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Distinct Distribution of the Tensin Family in the Mouse Kidney and Small Intestine

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Abstract: Tensin family members are cytoplasmic proteins that are localized to the integrin-mediated cell-basement membrane junctions and implicated in cytoskeletal organization, cell migration, and proliferation. The mammalian genome contains four paralogs, Tns1, Tns2, Tns3, and Tns4. Murine mutations in the *Tns1* and *Tns2* genes cause polycystic kidney disease and glomerular sclerosis, respectively, and *Tns3*-null mice exhibit an impaired intestinal epithelial development. However, the knowledge concerning the localization of each tensin is still fragmentary. In this study, the cellular and subcellular distributions of tensin members were defined and compared with each other. RT-PCR analysis indicated that *Tns2* and *Tns3* were more abundant in isolated glomeruli and that *Tns1* was highly expressed in areas other than the glomeruli, but no *Tns4* expression was observed in the kidney. All tensin members were detected in the small intestine. Immunohistochemical staining revealed that Tns1 was predominantly localized to the mesangium of glomeruli and renal tubules. In contrast, Tns2 and Tns3 were highly expressed in the podocytes and the partial collecting system. In the small intestine, Tns2 and Tns3 were highly expressed in crypt and villous epithelial cells. Furthermore, we found that Tns3 was colocalized with TJ protein ZO-1 in renal tubules. These results indicate distinct differences in the cellular expression of Tns1, Tns2, and Tns3, and suggest that they may be able to function independently of each other in the kidney and the small intestine.

Key words: cellular distribution, kidney, mouse, small intestine, tensin

Introduction

Tensin (Tns) is a family of multidomain scaffold proteins that bind the cytoplasmic tail of β -integrins, and localize to adhesions that anchor stress fibers in cells [13], and are thought to be an important component linking the extracellular matrix, the actin cytoskeleton, and signal transduction. The tensin family of proteins includes Tns1, Tns2, Tns3, and Tns4, which lacks the N-

terminal PTP-related region found in the other tensin proteins (Fig. 1). Important functions of Tns have been investigated in cell adhesion modulation, cell migration and differentiation, and pathological conditions such as wound healing and metastatic cancer [14, 19].

Tns1 knockout (KO) mice developed normally and appeared healthy postnatally for at least several months. Over time, Tns1 KO mice became frail because of abnormalities in their kidneys. Tns1-null mice develop

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Fig. 1. Schema of the tensin family members. C1, protein kinase C conserved region 1 domain; PTPc, protein tyrosine phosphatase catalytic domain; PTEN C2, C2 domain of PTEN tumor-suppressor protein; SH2, Src homology 2 domain; PTB, phosphotyrosine binding.

polycystic kidney disease (PKD) in the proximal renal tubules as cell-matrix junctions are disrupted and tubule cells lack polarity [14]. Furthermore, Tns1 KO mice have demonstrated the critical role of Tns1 in wound healing [11]. On the other hand, ICGN mice, a Tns2 deletion mutant, exhibit severe proteinuria and glomerulosclerosis. Genetic studies have shown that glomerulosclerosis is a disease of the podocytes, and various slit diaphragm and cytoskeletal proteins contribute to the maintenance of podocyte permeability. Therefore, Tns2 is considered to be involved in the organization of the cortical cytoskeleton adjacent to the adherent junctions in podocytes [6, 17]. Tns3 KO results in growth retardation and death within 3 weeks after birth. Histological analysis of this mutant showed smaller digestive tracts with defects in both villi and enterocyte differentiation as well as abnormal lung morphology. These observations indicate that Tns3 is essential for the normal development and functions of the small intestine [5]. In these mutant mice, all other tissues appeared normal, suggesting that the functions of Tns1-3 are redundant and may compensate for each other in these tissues. On the other hand, they appear to have individual roles critical for specific tissues. The tissue distribution and KO phenotype of Tns4 have not yet been reported.

Northern blot analysis showed that *Tns1-3* genes are ubiquitously expressed in adult mice [3, 5, 14]; however, the details of Tns1-3 localization in mouse tissues require clarification. In the present study, we compared the immunohistochemistry of Tns1-3 in the mouse kidney and small intestine, both of which are severely affected in Tns1-3 KO mice. Subsequently, to verify the reliability of our results regarding Tns1-3 localization,

we compared their localization in relation to each other in the corresponding regions. Distinct differences in the cellular expression of Tns1-3 suggest that they may be able to function independently of each other in the kidney and small intestine.

