Deletion of the Tensin2 SH2-PTB domain, but not the loss of its PTPase activity, induces podocyte injury in FVB/N mouse strain

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Abstract: Tensin2 (TNS2) is a focal adhesion-localized protein possessing N-terminal tandem protein tyrosine phosphatase (PTPase) and C2 domains, and C-terminal tandem Src homology 2 (SH2) and phosphotyrosine binding (PTB) domains. Genetic deletion of Tns2 in a susceptible murine strain leads to podocyte alterations after birth. To clarify the domain contributions to podocyte maintenance, we generated two Tns2-mutant mice with the genetic background of the susceptible FVB/NJ strain, Tns2ΔC and Tns2CS mice, carrying a SH2-PTB domain deletion and a PTPase domain inactivation, respectively. The Tns2ΔC mice developed massive albuminuria, severe glomerular injury and podocyte alterations similarly to those in Tns2-deficient mice. In contrast, the Tns2CS mice showed no obvious phenotypic abnormalities. These results indicate that the TNS2 SH2-PTB domain, but not its PTPase activity, plays a role in podocyte maintenance. Furthermore, in a podocyte cell line, the truncated TNS2 mutant lacking the SH2-PTB domain lost the ability to localize to focal adhesion. Taken together, these data suggest that TNS2 recruitment to focal adhesion is required to maintain postnatal podocytes on a susceptible genetic background.

Key words: chronic kidney disease, focal adhesion, nephropathy, podocyte, tensin

Introduction

Tensin2 (TNS2, also known as TENC1 or C1-TEN) is a member of the tensin family that includes Tensin1 (TNS1), Tensin3 (TNS3) and Tensin4 (TNS4) in mammals. All tensins possess C-terminal tandem Src homology 2 (SH2)-phosphotyrosine binding (PTB) domains, which bind short peptide motifs containing phosphorylated tyrosines and the cytoplasmic tails of cell surface receptors (including receptor tyrosine kinases and integrins) through NPXY motifs [8, 24, 26]. An additional characteristic of tensins, except for TNS4, is the presence of a protein tyrosine phosphatase (PTPase) domain paired with the C2 domain, resembling that of PTEN [6, 8]. However, TNS1 lacks an essential catalytic cysteine residue for PTPase enzymatic activity, which catalyzes the dephosphorylation of phosphotyrosyl proteins [6]. Moreover, structural analyses suggest that the TNS3 catalytic pocket is structurally different from those of PTEN and TNS2 [7]. Indeed, only TNS2 appears to have PTEN-like PTPase activity [7]. It is easy to imagine that these proteins, which possess SH2-PTB and PTPase domains, interact with tyrosine phosphorylation signaling pathways.

Tensin functions have been well investigated in cancer cells with a focus on focal adhesion, migration, prolif-
eration, cytoskeleton, integrin-mediated signal transduction, and so on. Structurally resembling PTEN and binding to Rho GTPase-activating protein DLC1 [8], both tumor suppressors, makes tensins an appealing target for cancer research. However, their biological functions remain poorly understood. Tns2-null mutant (called Tns2<sup>2<sub>npb</sub></sup>) mice develop renal failure caused by postnatal alterations in podocytes, although this occurs in a strain-dependent manner. Tns2<sup>2npb</sup> induces podocyte foot process effacement accompanied by glomerular basement membrane (GBM) thickening, and subsequent glomerular and tubulointerstitial injuries in ICGN, which is the original strain possessing the nph mutation, FVB/NJ (FVB) and DBA/2J strains, but only modest GBM thickening in C57BL/6J and 129<sup>1/2</sup>T<sup>129</sup>/J strains [15, 16, 22, 27, 28]. Recently, to clarify the domain contributions, we have generated TNS2 C-terminal deletion mutant (designated Tns2<sup>ΔC</sup>, Fig. 1) mice with a frameshift mutation in exon 22, resulting in truncation of the SH2 domain and deletion of the PTB domain [13]. These mice showed podocyte abnormalities, GBM thickening, glomerular injury and proteinuria, suggesting that the TNS2 SH2-PTB domains are involved in TNS2 function in podocytes [13]. However, we cannot conclude that the results obtained from Tns2<sup>ΔC</sup> in mice are equal to those from Tns2<sup>2npb</sup>; i.e., that TNS2 SH2-PTB domains play a critical role in maintaining podocytes, as it was impossible to compare the Tns2<sup>2Ac</sup> mice, which were F1 progeny from an N4 backcross (BDF1 × FVB), with the Tns2<sup>2npb</sup> mice in an FVB genetic background due to differences in genetic background. We also cannot exclude the possible contributions of other functional domains, such as the PTPase domain.

