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Expression of *Nephrin* Homologue in the Freshwater Planarian, *Dugesia japonica*

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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 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 eucoelomates 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 eucoelomates. 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 Neph1 [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 eucoelomates, 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: Turbellari (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 metazoan 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% OsO_4 in 0.1 M phospate 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 templeture (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/ gorf/orfig.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 TA-3'; *nephrin*-4410RT7: 5'-GGT AAT ACG ACT CAC

TAT AGG ATT ATT TGG ATC AAG GGT TCC AT-3'; *pcna*-107F: 5'-GCA ATT AAA GAT TTG ATC CAA GAA-3'; *pcna*-742RT7: 5'-GGT AAT ACG ACT CAC TAT AGG CAA AGA CAG TGA CAC GAT AGG-3'. The partial-length *nephrin* and *pcna* cDNA were subcloned into pCRII-Blunt-TOPO (Invitrogen, Carlsbad, CA), and verified by DNA sequencing. cDNA templetes for RNA probe synthesis were amplified from the pCRII-Blunt-TOPO plasmid using the same primer sets described above. Digoxigenin-labeled RNA probes were synthesized using T7 RNA polymerase (Roche Diagnostics, Mannheim, Germany) and DIG-RNA labeling kit (Roche Diagnostics).

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 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 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 TCC AAA AGC AAA CAG A-3'; *beta-actin*-829R: 5'-CAT GAA TAC CGA CTG ATT CCA A-3'.

III. Results

Ultrastructure and distribution of planarian flame cells

We first examined the ultrastructure of planarian flame cells in *D. japonica*. The flame cell is shaped like a test tube to form a flame bulb at the most proximal end of the protonephridial tubule (Fig. 1B). The cytoplasmic wall of flame bulb possessed numerous rectangular fenestrations, which were aligned in parallel and spanned by the SD-like structure (Fig. 1D, E) [13]. In comparison with the SD in mammalian podocytes, the SD-like structure is somewhat ambiguous. However, the SD-like structure was associated with an electron-dense cytoplasmic plaque, which was on the inner leaflet of the plasma membrane at the insertion site of SD-like structure (Fig. 1D), as seen in the podocyte SD (Fig. 1C).

Podocytes in eucoelomates possess a basement membrane. Especially in the vertebrate glomerulus, it is referred to as the glomerular basement membrane (GBM) (Fig. 1C) and serves as the glomerular filtration barrier together with the SD [16]. Unlike podocytes, planarian flame cells did not exhibit a distinct basement membrane (Fig. 1D), and appeared to be in direct contact with the interstitial space (Fig. 1B, D, E). Thus, the SD-like structure mainly formed a boundary between the interstitial space and the urinary space (the lumen of the flame bulb). The interstitial space contained amorphous materials, which were presumably aggregations of interstitial fluid proteins fixed by glutaraldehyde (Fig. 1B, D, E). Such materials were not observed in the urinary space (the lumen of the flame bulb), which the primary urine occupied, indicating that the SD-like structure played a role in the filtration barrier function in planarian flame cells.

Next, to determine the distribution of planarian flame cells, we visualized the cells using whole-mount immunohistochemical detection for acetylated α-tubulin (Fig. 2), since the flame bulb contained a bundle of motile cilia (arrows in Fig. 1B, E), whose axonemal α-tubulin was highly acetylated. The bundle of cilia in the flame bulb exhibited a characteristic fox-tail appearance (arrowheads in Fig. 2B–D), as reported in another planarian *Schmidtea mediterranea* [19]. The flame cells were situated beneath the epidermis and recognized to be distributed throughout the entire body.