Materials and Methods

Ethical statement

All experimental animal care and handling adhered to the Regulations for the Care and Use of Laboratory Animals, Hokkaido University. The animal experimentation protocol was approved by the President of Hokkaido University based on the judgment of the Institutional Animal Care and Use Committee of Hokkaido University (Approval ID: 11-0072).

Construction of Tns expression vectors and their transfection

The mouse *Tns2*-coding regions (NM_153533.2), mouse *Tns3* (NM_001083587.1) and human *TNS1* (NM_022648.4) were generated using PCR amplification. Human TNS1 is more than 90% identical to the corresponding mouse Tns1; thus we used human cDNA in this study. The sequence coding for the FLAG peptide was added to the C-terminals, and the resultant cDNAs were subcloned in a pTriEx 1.1 vector (EMD Millipore Corporation, Billerica, MA, USA). These plasmids were transfected into 293FT and BHK21 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The human TNS1 cDNA was kindly gifted by Dr. Su Hao Lo (University of California Davis).

Isolation of glomeruli and semiquantitative RT-PCR

The glomeruli were isolated from 18-week-old C57BL/6J mice (CLEA Japan, Inc., Tokyo, Japan) via Dynabead (Invitrogen) perfusion [21], while the other areas were retained in the supernatant of this suspension. Quantitation of *Tns1* (NM_027884.3), *Tns2* (NM_153533.2), *Tns3* (NM_001083587.1), *Tns4* (NM_172564.3), and *Actb* (NM_007393.3) expression was performed by quantitative RT-PCR. Primers were designed for *Tns1* (ctcaacctgacctgctga and tggagacgaagttgacgatg), *Tns2* (tacctgacctgggtggagac and tgtg-gcttctctcatttc), *Tns3* (ttcagaagtggcctcacctt and gag-cagcttgagggttaaag), *Tns4* (cccaggctgagctaccaac and acagggtggtgacagtat), and the internal control, *Actb* (tgatggtgggaatgggtcag and gaaggctggaaaagagcctc). To determine the optimum PCR amplification conditions in the linear range, three amounts of cDNA (50 and 100 ng) were tested for each sample. Each reaction tube contained 2 μ l of 10 $\times$ Ex Taq buffer (Takara, Shiga, Japan), 2 μ l of 2.5 mM dNTP mixture, 0.1 μ l of Ex Taq polymerase (Takara), and 1 μ l (4 μ M) of each primer. The total reaction volume was 20 μ l. PCR consisted of denaturation at 94°C for 3 min; 25 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 30 s; and a final extension at 72°C for 5 min. The PCR products were electrophoresed on 1.5% agarose gel. All assays were performed in duplicate.

Antibodies

For the generation of a polyclonal antibody against murine Tns2, amino acids 917–931 (VQGKESNRRQDT-TRS) and 1338–1353 (STD PQDRRWTPDGAT) were used to immunize rabbits. Antiserum was then collected and purified using a Melon Gel IgG Spin Purification Kit (Thermo Scientific, Rockford, IL, USA). The Tns2 antibody was diluted to 0.26 μ g/ μ l in sterile PBS. The primary antibodies (and their dilutions) used in this study were: Tns1 (1:50, sc-28542, Santa Cruz Biotechnology, Santa Cruz, CA, USA), Tns2 antibody (1:50 in immunofluorescence), Tns3 (1:50, sc-55153, Santa Cruz Biotechnology), ZO-1 (1:100, 40-2200, Invitrogen), and FLAG (1:2,000, F3165, Sigma-Aldrich, St. Louis, MO, USA). The secondary antibodies were: Alexa 568 α -rabbit IgG (1:1,000 for Tns1 and Tns2, A21069, Invitrogen), DyLight 549 α -goat IgG (1:1,000 for Tns3, 605-742-002, Rockland, Gilbertsville, PA, USA), DyLight 488 α -rabbit IgG (1:1,000 for ZO-1, 611-741-127, Rockland), Alexa 568 α -mouse IgG (1:5,000 for FLAG, A11031, Invitro-

gen), Alexa 488 α -rabbit IgG (1:5,000, for Tns1 and Tns2, A11034, Invitrogen), and DyLight 488 α -goat IgG (1:5,000 for Tns3, 605-741-125, Rockland). All secondary antibodies were diluted with PBS. Western blotting was carried out as described previously [20].

