Expression of Nephrin Homologue in the Freshwater Planarian, Dugesia japonica

Tomomi Nakamura¹,², Sota Takagi³, Midori Matsumoto³, Fumio Tashiro², Tatsuo Sakai¹ and Koichiro Ichimura¹

¹Department of Anatomy and Life Structure, Juntendo University School of Medicine, 2–1–1 Hongo, Bunkyo-ku, Tokyo 113–8421, Japan, ²Department of Biological Science and Technology, Graduate School of Industrial Science and Technology, Tokyo University of Science, 6–3–1 Niijuku, Katsushika-ku, Tokyo 125–8585, Japan and ³Department of Biological Sciences and Informatics, Keio University, 3–14–1 Hiyoshi, Kohoku-ku, Yokohama, Kanagawa 223–8522, Japan

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Excretory organs contain epithelial cells that form a filtration membrane specialized for ultrafiltration to produce primary urine. In vertebrates, the filtration membrane is made up of slit diaphragm (SD) formed by glomerular podocytes. Basal metazoans such as flatworms are also known to have filtration epithelial cells, called flame cells, which exhibit SD-like structures. The molecular components of podocyte SD have been studied in detail, while those of the SD-like structures in basal metazoans including flatworms remain to be clarified. To determine whether the SD-like structures in flatworms have molecular components common to the SD in vertebrate podocytes, we examined the expression of gene homologue for mammalian nephrin, which encodes an essential transmembrane protein that participates in the formation of the SD, in a species of flatworms, planarian (Dugesia japonica). Flame cells were distributed throughout the entire body of the planarian, but the nephrin-expressing cells identified by in situ hybridization were mainly detected at body periphery excluding head region. The distribution pattern of nephrin-expressing cells was similar to that of proliferating cell nuclear antigen-expressing neoblasts, which are pluripotent stem cells characteristic to planarians. These findings indicated that the SD-like structures can be formed without the Nephrin protein in planarian flame cells.

Key words: nephrin, slit diaphragm, flame cell, protonephridium, planarian

I. Introduction

In multicellular organisms the excretory organ is an essential visceral system to maintain the homeostasis in virtually all metazoans. This excretory system is functionally divided into two compartments: primary-urine producing apparatus and modulating tubule. The primary-urine producing apparatus filters the primary urine from the body fluid (interstitial fluid or blood plasma) for the excretion of excess water with metabolites. The primary urine is subsequently transferred to the modulating tubule, where it is modified by epithelial secretion and reabsorption. The modified urine is finally discharged outside the body as terminal urine.

The main portion of the primary-urine producing apparatus is made up of an epithelium layer specialized for ultrafiltration. In eucelomates including vertebrates, which possess a coelom lined with mesothelium, the filtration epithelium consists of podocytes and their basement membrane [20, 21]. These podocytes, together with several capillary loops, form a number of glomeruli within the mesonephric and metanephric kidneys in vertebrates. This basic cytoarchitecture of the podocyte is highly conserved among the taxonomic groups of eucelomates. Neighboring podocytes, which are interdigitated with each other by their numerous foot processes and separated by filtration slits,
are bridged by specialized intercellular junctions called slit diaphragms (SD) (Fig. 1A, C). The SD in the vertebrate podocytes serve as a highly selective filtration barrier in the glomerulus. Three kinds of SD-specific membrane proteins are essential to form and maintain the SD in vertebrate podocytes: Nephrin, Podocin, and Nephr1 [3, 6, 12, 16]. Mutations in nephrin and podocin genes cause congenital nephrotic syndrome of the Finnish type and autosomal recessive steroid-resistant nephrotic syndrome, respectively [1, 10].

In acoelomates and pseudocoelomates, both of which are invertebrate animals without the coelom lined with mesothelium, the primary-urine producing apparatus is also comprised of epithelial cells specialized for ultrafiltration of interstitial fluid at the most proximal end of the protonephridial tubules. Unlike podocytes in eucelomates, the filtration epithelial cells exhibit diversity in shape among the acoelomates and pseudocoelomates. However, the basic cytoarchitecture of the filtration site is well conserved and similar to that of the podocyte. The filtration epithelial cells form a SD-like structure at the intercellular space between neighboring cells or at the fenestrations in the cells [11, 29]. Although gene homologues for mammalian nephrin and podocin are found in acoelomates and pseudocoelomates, it is unclear whether Nephrin and Podocin proteins are contained in the SD-like structures of any of the species.

