

Genetic background-dependent diversity in renal failure caused by the *tns2* gene deficiency in the mouse

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ABSTRACT

Tensin2 (*Tns2*) is thought to be a component of the cytoskeletal structures linking actin filaments with focal adhesions and is known to play a role as an intracellular signal transduction mediator through integrin in podocytes, although the mechanism by which it functions remains unclear. A *Tns2*-null mutation (*nph*) leads to massive albuminuria following podocyte foot process effacement in the ICGN mice, the origin of the mutation, and the DBA/2J (D2) mice, but not in the C57BL/6J (B6) mice or 129^{+Ter}/SvJcl (129T) mice. Elucidating the reasons for these differences in diverse genetic backgrounds could help in unraveling *Tns2* function in podocytes. We produced congenic mice in which *Tns2*^{*nph*} was introgressed into a FVB/NJ background (FVB-*Tns2*^{*nph*}), and evaluated the progression of kidney disease. FVB-*Tns2*^{*nph*} mice developed albuminuria, renal fibrosis and renal anemia as seen in ICGN mice. The FVB-*Tns2*^{*nph*} mice demonstrated podocyte foot process alteration under an electron microscope by as early as 4 weeks of age. This revealed that FVB strain is susceptible to *Tns2*-deficiency.

Tensins are a family of multidomain proteins that bind to the cytoplasmic tails of β -integrins (8). Integrins are heterodimeric transmembrane cellular adhesion receptors that link the extracellular matrix (ECM) to the actin cytoskeleton and transmit signals from the ECM to the cytoplasm (2). In vertebrates, the tensin family consists of four members, TNS1, TNS2, TNS3 and TNS4. All isoforms conserve the C-terminal adjacent domains of a phosphotyrosine-binding (PTB) domain that can bind the β -integrin cytoplasmic tails and a Src homology 2 (SH2) do-

main that can bind tyrosine-phosphorylated proteins, including PI3 kinase, CAS and focal adhesion kinase, allowing them to mediate integrin-associated signaling cascades (8). These isoforms, except for TNS4, are also characterized by their possession of N-terminal adjacent domains consisting of a protein tyrosine phosphatase (PTP) domain and a protein kinase C conserved region 2 (C2) domain (5). In addition, as TNS2 has a protein kinase C conserved region 1 (C1) domain at the N-terminus, it is also referred to as tensin-like C1 domain containing phosphatase (TENC1). Tensins have been reported to be associated with a number of functions other than signal transduction, such as cell attachment and migration, cytoskeleton reorganization and tumor suppression (5). Due to their similar protein structures and gene expression patterns, tensins (excluding TNS4) appear to play redundant roles, although

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Table 1 *Tns2*-deficient mice

<i>Tns2</i> -deficient mice	Original name	Genetic background	Albuminuria	Reference
ICGN	ICGN	ICGN	+	Ogura <i>et al.</i> , 1989 This study
B6- <i>Tns2</i> ^{nph}	B6- <i>Tns2</i> ^{nep}	C57BL/6J	–	Nishino <i>et al.</i> , 2010
129T- <i>Tns2</i> ^{nph}	129T- <i>Tns2</i> ^{nep}	129 ^{+Ter} /SvJcl	–	Nishino <i>et al.</i> , 2012a
D2- <i>Tns2</i> ^{nph}	D2GN	DBA/2J	+	Uchio-Yamada <i>et al.</i> , 2013
FVB- <i>Tns2</i> ^{nph}	–	FVB/NJ	+	This study

All congenic strains were generated by backcrossing for ten and more generations.

they also have individual roles critical for specific tissues (3, 4, 7).