Protein structure of planarian Nephrin

Amino acid sequences of Nephrin homologues were compared among planarian (*D. ryukyuensis*), blood fluke (*Schistosoma mansoni*), and human. Identity and similarity of the overall amino acid sequences are lower between planarian and blood fluke (6.3% and 16.0%, respectively), and between planarian and human (6.4% and 12.2%). How-
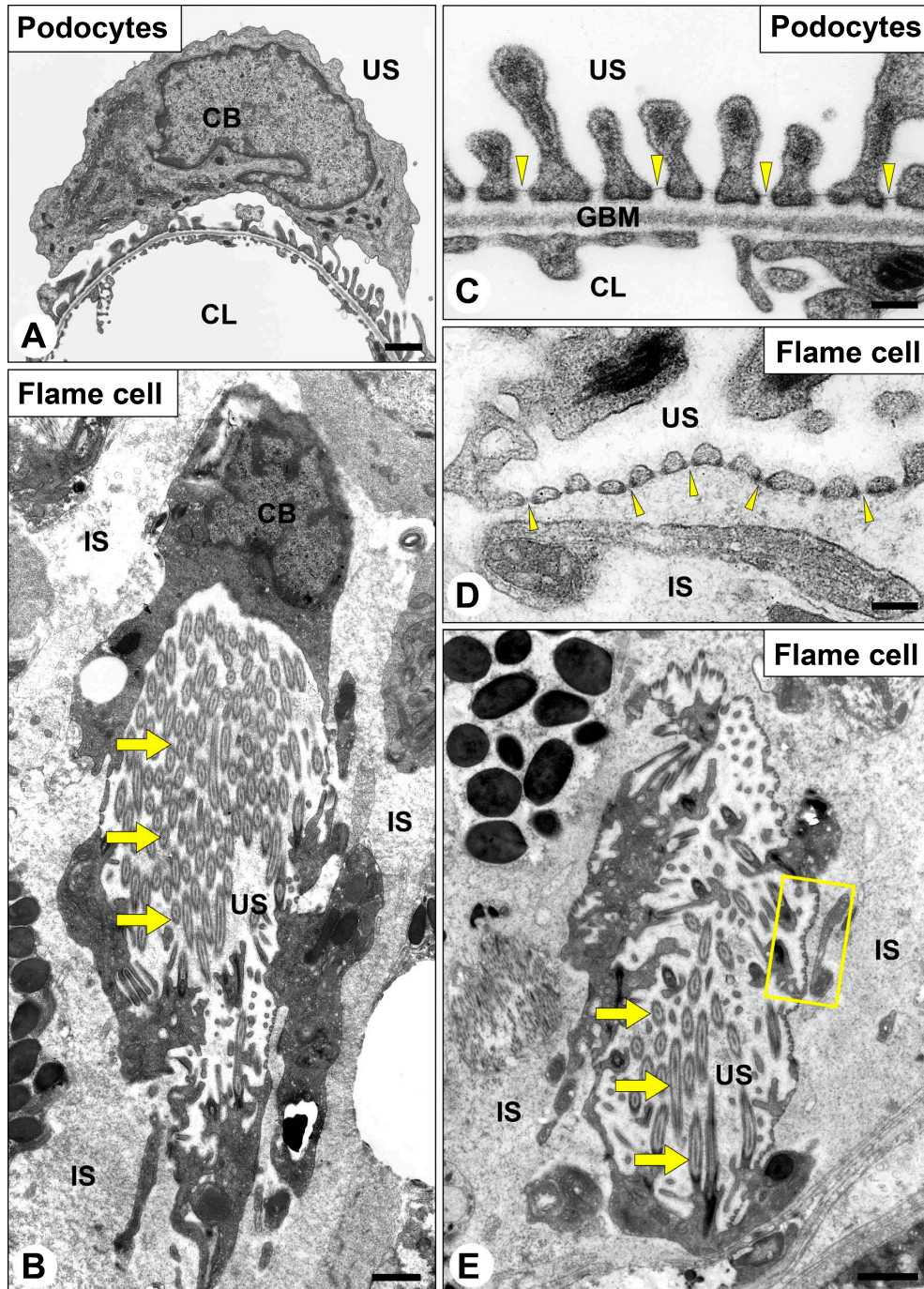
Fig. 1. Ultrastructure of flame cells in the planarian *D. japonica*. Transmission electron micrographs of rat podocytes (**A, C**) and planarian flame cells (**B, D, E**), both of which form a primary-urine producing apparatus. The cell bodies of podocyte (**A**) and flame cell (**B**) are shown in low magnification view. Flame cell forms a flame bulb at the most proximal blunt end of protonephridial tubule, and possesses a bundle of motile cilia in the lumen (arrows in **B**, **E**). The slit diaphragm and slit diaphragm-like structure are recognized in podocytes and flame cells, respectively (arrowheads in **C**, **D**). Magnification of an inset in **E** is shown as **D**. CL, capillary lumen; GBM, glomerular basement membrane; IS, interstitial space; US, urinary space. Bars=1 μm (**A, B, E**); 200 nm (**C, D**).