In this study, we generated a novel mutant mouse carrying a missense mutation (p.Cys231Ser) in TNS2 (designated Tns2<sup>CS</sup>, Fig. 1), resulting in the catalytic inactivation of the PTPase domain [6, 11]. We then assessed the effects of these Tns2 mutations (Tns2<sup>2npb</sup>, Tns2<sup>ΔC</sup> and Tns2<sup>2CS</sup>) on podocytes in mice with an FVB genetic background to investigate the contribution of each of the SH2-PTB and PTPase domains to podocyte maintenance.

**Materials and Methods**

**Ethical statement**

All research was conducted according to the Regulations for the Care and Use of Laboratory Animals of Kitasato University. The animal experimentation protocol was approved by the President of Kitasato University based on the judgment made by the Institutional Animal Care and Use Committee of Kitasato University (Approval ID: 18-072, 17-100, 16-074, 15-049).

**Animals**

Tns2<sup>2npb</sup> mice with an FVB genetic background (hereafter simply, Tns2<sup>2npb</sup> mice) were generated as described previously [22]. Tns2<sup>2Ac</sup> mice with an FVB genetic background (hereafter simply, Tns2<sup>2Ac</sup> mice) were generated by further backcrossing the previously described Tns2<sup>2Ac</sup> mice [13] 7 times onto FVB for 11 generations in total. Tns2<sup>2CS</sup> mice with an FVB genetic background (hereafter simply, Tns2<sup>2CS</sup> mice) were generated by CRISPR/Cas9-mediated genome editing as described previously [17]. Briefly, the guide RNA (gRNA) guiding sequence for the catalytic site of the PTPase domain (NCBI accession numbers NM_001355636 and NM_153533) was designed as follows: 5’-CGTTGTTGTGTTGTTACTGCA-3’ (where the underlined residues correspond to an essential catalytic residue Cys231 [6, 11]). From the DNA oligonucleotides consisting of tandemly arranged T7 promoter and gRNA sequences, gRNA was transcribed <i>in vitro</i> using the MEGAscript T7 kit (Thermo Fisher Scientific, Waltham, MA, USA). A single-stranded oligodeoxynucleotide (ssODN) for the targeted insertion (5’-GGCAAGTGAAGGCTGCCACCTTGA-3’) was digested with NsiI and ligated to the vector.

![Fig. 1. Schematic illustrations of the mouse TNS2 protein and the mutant proteins. Asterisk indicates catalytic inactivity.](image-url)
GGGGTCACAG-3', where underlined residue indicates a point mutation in the reference genome sequence, NCBI accession number NC_000081, resulting in a single amino acid alteration Cys231Ser) was synthesized artificially (Eurofins Genomics, Brussels, Belgium). Cas9 mRNA was synthesized by mMESSAGE mMACHINE T7 Ultra transcription kit (Thermo Fisher Scientific). The Cas9 mRNA (20 ng/µl), gRNA (10 ng/µl) and ssODN (50 ng/µl) were delivered into fertilized eggs from FVB mice by microinjection. After overnight culture, two-cell embryos were transferred into pseudopregnant female mice. The TNS2 PTPase domain-coding region of the genome from the offspring was amplified by PCR and sequenced with the following primers: Tns2CS forward, 5'-GCAAGACTTTGGTTGCGCTG-3' and Tns2CS reverse, 5'-GGGACAGATGAGGGAAGGCC-3'. All mutant strains were maintained by back-crossing to FVB mice. Homozygous mutant mice were generated by crossing between the heterozygous mutant mice themselves and used for experiments.