Immunofluorescence microscopy

The tensin-transfected BHK cells were cultured on chamber slides, fixed with acetone for 5 min at 4°C, and incubated with diluted primary antibody overnight at 4°C. After washing with PBS three times, the cells were incubated with the secondary antibody mixture for 1 h at room temperature. For tissue staining, male and female C57BL/6J mice (8 weeks old, CLEA Japan, Inc.) were used for immunohistochemistry. The frozen sections (4 μ m) were fixed with acetone for 10 min at 4°C. The slides were washed with PBS three times, treated with 5% normal goat (for Tns1 and Tns2 staining), rabbit (for Tns3 staining) or bovine (for Tns3 and ZO-1 double staining) serum for 30 min at room temperature, and then incubated with diluted primary antibodies overnight at 4°C. After washing with PBS three times, the sections were incubated with secondary antibodies for 1 h at room temperature. DAPI was used for nuclear staining. The sections were enclosed and observed with a Carl Zeiss LSM.

Results

Previously, Northern blot analysis showed that *Tns1-3* genes are ubiquitously expressed in adult mice [3, 5, 14]; however, the details regarding Tns1-3 localization in mouse tissues remain to be clarified. To examine the tissue specificity of Tns1-3 in more detail, we first examined Tns1-3 expressions using RT-PCR. We detected the presence of *Tns1-3* mRNAs, but not *Tns4* mRNA, in the mouse whole kidney (Fig. 2). To examine the differential expression of *Tns1-3* in the glomeruli and other areas, including the cortex, medulla, and renal pelvis, we isolated mRNAs from isolated glomeruli and the other areas. The *Tns3* expression level was greater in the glomeruli than in the other areas; however, *Tns1* transcripts were more abundant in the other areas. *Tns2* was detected in both the glomeruli and other areas to an equivalent extent. All tensin members were detected in the small intestine (Fig. 2).

To characterize the cellular localization of the Tns family in the kidney and small intestine, we developed

Fig. 2. Semiquantitative RT-PCR for tensin family members. The glomeruli were isolated via Dynabead perfusion, while the other areas were retained in the supernatant (Sup) of this suspension. *Tns1* was strongly expressed in all areas except the glomeruli. On the other hand, *Tns3* was strongly expressed in the glomeruli. *Tns2* was expressed in both fractions. *Tns4* was not detected in the kidney. All *Tns* members were detected in the small intestine.

a novel antibody against Tns2. The Tns2 antibody (Tns2-Ab) was shown to specifically recognize a single 170-kD band in the Tns2 cDNA-transfected cell lysate by Western blotting (Fig. 3a). To verify the reactivity and specificity of Tns2-Ab, FLAG tagged-Tns family proteins were expressed in BHK cells, and immunofluorescent staining was performed. FLAG staining in Tns-expressed cells revealed focal adhesion-like spots that were consistent with those described in earlier reports (Fig. 3b) [7]. Tns2-Ab also recognizes the same focal adhesion-like spots by double staining with the FLAG. In addition, commercially available antibodies of Tns1 and Tns3 also showed similar signals for FLAG-Tns1 and FLAG-Tns3 proteins, respectively (data not shown). Since no cross-reactivity was observed among these antibodies, we performed an immunohistochemical analysis for the specific detection of the Tns family proteins in the kidney and small intestine.

Tns1-Ab staining was observed in the proximal and distal tubules in the cortex area, as well as in mesangial cell cytoplasm and Bowman's capsules, which is consistent with the results from previous reports [22]. Clear signals were detected along the basement membrane of the tubule epithelia, in particular, where major patho-

Fig. 3. Verification of the reactivity and specificity of a novel anti-Tns2 antibody. a) Western blotting for 293FT expressing murine Tns2; b) Double immunofluorescence staining was performed on BHK21 cells overexpressing FLAG-human TNS1 (upper panels), FLAG-murine Tns2 (middle panels), and FLAG-murine Tns3 (lower panels). FLAG signals (red) and Tns2 signals (green) were colocalized only in the FLAG-Tns2 over-expressing cells. The nuclei were stained with DAPI (blue).