Tns3- and *Tns1*-deficient 129Sv/C57BL6 hybrid mice, for example, show abnormal morphologies in the small intestine and lung, and cyst formation in renal tubule, respectively, whereas *Tns2*-deficient C57BL/6J (B6) or 129^{+Ter}/SvJcl (129T) mice show no such defects (3, 7, 11, 12). On the other hand, in DBA/2J (D2) or ICGN genetic background, which is the origin of the *Tns2*-deficient mutation, *Tns2*-deficient mice display kidney glomerular alterations including excessive accumulation of the glomerular basement membrane (GBM) materials and loss of podocyte foot processes with age from 2 to 4 weeks after birth (15, 20). Eventually, they develop massive albuminuria, glomerulosclerosis, tubulointerstitial fibrosis and anemia (10, 14, 20, 22). In the ICGN mouse, there is a deletion of eight nucleotides situated in exon 18 of *Tns2*, leading to a frameshift and premature termination codon (4). The truncated transcript lacking the SH2 and PTB domains also loses its expression in podocytes and tubular epithelial cells, which are the intrinsic expression sites of *Tns2* in the normal kidney, likely due to nonsense-mediated mRNA decay (4, 13). Although all the previously reported *Tns2*-deficient mice carry this recessive mutation, called *Tns2*^{nph} (*nph*), these mouse strains can be sharply divided into two groups, susceptible and resistant to *Tns2*-deficiency, based on whether they develop albuminuria, likely induced by the alteration of glomerular podocytes (Table 1) (11, 12, 20).

Studies with inbred mouse strains have identified a genetic influence on the *nph* mutation: C57BL/6 and 129T mice are highly resistant to renal phenotype, in contrast to the susceptibility demonstrated in ICGN and D2 mice (11, 12, 20). This disparity among strains indicates the presence of modifier genes. Assuming that a key molecule plays a decisive role in triggering podocyte foot process effacement (i.e., albuminuria) in *Tns2*-deficiency, a comparison of the differences among diverse strain genetic back-

grounds (e.g., SNP analysis) helps in identifying the modifier gene. It also helps in unraveling *Tns2* function in podocytes. In this study, we investigated the renal phenotype of *Tns2*-deficient FVB/NJ (FVB) mice to increase the resources available for the comparative analysis.

MATERIALS AND METHODS

Animals. We produced a congenic strain, FVB.ICGN-*Tns2*^{nph} (FVB-*Tns2*^{nph}). The FVB-*Tns2*^{nph} strain was generated by backcrossing the *nph* allele from ICGN mouse into a FVB background for 10 generations. *nph* genotypes were determined as described previously (19). FVB, B6, D2 and 129T mice were purchased from CLEA Japan (Tokyo, Japan). The animal facility was air-conditioned at 22 ± 2°C, maintained at 40–60% relative humidity, and mice were maintained under a 12 h light-dark cycle. A standard laboratory diet, Labo MR Standard (Nosan, Kanagawa, Japan), and tap water were available *ad libitum*. A humane end point was applied when the mice with severe anemia became moribund.

Ethical statement. All research was conducted according to the Regulation for the Care and Use of Laboratory Animals of Kitasato University. The experimental protocols were approved by the president of Kitasato University following to the review of the institutional animal care and use committee (Approval ID: No. 14033).

Measurements of blood and urine. Blood and urinary samples were collected from the inferior vena cava and bladder, respectively under isoflurane anesthesia. Hemoglobin concentration, hematocrit, red blood cell count (RBC) and blood urea nitrogen (BUN) were measured as described previously (11). Urinary excretion of albumin was determined using an Albuwell M ELISA kit (Exocell, Philadelphia, PA, USA).