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

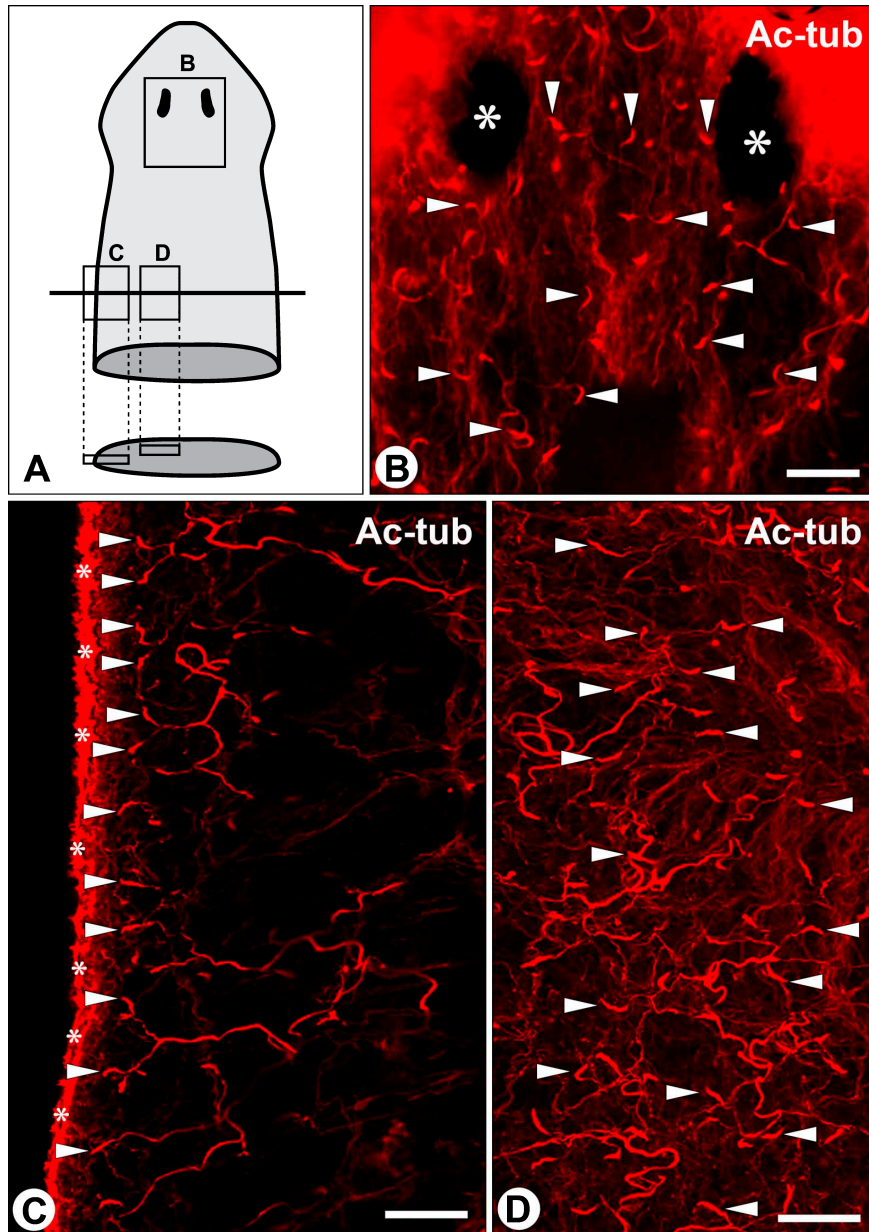The expression of *nephrin* was confirmed by PCR

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.