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, CE-2 (Clea Japan), and tap water were available ad libitum. The animals’ microbiological status was monitored periodically according to the Japanese Association of Laboratory Animal Facilities of Public and Private Universities guidelines. A humane endpoint was applied when the mice with severe anemia became moribund.

Measurement of urinary albumin excretion

Urine samples were collected from 8-week-old mice by gentle manual compression of the abdomen. To measure albumin, urine samples were diluted with sample buffer containing 2% sodium dodecyl sulfate (SDS), 5% 2-mercaptoethanol, 10% glycerol, 60 mM Tris-HCL (pH 7.4), and then fixed in buffered 1% osmium tetroxide for 2 h. The fixed tissue was dehydrated by graded alcohol, and embedded in epoxy resin. The epoxy resin-embedded specimens were sectioned at a thickness of 70 nm and stained with uranyl acetate and lead citrate, and observed under an H-7650 transmission electron microscope (Hitachi High-Technologies, Tokyo, Japan).

Histology

Kidneys from 8-week-old mice were fixed with 4% paraformaldehyde (PFA) at 4°C overnight. The PFA-fixed paraaffin sections (2 µm in thickness) were subjected to normal histological processes and stained with periodic acid-Schiff (PAS) solution. To quantify the severity of glomerular damage, we used a histological injury score as previously described [22], with a slight modification. We subdivided the severity of glomerular histopathology into seven stages characterized by the following observations: 0, no abnormality; 1, mild expansion of the mesangial matrix; 2, partial thickening of the GBM; 3, vascular stenosis (partial expansion of the mesangial matrix); 4, entire expansion of the mesangial matrix; 5, abnormal dilation of the capillary lumen; and 6, retraction and collapse of the glomerular tuft. Glomerular histopathology was scored blindly and independently by 2 observers. The glomerular injury score was calculated as a mean of these ratings for twenty-five randomly selected cortical glomeruli averaged for the 2 independent observers.

Transmission electron microscopy

Kidneys from 8-week-old mice were cut into small pieces (1 mm³) and prefixed in Karnovsky solution (2% glutaraldehyde, 2% PFA in 50 mM cacodylate buffer, pH 7.4), and then fixed in buffered 1% osmium tetroxide for 2 h. The fixed tissue was dehydrated by graded alcohol, and embedded in epoxy resin. The epoxy resin-embedded specimens were sectioned at a thickness of 70 nm and stained with uranyl acetate and lead citrate, and observed under an H-7650 transmission electron microscope (Hitachi High-Technologies, Tokyo, Japan).

Plasmid construction

The full-length cDNA fragment encoding TNS2 (1400 amino acid, NCBI accession number NM_153533) from FVB mice was subcloned into pCMV-SPORT6 (Invitrogen). The initiation codon and FLAG-encoding sequence were then attached at the 5' end of the open reading frame of Tns2. To generate a truncated TNS2 mutant lacking the SH2-PTB domain (Fig. 1), we inserted tandem stop codons after gln1125, which is the first residue of the TNS2 SH2 domain [3]. The mutant DNA fragment was synthesized from the wild-type sequence by fusion PCR using the following primers: outside forward, 5'-ACTTCAGATGCCCCAGTGGC-TC-3', outside reverse, 5'-AAATAGGGCTCAGTG-GGACAAC-3', inside forward, 5'-TAATAGGAATTC-
GATACTCAAGTTCTGGTACAA-3’ and inside reverse, 5’-GAATTCTATTACGTGGACAAACTTGACATTGCT-3’ (where the underlined residues correspond to overlap mutant sequence including the tandem stop codons). The SacI-BamHI-digested mutant fragment was substituted for the SacI-BamHI-digested wild-type fragment in the FLAG-tagged TNS2 in the expression plasmid by DNA ligation.