Platyhelminthes (flatworms) are primitive acoelomate invertebrates, which possess flame bulbs as a primary-urine producing apparatus, and exhibit a basket-like structure formed by flame cells, which is one type of filtration epithelial cells [29]. The Platyhelminthes phylum is traditionally divided into four taxonomic classes: Turbellaria (including planarians), Cestoda (tapeworms), Trematoda (flukes), and Monogenea [21], all of which possess flame cells. In freshwater and land planarians, the SD-like structures bridge numerous rectangular fenestrations perforating the cytoplasmic wall of the flame bulb [7, 13, 24]. To explore whether the filtration epithelial cells utilize Nephrin to make the SD-like structure in the basal metazoon such as flatworms, we examined and identified the nephrin-expressing cells in the freshwater planarian, Dugesia japonica.

II. Materials and Methods

Animals

Planarians D. japonica were collected at a branch of the Tedori River in Hakusan City, Ishikawa, Japan (GPS coordinates: 36.42960, 136.63722). No specific permissions were required for the collection in this place, and D. japonica was not endangered or protected. Planarians were maintained at 20°C in dechlorinated tap water and fed chicken liver. All animal works have been conducted according to the national guidelines. Approval of an ethics committee was not required in case of the planarians.

Transmission electron microscopy

Planarians were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer at 4°C for 24 hours (hr). The fixed samples were processed by modified cold dehydration method. This method enables detailed morphological observation of the extracellular matrices and cytoskeletons, as previously reported [5]. In brief, the samples were successively immersed in 0.4% OsO₄ in 0.1 M phosphate buffer for 1 hr, 2% low molecular weight tannic acid (Electron Microscopy Sciences, Hatfield, PA) in 0.05 M maleate buffer for 4 hr, and 1% uranyl acetate in 0.05 M maleate buffer for 3 hr. The samples were then dehydrated with a graded series of ethanol and embedded in Epoxy resin. The ultrathin sections were stained with uranyl acetate and lead citrate and observed with a JEM1230 transmission electron microscope (JEOL, Tokyo, Japan).

Immunohistochemistry

Planarians were treated with cold 2% HCl in 5/8 Holtfreter’s solution for 5 min, and then fixed in Carnoy’s fixative at 4°C for 3 hr. To remove pigmentation, the specimens were immersed in 5% hydrogen peroxide in methanol under fluorescent light at room temperature (RT) for 15 hr. Bleached samples were rehydrated with a graded series of methanol, and washed with phosphate buffer saline (PBS) containing 0.5% Triton X-100 (PBSTx). Subsequently, the samples were blocked with incubation solution (PBSTx containing 1% BSA) for 2 hr at RT, and incubated with the anti-acetylated α-tubulin antibody (clone 6-11B-1, Sigma-Aldrich, St. Louis, MO) diluted with the incubation solution (working dilution 1:100) for 10–15 hr at 4°C. After washing with PBSTx, the samples were incubated with tetramethylrhodamine (TRITC)-conjugated donkey anti-mouse IgG F(ab’)$_2$ (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted with the incubation solution (1:500) for 2 hr at RT. After being mounted in anti-fading mounting medium (90 ml glycerol, 10 ml PBS, 100 mg p-phenylenediamine), the samples were imaged with a LSM510 confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany).

Synthesis of digoxigenin-labeled RNA probes

One clone of gene homologue to mammalian nephrin (comp12709_c0_seq1) was selected from a previously established D. ryukuensis expressed sequence tags (EST) database [8]. This EST sequence is predicted to contain the full open reading frame (ORF) sequence by searching for it with the ORF prediction tool (http://www.ncbi.nlm.nih.gov/ orf/ orf/ cgi). The full length cDNA sequence for proliferating cell nuclear antigen (pcna) homologue of D. japonica was as previously reported [15]. Partial-length nephrin and pcna of D. japonica were amplified from cDNA of adult D. japonica using KOD-Plus-Neo-DNA polymerase (TOYOBO, Osaka, Japan) and the following primer sets: nephrin-3698F: 5’-AAA GAT CAC GAA CTG CCA AAT CTA-3’; nephrin-4410RT7: 5’-GGT AAT ACG ACT CAC
SuperScript III reverse transcriptase (Invitrogen), and was irradiated planarians seven days after irradiation (n=6, for reduce the blue staining.