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*.
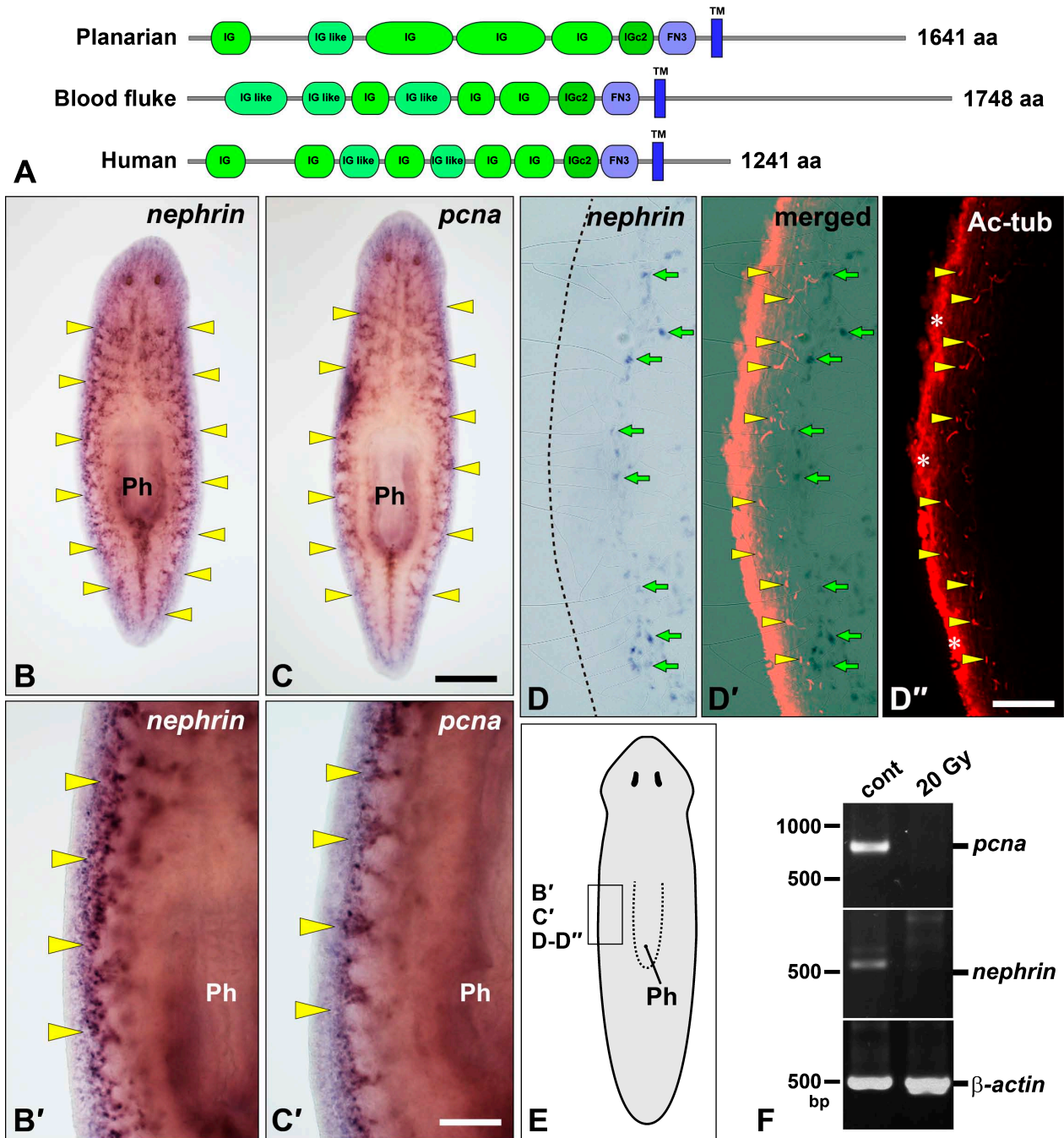
Fig. 3. Gene expression of planarian *nephrin*. (**A**) Structural similarity of Nephrin protein homologues among planarian (*D. ryukyuensis*, comp12709_c0 seq1), blood fluke (*S. mansoni*, Gene Bank Accession No. CCD77361), and human (No. AAG17141). All of the Nephrin homologues are a type-1 integral 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''**).