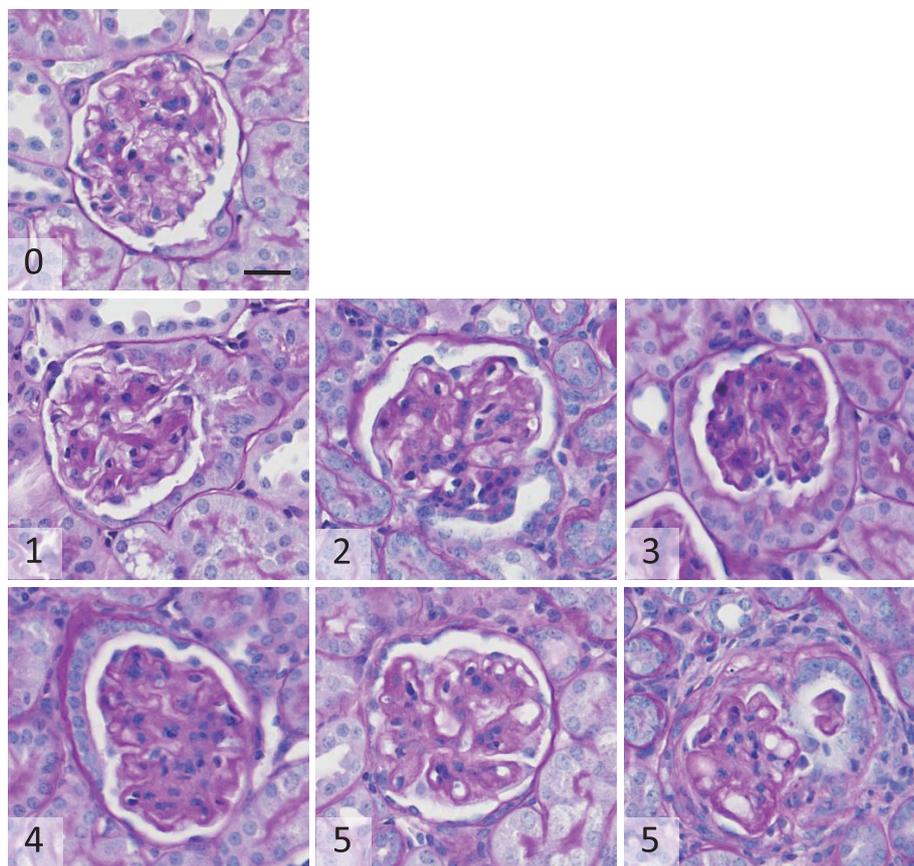


Fig. 1 Representative examples of modified glomerular score. Bar indicates 25 μ m.

Histological analysis. Formalin-fixed and paraffin-embedded kidney blocks were sectioned at a thickness of 2 μ m, and stained with periodic acid-Schiff (PAS) solution. To quantify the severity of renal damage, we used two types of histological injury score (glomerular score and tubular score) based on the progression of kidney pathology in ICGN mice, as previously described (19). To provide a more precise definition of the progression of glomerular damage, we subdivided the previously described severity of glomerular pathology into six stages characterized by the following additional observations: 0, no abnormality; 1, mild expansion of the mesangial matrix; 2, partial thickening of the GBM; 3, vascular stenoses (partial expansion of the mesangial matrix); 4, entire expansion of the mesangial matrix; 5, abnormal dilation of capillary lumen or retraction and collapse of the glomerular tuft (Fig. 1). The modified glomerular score was calculated as an average of these ratings for twenty randomly selected glomeruli.

Ultrastructural analysis. Kidneys from 4-week-old

mice were cut into small pieces (1 mm³) and prefixed in buffered 2.5% glutaraldehyde for 4 h, and then fixed with buffered 1% osmium tetroxide for 2 h. Fixed tissue was dehydrated by a graded alcohol, and embedded in epoxy resin (Quetol 812 Mixture; Nisshin EM, Tokyo, Japan). Epoxy resin-embedded specimens were sectioned at a thickness of 70 nm and stained with uranyl acetate and lead citrate, and observed under a JEOL transmission electron microscope (JEM-1210; JEOL, Tokyo, Japan).

Immunohistochemical analysis. Kidneys were fixed with 4% paraformaldehyde (PFA) at 4°C overnight. The PFA-fixed paraffin sections (2 μ m thick) were subjected to normal histological processes and antigen retrieval with sodium citrate buffer (pH 6.0). After treatment with 3% H₂O₂ and blocking with normal goat serum for 1 h, sections were incubated with 1 : 1000 diluted rabbit primary antibodies at 4°C overnight. Primary antibodies against Wilm's tumor suppressor gene 1 (WT1) (sc-192; Santa Cruz Biotechnology, Dallas, TX, USA) and podocin (P0372; Sigma-Aldrich, St. Louis, MO, USA) were used.