Table 1. Summary of distribution of the tensin family in the mouse kidney

			Tns1	Tns2	Tns3	Tns4
Cortex	Glomerulus	Podocyte	–	+	+	–
		Mesangium	+	+	–	–
	Bowman's capsule		+	–	–	–
	Tubule		+	+	+	–
Medulla	Collecting duct		–	+	+	–
	Renal pelvis epithelium		–	+	+	–

Plus (+) or minus (–) indicates the presence or absence of a signal in the immunohistochemical analysis.

Fig. 4. Immunofluorescence for Tns1, Tns2, and Tns3 in the mouse kidney. a–c) Tns1 was detected in the mesangial area, Bowman's capsule, and basement membrane of the tubules, but not in the renal medulla. d–f) Tns2 was detected in the podocytes, mesangial area, intercellular junctions in some tubules, collecting ducts, and renal pelvis epithelium. g–i) Tns3 was detected in the podocytes, intercellular junctions in some tubules, collecting ducts, and renal pelvis epithelium. The nuclei were stained with DAPI (blue). White arrow, mesangial area; white arrowhead, podocyte; black arrow, tubular basement membrane; black arrowhead, intercellular junction of the tubule; green arrowhead, collecting duct; asterisk, renal pelvis; dotted line, tubule.

logic lesions were detected in Tns1 KO mice (Fig. 4a and 4b). In contrast, Tns2 was detected in the podocytes in the glomeruli (Fig. 4d), intercellular junctions in some of the tubules and collecting ducts, and the renal pelvis epithelium (Fig. 4e and 4f). Weak Tns2 signals were also detected in mesangial cell cytoplasm (Fig. 4d). The distribution of Tns3 was similar to that of Tns2, although

Tns3 signals were not detected in the mesangial area (Fig. 4g–i). Compared with Tns2, Tns3 staining was detected more widely in the tubules (Fig. 4e, 4h and summary in Table 1). In the small intestine, Tns1 was only detected in villous smooth muscle. On the other hand, Tns2 and Tns3 were detected in the villous smooth muscle, and in the villous and crypt epithelial cells, with no intensity gradients apparent along the crypt-to-villus axis (Fig. 5). Tns2 expression was broadly cytoplasmic, whereas, the expression profile of Tns3 was characterized by an intense granular cytoplasmic staining. Additionally, Tns3 showed the characteristic subcellular localization in the closely associated areas of two cells similar to the tight junctions (TJs). To verify whether Tns3 is localized at the TJs, we compared the localization of Tns3 with that of the ZO-1 TJ protein in the corresponding regions. In renal tubules, Tns3 was colocalized with ZO-1, which appeared to be localized at the TJs, whereas Tns3 was localized at the cytoplasmic portion of the cells (Fig. 6a–c). Interestingly, Tns3 was not colocalized with ZO-1 in small intestinal crypt epithelial cells, and showed a rather strong granular cytoplasmic staining (Fig. 6d–f).

Discussion

In the present study, we investigated the mRNA expression of four Tns species in the kidney and small intestine by RT-PCR, and the subcellular localization of Tns1–3 by immunofluorescence microscopy. We found strong correlations between the mRNA and protein levels of Tns1–3 in both the kidney and small intestine. In addition, we identified novel localization patterns for the Tns family. Tns1 was localized along the tubular basement membrane, in the Bowman's capsule and in the mesangial area in the kidney, and in the villous smooth muscle. Tubule epithelial cells are attached to the tubu-

Fig. 5. Immunofluorescence for Tns1, Tns2, and Tns3 in the mouse small intestine. Tns1 was detected in only the villous smooth muscles (left panel, white arrow). Tns2 (middle panel) and Tns3 (right panel) were localized in the villous smooth muscle (white arrow), and crypt (arrowhead) and villous epithelial cells (black arrow).