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'). *pcna* is predominantly expressed in neoblasts, which are

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.

V. Acknowledgments

VI. References

1. Boute, N., Gribouval, O., Roselli, S., Benessy, F., Lee, H., Fuchshuber, A., Dahan, K., Gubler, M. C., Niaudet, P. and Antignac, C. (2000) NPHS2, encoding the glomerular protein podocin, is mutated in autosomal recessive steroid-resistant nephrotic syndrome. *Nat. Genet.* 24; 349–354.

2. Dworak, H. A., Charles, M. A., Pellerano, L. B. and Sink, H. (2001) Characterization of Drosophila hibris, a gene related to human nephrin. *Development* 128; 4265–4276.

3. Fukuyo, Y., Nakamura, T., Bubenshchikova, E., Powell, R., Tsuji, T., Janknecht, R. and Obara, T. (2014) Nephrin and Podocin functions are highly conserved between the zebrafish pronephros and mammalian metanephros. *Mol. Med. Rep.* 9; 457–465.

4. Ichimura, K., Kurihara, H. and Sakai, T. (2003) Actin filament organization of foot processes in rat podocytes. *J. Histochem. Cytochem.* 51; 1589–1600.

5. Ichimura, K., Kurihara, H. and Sakai, T. (2010) Primary cilia disappear in rat podocytes during glomerular development. *Cell Tissue Res.* 341; 197–209.

6. Ichimura, K., Fukuyo, Y., Nakamura, T., Powell, R., Sakai, T., Janknecht, R. and Obara, T. (2013) Developmental localization of nephrin in zebrafish and medaka pronephric glomerulus. *J. Histochem. Cytochem.* 61; 313–324.

7. Ishii, S. (1980) The ultrastructure of the protonephridial flame cell of the freshwater planarian Bdellocephala brunnea. *Cell Tissue Res.* 206; 441–449.

8. Ishizuka, H., Maezawa, T., Kawauchi, J., Nodono, H., Hirao, Y., Nishimura, O., Nakagawa, H., Sekii, K., Tasaka, K., Tarui, H., Agata, K., Hoshi, M., Kobayashi, K., Sakakibara, Y. and Matsumoto, M. (2007) The Dugesia ryukyuensis database as a molecular resource for studying switching of the reproductive system. *Zoolog. Sci.* 24; 31–37.

9. Kaplan, J. M., Kim, S. H., North, K. N., Rennke, H., Correia, L. A., Tong, H. Q., Mathis, B. J., Rodriguez-Perez, J. C., Allen, P. G., Beggs, A. H. and Pollak, M. R. (2000) Mutations in ACTN4, encoding alpha-actinin-4, cause familial focal segmental glomerulosclerosis. *Nat. Genet.* 24; 251–256.

10. Kestila, M., Lenkkeri, U., Mannikko, M., Lamerdin, J., McCready, P., Putaala, H., Ruotsalainen, V., Morita, T., Nissinen, M., Herva, R., Kashtan, C. E., Peltonen, L., Holmberg, C., Olsen, A. and Tryggvason, K. (1998) Positionally cloned gene for a novel glomerular protein—nephrin—is mutated in congenital nephrotic syndrome. *Mol. Cell* 1; 575–582.

11. Kieneke, A., Ahlrichs, W. H., Arbizu, P. M. and Bartolomaeus, T. (2008) Ultrastructure of protonephridia in Xenotrichula carolinensis syltensis and Chaetonotus maximus (Gastrotricha: Chaetonotida): comparative evaluation of the gastrotrich excretory organs. *Zoomorphology* 127; 1–20.

12. Kramer-Zucker, A. G., Wiessner, S., Jensen, A. M. and Drummond, I. A. (2005) Organization of the pronephric filtration apparatus in zebrafish requires Nephrin, Podocin and the FERM domain protein Mosaic eyes. *Dev. Biol.* 285; 316–329.