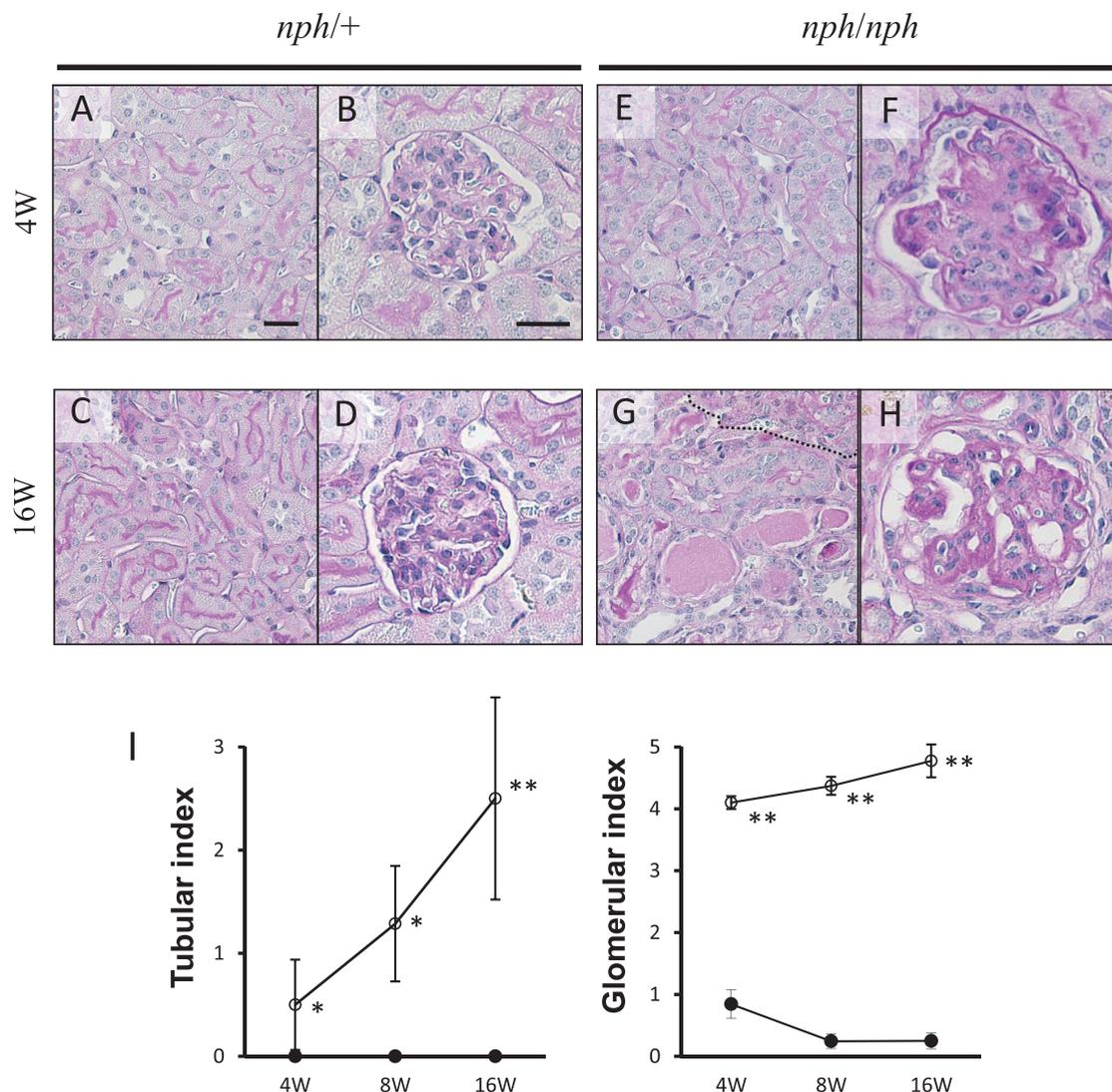


Fig. 2 Histology of the kidney in FVB congenic mice. (A–H) Representative PAS-stained kidney sections of FVB-*neph/+* and FVB-*Tns2^{nph}* mice. *neph* heterozygous kidneys are normal (A–D). *neph* homozygous glomeruli display mesangial matrix expansion at 4 weeks old (F), and eventually go into “collapse” at 16 weeks old (H). Tubular dilations and tubulointerstitial lesions (surrounded by a dotted line) are also observed in the homozygous kidney at 16 weeks old (G). (I) Histological scores for FVB-*neph/+* (closed circle) and FVB-*Tns2^{nph}* mice (open circle). Bars indicate 25 μ m. Asterisks indicate *P*-values, ** < 0.01 and * < 0.05. *n* = 4.

Sections were then incubated with biotin-conjugated goat anti-rabbit IgG secondary antibody (Histofine; Nichirei Biosciences, Tokyo, Japan) for 30 min at room temperature, and treated with horseradish peroxidase (HRP)-conjugated streptavidin complex (Histofine; Nichirei Biosciences) for 3,3-diaminobenzidine (DAB) staining. For quantitation of the loss of slit diaphragms and podocyte foot processes (podocin), and podocytes (WT1), the signal intensity of DAB was measured for seven randomly selected glomeruli for each sample using ImageJ software (<http://rsb.info.nih.gov/ij/>).