Whole-mount in situ hybridization
Whole mount in situ hybridization was performed as described previously [26]. In brief, planarians were treated with cold 2% HCl in 5/8 Holfrerter's solution for 5 min, and then fixed in Carnoy's fixative at 4°C for 3 hr. To remove pigmentation, the specimens were immersed in 5% hydrogen peroxide in methanol under fluorescent light at RT for 15 hr. The specimens were incubated in a prehybridization solution of 50% formamide, 5×saline sodium citrate buffer (SSC), 10 μg/ml yeast tRNA, 10 μg/ml heparin, 0.1% Tween 20, and 10 mM dithiothreitol at 55°C for 1.5 hr, and hybridized with a digoxigenin-labeled anti-sense RNA probe (about 40 ng/ml) in the prehybridization solution supplemented with 10% dextran sulfate at 55°C for 15 hr. After hybridization, the specimens were washed three times with SSC buffer at 55°C for 1 hr. They were then incubated in a blocking solution (pH 7.5) consisting of 0.1 M maleic acid, 0.15 M NaCl, 0.1% Triton X-100, and 1% Blocking Reagent (Roche Diagnostics) at RT for 1 hr and incubated in the blocking solution with an alkaline phosphatase-conjugated Fab fragment against digoxigenin (Roche Diagnostics) at RT for 3 hr. NBT/BCIP (Roche Diagnostics) was used as the chromogenic substrate to produce the blue staining.

X-ray irradiation
To extinguish neoblasts from planarians, they were exposed to a single 20-Gy dose of soft X-rays using an MBR-1505R2 X-ray generator (Hitachi Medical Corp., Tokyo, Japan). The planarians which were cut at seven-days after irradiation could not regenerate at least for seven days.

RT-PCR
Total RNA was individually isolated from control and irradiated planarians seven days after irradiation (n=6, for each group). cDNA was synthesized from total RNA using SuperScript III reverse transcriptase (Invitrogen), and was used as a template for PCR using KOD -Plus- Neo-DNA polymerase. The primer set for nephrin, pcna, and beta-actin is following: nephrin-3698F; nephrin-4181R: 5'-CCT CCT TCA TGA ATT GGT GAT AA-3'; pcna-107F; pcna-817R: 5'-TTT CGG AGC CAG ATA ATA ACG TA-3'; beta-actin-330F: 5'-TCT CAA TTC AAA AGC AAA CAG A-3'; beta-actin-829R: 5'-CAT GAA TAC CGA CTA CTG ATT CCA A-3'.
ever, the alignment pattern of functional protein domains was highly conserved among the three organisms (Fig. 3A). Nephrin homologues consisted of a type-1 integral membrane protein with several immunoglobulin and immunoglobulin-like domains and all three organisms had a fibronectin domain adjacent to the transmembrane domain in common.

**Gene expression of planarian nephrin**

The expression of *nephrin* was confirmed by PCR...
using cDNA templates of *D. japonica* planarians (Fig. 2F). Whole-mount in situ hybridization for *nephrin* mRNA was performed on *D. japonica*, and the *nephrin*-expressing cells were found mainly at body periphery excluding head region (arrowheads in Fig. 3B, B'). Some *nephrin*-expressing cells were also scattered at the central region of the trunk. The distribution pattern of *nephrin*-expressing cells was almost same among more than 50 planarians examined in this study.

This distribution pattern of *nephrin*-expressing cells was different from that of flame cells as shown in Figure 2. In particular, although numerous flame cells were found at the head region, the *nephrin*-expressing cells were not observed in the same region. Moreover, the double labeling for *nephrin* mRNA and acetylated α-tubulin protein clearly confirmed that most of the characteristic fox-tail signals for acetylated α-tubulin, which represented the bundle of motile cilia in flame bulb, was not colocalized with the *nephrin* signals (Fig. 3D–D''). The flame cells were predominantly found beneath the epidermis (arrowheads in Fig. 3D', D''), while the *nephrin* signals were recognized at the more medial region (arrows in Fig. 3D, D'). The above findings clearly indicated that flame cells did not express *nephrin*.

**Fig. 2.** Distribution of flame cells in the planarian *D. japonica*. Distribution of flame cells is shown in the three regions which are indicated by rectangles in A. Motile cilia of flame cells were detected by whole-mount immunostaining with anti-acetylated α-tubulin (Ac-tub) antibody, and visualized as characteristic fox-tail-like signals (arrowheads in B, C, D). The flame cells were distributed throughout the entire body including head region. Asterisks in B, eyes; asterisks in C, motile cilia of epidermis. Bar=50 μm.
As to which cell type expressed nephrin in planarians, as described above, nephrin was expressed at the body periphery excluding head region. This characteristic expression pattern is similar to that of pcna as previously reported in D. japonica [15] (arrowheads in Fig. 3C, C').