Lipofection and western blotting

Human embryo kidney cell line 293FT (Thermo Fisher Scientific) was maintained in DMEM containing 10% fetal bovine serum. Each of the Tns2 expression plasmids was introduced into 293FT cells using Lipofectamine 3000 transfection reagent (Thermo Fisher Scientific) according to the manufacturer’s instructions. At 48 h after transfection, cells were lysed in RIPA buffer and boiled with 2% sodium dodecyl sulfate (SDS) and 5% 2-mercaptoethanol. Lysates were electrophoresed in a SDS-polyacrylamide gel and blotted on polyvinylidene difluoride membranes (GE Healthcare, Chicago, IL, USA). For blocking non-specific binding, the membranes were incubated in Blocking One reagent (Nacalai Tesque) for 1 h at room temperature. The membranes were incubated with mouse monoclonal antibody against the N-terminal DYKDDDDK peptide (clone number 2H8, Trans Genic, Kobe, Japan) or rabbit polyclonal antibody targeting the phosphorylated Tyr483 of TNS2 (1:1,000, Aviva Systems Biology, San Diego, CA, USA) for 1 h at room temperature. Concomitantly, rabbi ty polyclonal antibody against GAPDH (1:10,000, Abcam, Cambridge, UK) was used for a loading control. Thereafter, the membranes were incubated with secondary horseradish peroxidase (HRP)-conjugated antibodies against mouse IgG (1:30,000, Fujifilm Wako Pure Chemical, Osaka, Japan) or rabbit IgG (1:5,000, Immuno Reagents, Raleigh, NC, USA) for 1 h at room temperature. After incubation with ECL Prime detection reagent (GE Healthcare), the blots were imaged using an Omega Lum C imaging system (Gel Co., San Francisco, CA, USA).

Electroporation and immunofluorescent staining

A podocyte cell line [23] derived from mice carrying a temperature-sensitive mutant of the immortalizing SV40 large T antigen under control of the interferon-γ (IFN-γ)-inducible H-2Kβ promoter was kindly provided by Dr. Karlhans Endlich. Cells were cultured in RPMI1640 medium containing 10% fetal bovine serum and 100 U/ml recombinant mouse IFN-γ (Merck, Darmstadt, Germany) in a 33°C incubator supplied with 5% CO2. For transfection, cells were collected by TrypLE Express dissociation reagent (Thermo Fisher Scientific), centrifuged and resuspended in reduced serum medium Opti-MEM (Thermo Fisher Scientific). Then, 100 µl of the cell suspension containing 2 × 105 cells and 10 µg of the Tns2 expression plasmid was placed into an electroporation cuvette with a 2-mm gap. Electric pulses were generated using an electroporator NEPA21 (Nepa Gene, Ichikawa, Japan). Parameters for the poring pulse were 180 V × 2 times with a 7.5 ms pulse length, a 50 ms pulse interval, and a 10% decay rate. Those for the transfer pulse were 20 V × 10 times with a 50 ms pulse length, a 50 ms pulse interval, polarity-exchanged, and a 40% decay rate. After electroporation, the cell suspension was transferred into growth medium, seeded in a chamber slide (Nunc Lab-Tek II, Product number 154534, Thermo Fisher Scientific) and cultured in growth condition for 48 h. Cells were washed three times in PBS, fixed in 4% PFA for 20 min at room temperature, and incubated with PBS and 0.3% Tween 20 (PBST) for 20 min at room temperature. For blocking non-specific binding, cells were incubated with 5% goat serum in 0.3% PBST for 1 h at room temperature. After blocking, cells were incubated with mouse primary antibody against the N-terminal DYKDDDDK peptide (1:400) at 4°C overnight, followed by incubation with Alexa Fluor 488-conjugated goat anti-mouse IgG secondary antibody (Cell Signaling Technology, Danvers, MA, USA) for 30 min at room temperature. Cells were mounted in Pro-Long Diamond Antifade Mountant with DAPI (Thermo Fisher Scientific). The sample was imaged using an LSM 710 confocal microscope (Carl Zeiss, Oberkochen, Germany).

Statistics

Data are expressed as means ± standard deviation. Bonferroni’s multiple comparison test was used for calculating statistical significance and carried out using GraphPad Prism 5 software (MDF, Tokyo, Japan). A P value <0.05 was considered to be statistically significant.