RESULTS

We produced a congenic strain carrying *Tns2^{nph}* on a FVB genetic background. The *neph*-homozygous (*neph/neph*) and *neph*-heterozygous (*neph/+*) FVB mice were analyzed at 4, 8 and 16 weeks old, from the time when ICGN mice show glomerulosclerosis to the time when they begin to show tubulointerstitial fibrosis. First, histological analysis with PAS staining revealed that almost all glomeruli in the *neph/neph* FVB mice (FVB-*Tns2^{nph}*) showed entire expansion of the mesangial matrix at 4 weeks old (Fig. 2F, I).

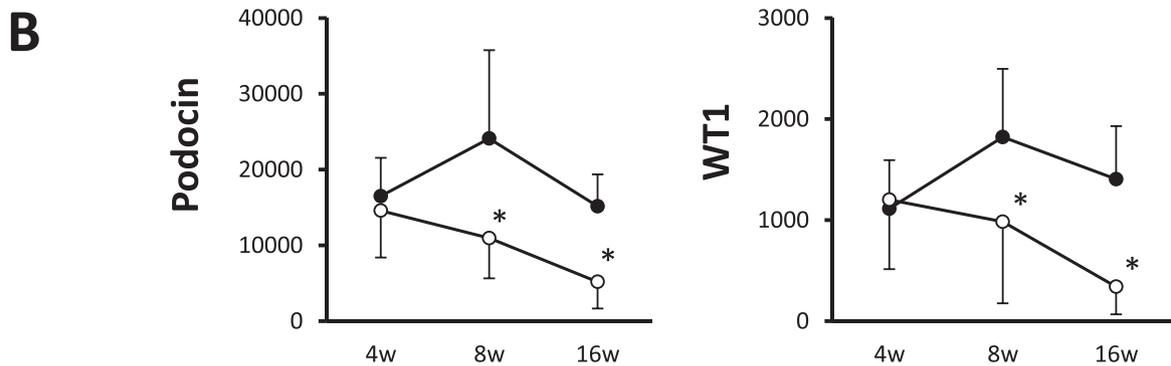
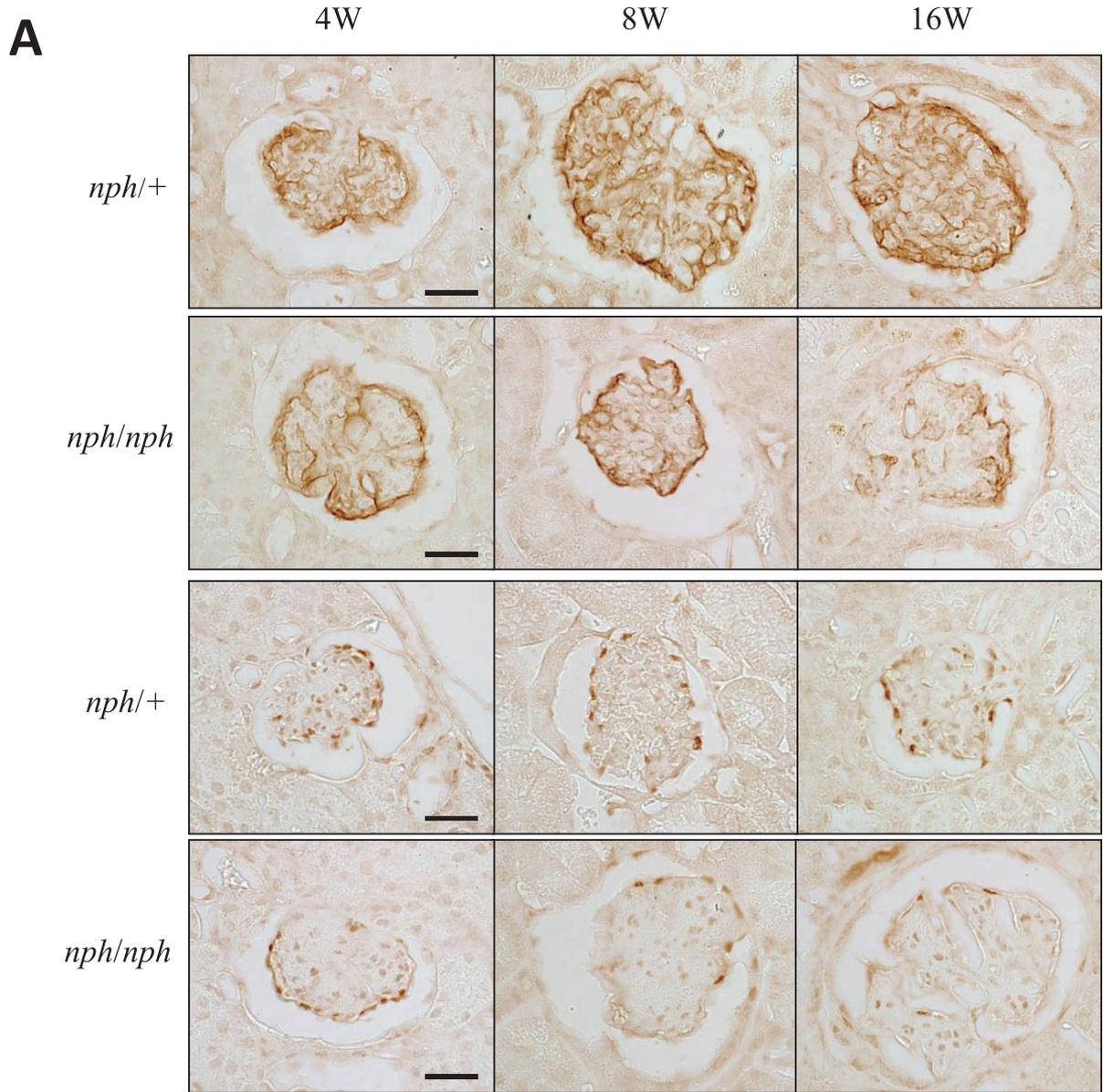