Fig. 6. Double immunofluorescence for Tns3 and ZO-1 in the mouse kidney and small intestine. a–c) In the renal tubules, Tns3 was colocalized with ZO-1, and a fraction of Tns3 existed in the cytoplasm (white arrowhead). d–f) Tns3 was not colocalized with ZO-1 in the small intestinal crypt epithelial cells.

lar basement membrane, which overlaps the subjacent interstitial connective tissue. Tns1 deletion may cause the disruption of basement membrane adhesion, resulting in abnormal cell function, and leading to PKD. On the other hand, Tns2 and Tns3 were distributed in podocytes, intercellular junctions of the renal tubules and some of collecting ducts in the kidney, and the villous smooth muscle, and villous and crypt epithelial cells in the small intestine. In both podocytes and small intestinal epithelial cells, the actin-based cytoskeleton and cell junctions have important roles in maintaining normal morphology

and physiology. Taking into consideration of the localization patterns and structures of Tns2 and Tns3, the Tns2 and Tns3 KO phenotypes may be caused by the disturbance of the cytoskeleton and/or the intercellular junction formation. In spite of the similarities in their distribution in the kidney and small intestine, Tns2 and Tns3 cannot compensate for each other in the respective KO mice [5, 6]. Further, despite the high sequence similarities between Tns2 and Tns3 at the N- and C-termini, the middle regions are highly divergent, suggesting that they may have acquired unique biological functions and may

be associated with different, though related, molecules in the kidney and small intestine. Renal epithelial cells and small intestinal epithelial cells have different types of intercellular junctions. For example, podocytes have a specific intercellular junction known as a slit diaphragm, which is comprised of podocyte-specific molecules such as nephrin and podocin. Slit diaphragms and the apical and basal membrane domains of podocytes are connected to each other by an actin-based cytoskeleton that is critical to the maintenance of the glomerular filtration barrier [8]. As ICGN mice have abnormal podocytes, Tns2, but not Tns3, may interact with the podocyte-specific molecule(s) that forms the slit diaphragms. Furthermore, Tns3 was localized in the TJs in the renal tubules, but not in the intestinal epithelial cells. This result indicates that Tns3 has distinct roles in the kidney and small intestine, underpinning the notion that Tns3 has crucial roles in the small intestine, but not in the kidney. In addition to their cellular distribution, we also revealed their subcellular localization: Tns1 localizes along the basolateral surface, and Tns2 and Tns3 localize along the lateral wall or in the cytoplasm. These subcellular localizations may depend on the presence of interaction partners in each cell type. For example, nephrocystin, a causative protein for PKD, has been shown to interact with a number of proteins involved in focal adhesion complexes and at cell-to-cell contact sites in renal tubule cells [10]. It has also been reported that nephrocystin is able to associate with Tns1 [1]. This finding implies that the disruption of protein complex formation may result in abnormalities in specific cell types.

Overexpression or knockdown of Tns, leads to the respective impairment or enhancement of cell migration [2, 9, 12, 16]. Expression of Tns2 and Tns3 at the mRNA and protein levels was largely absent in a panel of diverse human cancer cell lines [16]. Tns may represent a novel group of metastasis suppressors, the loss of which leads to greater tumor cell motility and consequent metastasis. Similarly, Claudin1 is localized in the TJs, and its expression has been shown to be decreased in breast and colon carcinomas [15]. Cancer progression can be explained partly by the loss of TJs. Thus, a decrease in Tns3 may contribute to the metastasis via TJ regulation. Focal adhesion kinase (FAK) is a known modulator of the integrin-based signal transduction pathway usually restricted to the cell-extracellular matrix interface at focal contacts [18]. Recently, FAK was shown to be an inte-

grated component of the occludin-ZO1 multiprotein complex in Sertoli cells [4]. Thus, it is not surprising that Tns3 was detected at the TJs in the present study, though we need to further examine its precise localization using immunoelectron microscopy.

This is the first report to show that Tns may associate with not only the cell basement membrane but also intercellular junctions *in vivo*. Previous reports on Tns may have mislead the discussion regarding their physiological roles, as most of the studies were carried out using *in vitro* approaches and were focused on their roles in cell-basement membrane adhesion. To understand how adhesion and signals are coordinated, more information is required on the specific molecular interactions of Tns proteins, particularly the proteins that coordinate interactions among the transmembrane and cytosolic proteins. Nevertheless, the present study provides the first step in delineating the physiological and pathological roles of Tns in the kidney and small intestine.

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