Fig. 3. Gene expression of planarian nephrin. (A) Structural similarity of Nephrin protein homologues among planarian (D. ryukyuensis, comp12709_c0 seq1), blood fluke (S. mansoni, Gene Bank Accession No. CCD77361), and human (No. AAG17141). All of the Nephrin homologues are a type-1 integral membrane protein with immunoglobulin domains (IG), immunoglobulin-like domains (IG like), immunoglobulin C2-type domain (IGc2), and fibronectin type-3 domain (FN3) close to the transmembrane domain (TM). To predict the protein domains, SMART was used. Whole-mount in situ hybridization for nephrin (B, B') and pcna (C, C') show that the localization pattern of nephrin mRNA is similar to that of pcna (arrowheads). Ph, pharynx. (D–D'') Double labeling for nephrin mRNA and acetylated α-tubulin (Ac-tub) protein. Longitudinal section of whole-mount double-labeled specimen. Motile cilia in flame cells are visualized as a fox-tail appearance of Ac-tub signals (arrowheads). The signals for nephrin (arrows) are not colocalized with the fox-tail signals of Ac-tub. (E) B', C', and D–D'' show the left-lateral peripheral region indicated by the rectangle. (F) RT-PCR for pcna, nephrin, and β-actin in control and X-irradiated planarians. The expression of nephrin is remarkably reduced in the planarian which exhibits the marked reduction of pcna expression. Bars=1 mm (C); 500 μm (C', D'').
pluripotent stem cells peculiar to planarians. The neoblasts possess high radiation sensitivity and can be extinguished from planarians using X-ray irradiation [15]. We thus examined the expression of nephrin in the planarians where neoblasts were extinguished using a single 20-Gy irradiation. The expression levels of pcna and nephrin corresponded well with one another, and in planarians which exhibited marked reduction of pcna expression, the expression of nephrin was also remarkably reduced (Fig. 3F). The above findings suggested that nephrin was expressed in the neoblasts, although further immunohistochemical examination using the specific antibody for planarian Nephrin protein is required to identify the Nephrin-expressing cell more exactly.

IV. Discussion

Planarian SD-like structure can be made without Nephrin protein

While vertebrate podocytes have a SD in the selective filtration barrier of its filtration membrane, planarian flame cells incorporate a SD-like structure. The SD-like structure was ambiguous in transmission electron microscopy in comparison with the SD, which allows for the possibility that the molecular components of the SD-like structure in flame cells may be markedly different from those of the SD in podocytes. In fact, planarians possess the gene homologue of nephrin, which encodes one of an essential transmembrane protein that participates in the formation of the SD in vertebrate podocytes, but it was expressed by a cell type other than the flame cell in planarians. This finding inevitably indicated that the SD-like structures can be formed without the Nephrin protein in planarian flame cells, unlike the SD in vertebrate podocytes.

Several instances of molecular diversity in podocyte-expressing proteins have been reported among vertebrate species. The most famous instances are α-actinin and drebrin, both of which are actin-associated proteins. In rodents, podocytes express α-actinin-2 and α-actinin-4, but not drebrin. On the other hand, in humans, podocytes express α-actinin-4 and drebrin, but not α-actinin-2 [4, 9, 17]. Recently, Volker and colleagues reported that the nephrin gene homologue was not registered in the chicken gene databases [27], indicating that the SD in chicken podocytes can be formed without the Nephrin protein [14]. It is highly possible that similar molecular diversity exists among the flame-cell-expressing proteins in the flatworm species. At the present time, we therefore cannot conclude that the SD-like structures in all classes of flatworms are formed without the Nephrin protein as found in planarians. The next issue is to determine whether flame cells express nephrin in flatworms other than planarians such as flukes, tapeworms, and monogeneans. Moreover, in the future study, we will investigate other SD-related genes such as podocin and neph-1 homologues in the flame cells of flatworms including planarians.

Diversity of nephrin-expressing cell type in invertebrate species

In some arthropods (insects and crustaceans), the podocyte-related cells, nephrocytes, serve to maintain the homeostasis of body fluid, and express two kinds of Nephrin homologue: Hibris and Sticks-and-stones (SNS) [28, 30, 31]. Both Nephrin homologues are essential components to form SD in the nephrocytes of fruit fly (D. melanogaster) as seen in the vertebrate podocytes, and are also involved in pupal eye development and axonal guidance [2, 18, 25]. Furthermore, in nematode worm (C. elegans), which do not possess the cell types performing ultrafiltration such as the podocyte, nephrocyte, and flame cell [21], the Nephrin homologue, SYG-2 is involved in synapse development and synaptic target recognition cooperatively with the Neph-1 homologue, SYG-1 [22, 23]. It is interesting that Nephrin homologue is also utilized for neural development in Arthropoda and Nematoda.

The present study revealed that the nephrin homologue was presumably expressed by neoblasts, but not by flame cells and neurons in planarians, although the function of Nephrin protein remains unclear in planarian neoblasts. On the basis of recent molecular taxonomy, Arthropoda and Nematoda belong to the superphylum Ecdysozoa, while Platyhelminthes including planarians belong to another superphylum Lophotrochozoa. Thus, it is possible that a great difference exists in the manner in which the Nephrin protein is utilized between Ecdysozoa and Lophotrochozoa.

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VI. References

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