Fig. 3 Protein expression of podocin and WT1 in FVB congenic mice. **(A)** Representative immunohistological staining of glomerular podocin: slit diaphragm (upper panel) and WT1: podocyte nucleus (lower panel) in FVB congenic mice at 4, 8 and 16 weeks old. Bars indicate 25 μ m. **(B)** Quantification of histological expression levels in glomeruli. Asterisk, $P < 0.01$. $n = 4$.

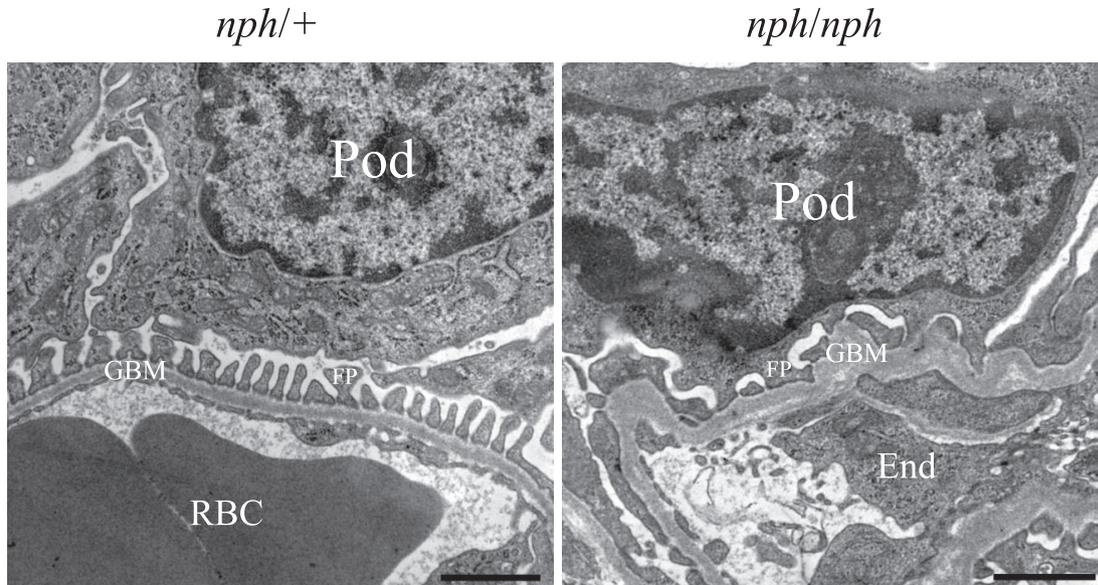


Fig. 4 Ultrastructural glomerular morphology in FVB congenic mice. In *nph* homozygous mice, podocyte foot process effacement and thickening and wrinkling of GBM are observed at 4 weeks after birth. Pod, podocyte; GBM, glomerular basement membrane; FP, foot process; RBC, red blood cell; End, endothelial cell. Bars indicate 1 μ m.