Results

Tensin2 SH2-PTB domain deletion induces albuminuria and podocyte injury in mice

Tns2CS mutant mice were born according to Mendelian rules without apparent defects (e.g., body size, behavior and fertility). We evaluated the severity of podocyte injury in three different Tns2 mutant strains, Tns2nph, Tns2MC and Tns2CS, on an FVB genetic background at 8 weeks old. Tns2nph and Tns2MC mice, but not Tns2CS mice, developed massive albuminuria (Figs. 2A and B). Urinary albumin excretion in Tns2MC mice was equal to...
that in Tns2nph mice (Fig. 2B). Urinary albumin excretion in Tns2CS mice was in the normal range (Fig. 2B). Histological analysis revealed that almost all glomeruli in the Tns2nph and Tns2ΔC mice showed entire expansion of the mesangial matrix, abnormal dilation of the capillary lumen or retraction and collapse of the glomerular tuft, while almost no glomeruli in the Tns2CS mice showed any abnormalities (Fig. 3A). The retraction and collapse of the glomerular tuft was more frequently observed in the Tns2nph than in the Tns2ΔC mice, resulting in a significant difference in glomerular injury score between the Tns2nph and Tns2ΔC mice (Fig. 3B). Ultrastructural analysis showed podocyte foot process effacement and GBM thickening in the Tns2nph and Tns2ΔC mice. Furthermore, the glomerular structures appeared more extraordinary in the Tns2nph than in the Tns2ΔC mice with respect to the thickness of GBM, the number of GBM protrusion and the morphology of foot process (Fig. 4). In the Tns2CS mice, no abnormalities in glomerular morphology were detected by transmission electron microscopy (Fig. 4).

**Discussion**

Tns2 deficiency leads to podocyte foot process effacement accompanied by GBM thickening, and subsequent glomerular and tubulointerstitial injuries in susceptible murine strains including FVB. In this study, to investigate whether each of the SH2-PTB and PTPase domains in TNS2 plays a role in podocyte maintenance, we compared the severity of glomerular injury in two different Tns2-mutant mice with an FVB genetic background, Tns2ΔC and Tns2CS mice, carrying a SH2-PTB domain deletion and PTPase domain inactivation, respectively, to that in Tns2 null mutant mice with an FVB genetic background, Tns2nph mice, or wild-type FVB mice. Our results revealed that the Tns2ΔC mice developed massive albuminuria, severe glomerular injury and podocyte alterations similarly to those in Tns2nph mice. In contrast, there were no significant differences in urinary albumin excretion or glomerular injury score between Tns2CS and wild-type FVB mice. Furthermore, electron microscopy showed that there were no abnormalities in the glomeruli from Tns2CS mice. Our results clearly suggest that deletion of the TNS2 SH2-PTB domain, but not the loss of its PTPase activity, induces podocyte injury.

Recently, TNS2 has been identified as activating the mammalian target of rapamycin complex 1 (mTORC1)
Fig. 3. Glomerular histology in the Tns2<sup>nph</sup>, Tns2<sup>CS</sup>, Tns2<sup>ΔC</sup> and wild-type FVB mice. Mice were analyzed at 8 weeks old (n=6). (A) Representative glomerular structures under PAS staining. Most glomeruli in Tns2<sup>ΔC</sup> and wild-type FVB mice were normal, and received a glomerular injury score of 0. In contrast, most glomeruli in Tns2<sup>nph</sup> and Tns2<sup>ΔC</sup> mice exhibited glomerular injury scores of 5. Glomeruli with a score of 6, which showed retraction and collapse of the glomerular tuft, were observed mostly in Tns2<sup>nph</sup> mice. (B) Glomerular injury scores. Asterisks indicate P values, **<0.0001 and *<0.05. NS, not significant. Scale bars, 50 μm.

