysis with the isolated glomeruli, transfected HEK cells, and cultured podocytes was performed with basically according to the method described previously[9]. Alkaline phosphatase-conjugated anti-rabbit immunoglobulins and alkaline phosphatase-conjugated anti-mouse immunoglobulins (Zymed Laboratory Inc. San Francisco, CA) were used as secondary antibodies. The reaction was developed with an alkaline phosphatase chromogen kit (Kirkegaard & Perry Laboratories, Inc. Gainthersburg, MD). The western blot analyses were performed at least 3 times, and the representative findings were shown.

Evaluation of the width of foot process

The width of foot process was evaluated by the method of Bechtel et al[10]. Randomly selected five glomeruli of n=5 mice each were evaluated. Electron microscopy images were analyzed using ImageJ software. From each picture, the mean width of the foot processes (FPWs) was calculated according to the following formula: $FPW=\pi/4 \times (\Sigma$ glomerular basement membrane length/Σ foot process). A foot process was defined as any

connected epithelial segment butting on the basement membrane between two neighboring filtration slits.

Urinalysis and calculation of Ccr

Twenty-four-hour urine samples were collected in metabolic cages just before sacrifice, and their protein concentrations were measured by the Bradford method as described previously.[5] Urinary creatinine (Ucr) and serum creatinine (Scr) level were measured. The 24-hour endogenous creatinine clearance (Ccr) was calculated using the following formula: [Ccr (mL/min/kg BW) = Ucr (mg/dL) × 24-hour urine volume (mL) × 1/Scr (mg/dL) × 1/1440 (min) × 1 /kg BW]. The amount of proteinuria was shown as urinary protein to creatinine ratio.

Plasmid constructs

The coding sequence of full-length of rat nephrin generated by PCR was subcloned into p3xFLAG-CMV vector (Sigma). The coding sequence of extracellular domain (ECD) of rat nephrin was subcloned into pCAGGS-IgG1Fc[11]. The coding sequence of nephrin ECD lacking the 1st Ig site and the coding sequence of nephrin ECD lacking a fibronectin-like site and the 8th Ig were subcloned into pCAGGS-IgG1Fc. The coding sequence of intracellular domain (ICD) of nephrin generated by PCR was subcloned into pcDNA3.1/V5-His vector (Invitrogen). The coding sequence of full-length of rat ephrin-B1 was generated by PCR and subcloned into pKH3 vector (Addgene). The substitution mutants of nephrin lacking the tyrosine residues (Y1204) and ephrin-B1 lacking the tyrosine residues (Y323 and Y328) were generated using the PrimeSTAR® Mutagenesis Basal Kit according to the manufacturer's instruction (TaKaRa, Shiga, Japan). To examine whether nephrin interacts with the EphB receptor biding region of ephrin-B1, and the truncated ephrin-B1 lacking the site of 25-166 amino acids, which contains a region essential for interaction with EphB receptors, and that lacking the site of 167-235 amino acids, a basal region of extracellular domain, were generated using KOD –Plus– Mutagenesis Kit according to the manufacturer's instruction (TOYOBO, Tokyo, Japan). All constructs were verified by restriction enzyme digestion, DNA sequencing and Western blot analysis.

Immunoprecipitation assay

The cell lysates were incubated with an anti-FLAG antibody (Sigma-Aldrich) or anti-HA antibody (Santa Cruz) at 4°C overnight and precipitated with Dynabeads Protein G (Invitrogen) in the tube for 1 hour. The tube was placed on the magnet, and

the supernatant was removed. The Dynabeads-antibody-antigen complex was washed 5 times with PBS containing 0.1% Triton X-100, and then the antigen was eluted with an SDS-PAGE sample buffer. The eluates were separated by SDS-PAGE followed by immunoblotting with anti-FLAG, anti-HA antibody, anti-intracellular site of nephrin antibody or anti-extracellular site of nephrin antibody.

In vitro phosphorylation assay

Transfected HEK cells, cultured podocytes or isolated glomeruli were treated with 50 μM pervanadate (protein tyrosine phosphatase inhibitors) for 10 minutes. After treatments, cells were stimulated with 50 μg/ml of mouse anti-nephrin antibody [12] or 2 μg/ml of EphB2-Fc for 10 min. After the stimulation, cells were lysed with 1% Triton in Tris-HCl buffer with 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium fluoride and proteinase inhibitor mixture[13].

Cell motility Assay

Wound healing assays with HEK 293 cells transfected with ephrin-B1 and/or nephrin and cultured podocytes were performed basically according to the method of Cho H. J. et al[13]. The cells were pretreated with 1 μg/ml Mitomycin C (Sigma) for 2 h. Cell surface was scratched using a pipette tip. After scratching, cells were washed with PBS and incubated with fresh medium containing 0.2% FBS for 24 h. The photographs of wound closure were taken using a phase-contrast microscope with a digital camera. The gap area was quantified using Image J software. The %wound closure is determined as the (gap area at 0 h-gap area at 24 h)/gap area at 0 h x 100.

Induction of anti-nephrin antibody-induced nephropathy

A total of 9 female Wistar rats were intravenously injected with 10 mg/rat murine monoclonal antibody against rat nephrin (mAb 5-1-6) [5, 12], and 3 rats each were killed under pentobarbital anesthesia at 1 h, 24 h and 5 days after the antibody injection. The kidneys were removed, cut into portions and used for IF studies. Glomeruli isolated from the kidney pooled from three rats were solubilized and used for Western blot analysis.

Statistical analysis

Statistical significance was evaluated using the unpaired t-test or the Mann-Whitney U-test. Values were expressed as the mean ± SD. Differences at $p < 0.05$ were considered significant. Data were analyzed using Graphpad Prism 5.0 software (Graphpad Software, San Diego, CA).

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