As time passed, some glomeruli in the FVB-*Tns2*^{nph} mice began to display abnormal dilation of the capillary lumen, and eventually displayed the retraction and collapse of the glomerular tuft (Fig. 2H, I). In FVB-*Tns2*^{nph} mice, tubular structures were nearly normal at 4 weeks old, but tubulointerstitial injury including renal interstitial fibrosis and tubular dilation with protein cast became remarkable at 16 weeks old (Fig. 2E, G, I). The kidneys of the FVB-*nph*/+ mice were normal up to 16 weeks old (Fig. 2C, D, I).

Immunohistological analyses showed that the glomerular expression of podocin and WT1 in the FVB-*Tns2*^{nph} mice decreased from 8 weeks after birth and had almost disappeared at 16 weeks old (Fig. 3). These decreases began in the glomerular central area, with subsequent decrease in the marginal area (Fig. 3A). The ultrastructural analysis revealed fused podocyte foot processes and GBM thickening in some glomeruli in the FVB-*Tns2*^{nph} mice at 4 weeks old (Fig. 4).

Last, the clinical parameters of blood and urine are shown in Fig. 5. Unexpectedly, the hemoglobin concentration and hematocrit of the FVB-*Tns2*^{nph} mice were lower than those of FVB-*nph*/+ mice at an early age (Fig. 5). These hematological parameters grew worse in 16-week-old FVB-*Tns2*^{nph} mice, and the decreases of this point were considered to be due to the development of tubulointerstitial fibrosis, that is, renal anemia (Fig. 2I, 5). Blood urea

nitrogen concentration was elevated in some 16-week-old FVB-*Tns2*^{nph} mice (~233 mg/dL). In the FVB-*Tns2*^{nph} mice, albuminuria was detectable at 4 weeks old, and the elevated urinary albumin excretion thereafter plateaued until 8 weeks after birth. On the other hand, the urinary albumin excretion in FVB-*nph*/+ mice was almost zero (< 0.017 g/dL) up to 16 weeks after birth. Overall, the FVB-*Tns2*^{nph} mice, like ICGN mice, were found to develop glomerulosclerosis, tubulointerstitial fibrosis, renal anemia and albuminuria with increased age following the ultrastructural alterations of the podocyte foot process and GBM.

DISCUSSION

Four *Tns2*-deficient murine strains have been reported previously, two susceptible to and two resistant to *Tns2*-deficiency (Table 1). However, because ICGN mice were derived from a spontaneous mutant found in ICR outbred stock, D2-*Tns2*^{nph} was the only susceptible strain for which genetic resources were available for a comparison of differences among diverse genetic backgrounds of whether or not *Tns2*-deficient mice develop renal failure. To increase the resources available for the comparative analysis, we therefore produced a congenic strain in which *Tns2*^{nph} was introgressed into FVB background, which is classically known to be sensitive to glomerular disease. For instance, FVB strains develop