Fig. 4. Ultrastructure of the podocytes and GBM in the Tns2<sup>nph</sup>, Tns2<sup>CS</sup>, Tns2<sup>ΔC</sup> and wild-type FVB mice. Mice were analyzed at 8 weeks old. Representative transmission electron microscopy images of glomeruli. Arrowheads, foot process. Stars, protrusion of the GBM. Scale bars, 2 μm.
signaling in podocytes through its action as a PTPase toward nephrin, which is a principal podocyte slit diaphragm component [11]. This mTORC1 activation is associated with podocyte dysfunction in diabetic nephropathy [5, 10] and, interestingly, TNS2 is upregulated in diabetic nephropathy model glomeruli [11]. Adenovirus-mediated Tns2 overexpression induces mTORC1 activation and podocyte dysfunction in a PTPase-dependent manner [11]. On the other hand, decreased mTORC1 activity also induces podocyte dysfunction after birth [5]. These data indicate that balanced mTORC1 activity is required for podocyte homeostasis. Additionally, the effect of mTORC1 activity on podocytes seems to be modified by the age and genetic background of the mice [5]. Thus, we expected that decreased mTORC1 activity by the embryonic inactivation of the TNS2 PTPase domain might induce podocyte injury in mice [11]. However, Tns2<sup>CS</sup> mice developed neither podocyte dysfunction nor proteinuria. These results suggest that in normal podocytes, endogenous TNS2 PTPase activity is not involved in podocyte homeostasis.

Tensin family members are known as focal adhesion proteins, and are known to be localized to focal adhesion in at least in some cell types [8]. In a podocyte cell line, the transfected full-length TNS2 displayed a peripheral dotted distribution pattern typical of a focal adhesion protein as in other cell types [2, 9, 14, 31]. On the other hand, the distribution of the TNS2<sub>ΔSH2-PTB</sub> in the podocyte cell line was distinct from that of the full-length TNS2; i.e., a focal adhesion protein. The results indicate that SH2-PTB domain deletion impairs the ability of TNS2 to localize to focal adhesion. In focal adhesion, integrin-based multiprotein complexes orchestrate the connection of actin bundles to the extracellular matrix through the cell membrane. These functions are essential for the formation of highly differentiated cells with an intricate cytoskeletal architecture; i.e., podocytes.
cytes have thick arms, major processes that extend toward the capillaries, and spread numerous projections, or foot processes, over the GBM. The foot processes connect to the matrix molecules of the GBM via transmembrane adhesion receptors (i.e., integrin α3β1), and interdigitate with the neighboring foot processes via intercellular junctions, known as the slit diaphragm, to form filtration slits. Dense actin bundles aligned longitudinally together with cortical thin actin filaments beneath the cell membrane shape the cytoskeleton of the foot processes [19]. Hence, failure of the actin bundles to connect with the GBM at the focal adhesions induces podocyte foot process effacement and detachment. These podocyte abnormalities have been observed in genetically modified mice lacking focal adhesion structural proteins, such as integrins α3β1, talin1 and CD151 [20, 21, 25]. Conversely, inhibition of focal adhesion turnover by the deletion of focal adhesion regulatory proteins, such as FAK and CRK, attenuates foot process effacement in murine models [4, 12]. FAK phosphorylation at Tyr397, which activates the signaling pathway required for focal adhesion turnover, is increased in podocyte injury [12]. Such activation of FAK is also observed in the glomeruli of susceptible Tns2nph mice [27, 28]. In focal adhesion, multiple reciprocal focal adhesion protein interactions, including those of FAK, are intricately accompanied by conformational changes related to function, which are caused by binding cofactors or ligands, phosphorylation and mechanical tension [18, 29].

Collectively, our results demonstrate that deletion of the TNS2 SH2-PTB domain induces podocyte alterations in the susceptible mice, most likely due to its impaired ability to localize to focal adhesion. The absence of TNS2 in focal adhesion is supposed to affect the conformational states of the binding partners in focal adhesion, and finally lead to FAK activation in the susceptible mice, while it was compensated for in the resistant mice. Recently, 6 novel homozygous or compound heterozygous missense mutations in TNS2 have been detected in multiple families with nephrotic syndrome [1]. Interestingly, all the mutations are situated in the PTPase domain (1 of 6) or an unknown TNS2 unique region adjacent to the C2 and SH2-PTB domains (5 of 6), but not in the SH2-PTB domain [1]. Amino acid substitutions at these domains may not impair the ability of TNS2 to localize to focal adhesion, but change its conformational and, therefore, functional state. These molecular mechanisms need further study to clarify the TNS2-interacting proteins.
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