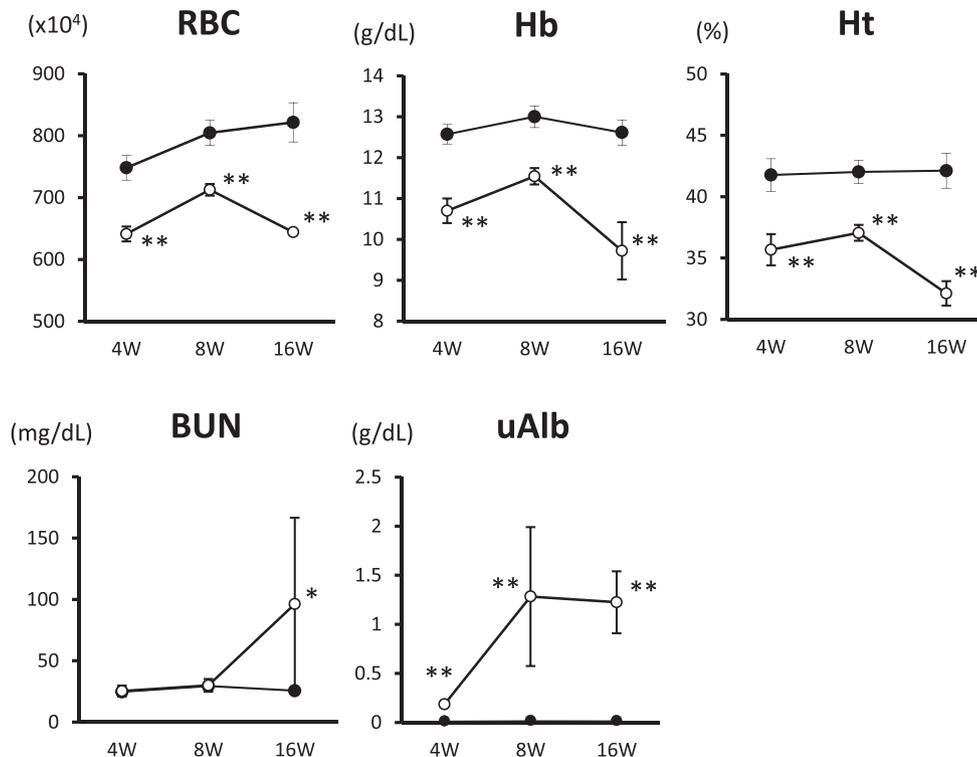


Fig. 5 Hematological parameters, BUN and urinary albumin concentration in FVB congenic mice. Phenotypic analysis between *nph* heterozygous mice (closed circle) and *nph* homozygous mice (open circle). RBC, number of red blood cells; Hb, hemoglobin concentration; Ht, hematocrit; BUN, blood urea nitrogen concentration; uAlb, urinary albumin excretion. Asterisk indicates P -values, ** < 0.01 and * < 0.05 . $n = 4$.

renal disease due to podocyte-specific knockout of tetraspanin CD151, whereas B6 strain is generally resistant to experimental glomerular disease, including HIV-associated nephropathy and adriamycin-induced nephropathy (16–18). Like Cd151, *Tns2* interacts with integrins and its deficiency leads to alterations in the podocyte foot processes (16–18). Thus, the FVB strain was predicted to be susceptible to *Tns2*-deficiency.

In fact, FVB-*Tns2*^{*nph*} mice, like ICGN mice, showed glomerulosclerosis, characterized by entire expansion of the glomerular mesangial matrix, and developed tubulointerstitial fibrosis (Fig. 2). In FVB-*Tns2*^{*nph*} mice, decreases in the glomerular expression of podocin and WT1, which localize in slit diaphragm area and podocyte nucleus respectively, were observed to begin from the glomerular central area with disease progression (Fig. 3). Similar pathological patterns in the glomerular expression of synaptopodin and nephrin were reported previously in ICGN mice (6). Reduction in the expression of these key podocyte proteins indicates podocyte dysfunction (9, 21). Alterations in the podocyte foot processes and GBM were actually observed under

an electron microscope in FVB-*Tns2*^{*nph*} mice at 4 weeks old (Fig. 4). These alterations probably led to proteinuria in FVB-*Tns2*^{*nph*} mice at 4 weeks old, and the resultant massive proteinuria induced tubular epithelial-mesenchymal transition, with the subsequent tubulointerstitial fibrosis, resulting in renal anemia (Fig. 2, 5) (1, 9). Therefore, the generation of FVB-*Tns2*^{*nph*} mice revealed that the FVB strain is susceptible to renal failure induced by *Tns2*^{*nph*} as expected. On the other hand, low values of the hematological parameters in FVB-*Tns2*^{*nph*} mice at 4 weeks were unexpected because remarkable tubulointerstitial lesions were not observed at this point (Fig. 2, 5). Early anemia is not observed in B6-*Tns2*^{*nph*} and ICGN mice (11). These results indicate the possibility that *Tns2*-deficiency leads to a decrease in the inherent function of renal erythropoietin-producing cells or a bone marrow failure in the FVB genetic background.

In conclusion, our study afforded two strains (B6 and 129T) resistant to and two strains (D2 and FVB) susceptible to nephropathy caused by *Tns2*-deficiency. Elucidation of the reasons for the distinct difference in susceptibility to *Tns2*-deficiency among

murine strains would help clarify the podocyte-specific function of Tns2 and provide a novel insight into the unique cytoskeleton of the podocyte foot processes.

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