13. McKanna, J. A. (1968) Fine structure of the protonephridial system in Planaria. I. Flame cells. *Z. Zellforsch. Mikrosk. Anat.* 92; 509–523.

14. Miner, J. H. (2012) Life without nephrin: it's for the birds. *J. Am. Soc. Nephrol.* 23; 369–371.

15. Orii, H., Sakurai, T. and Watanabe, K. (2005) Distribution of the stem cells (neoblasts) in the planarian Dugesia japonica. *Dev. Genes. Evol.* 215; 143–157.

16. Pavenstadt, H., Kriz, W. and Kretzler, M. (2003) Cell biology of the glomerular podocyte. *Physiol. Rev.* 83; 253–307.

17. Peitsch, W. K., Hofmann, I., Endlich, N., Pratzel, S., Kuhn, C., Spring, H., Grone, H. J., Kriz, W. and Franke, W. W. (2003) Cell biological and biochemical characterization of drebrin complexes in mesangial cells and podocytes of renal glomeruli. *J. Am. Soc. Nephrol.* 14; 1452–1463.

18. Ramos, R. G., Igloi, G. L., Lichte, B., Baumann, U., Maier, D., Schneider, T., Brandstatter, J. H., Frohlich, A. and Fischbach, K. F. (1993) The irregular chiasm C-roughest locus of Drosophila, which affects axonal projections and programmed cell death, encodes a novel immunoglobulin-like protein. *Genes Dev.* 7; 2533–2547.

19. Rink, J. C., Vu, H. T. and Sanchez Alvarado, A. (2011) The maintenance and regeneration of the planarian excretory system are regulated by EGFR signaling. *Development* 138; 3769–3780.

20. Ruppert, E. and Smith, P. (1988) The functional organization of filtration nephridia. *Biol. Rev.* 63; 231–258.

21. Ruppert, E., Fox, R. and Barnes, R. (2003) Invertebrate Zoology —A Functional Evolutionary Approach—, Thomson Learning, Belmont.

22. Shen, K. and Bargmann, C. I. (2003) The immunoglobulin superfamily protein SYG-1 determines the location of specific synapses in C. elegans. *Cell* 112; 619–630.

23. Shen, K., Fetter, R. D. and Bargmann, C. I. (2004) Synaptic specificity is generated by the synaptic guidepost protein SYG-2 and its receptor, SYG-1. *Cell* 116; 869–881.

24. Silveira, M. and Corinna, A. (1976) Fine structural observations on the protonephridium of the terrestrial triclad Geoplana pasipha. *Cell Tissue Res.* 168; 455–463.

25. Strunkelnberg, M., Bonengel, B., Moda, L. M., Hertenstein, A., de Couet, H. G., Ramos, R. G. and Fischbach, K. F. (2001) rst and its paralogue kirre act redundantly during embryonic muscle development in Drosophila. *Development* 128; 4229–4239.

26. Tazaki, A., Kato, K., Orii, H., Agata, K. and Watanabe, K. (2002) The body margin of the planarian Dugesia japonica: characterization by the expression of an intermediate filament gene. *Dev. Genes Evol.* 212; 365–373.

27. Volker, L. A., Petry, M., Abdelsabour-Khalaf, M., Schweizer, H., Yusuf, F., Busch, T., Schermer, B., Benzing, T., Brand-Saberi, B., Kretz, O., Hohne, M. and Kispert, A. (2012) Comparative analysis of Neph gene expression in mouse and chicken development. *Histochem. Cell Biol.* 137; 355–366.

28. Weavers, H., Prieto-Sanchez, S., Grawe, F., Garcia-Lopez, A., Artero, R., Wilsch-Brauninger, M., Ruiz-Gomez, M., Skaer, H. and Denholm, B. (2009) The insect nephrocyte is a podocyte-like cell with a filtration slit diaphragm. *Nature* 457; 322–326.

29. Wilson, R. A. and Webster, L. A. (1974) Protonephridia. *Biol. Rev. Camb. Philos. Soc.* 49; 127–160.

30. Zhang. F., Zhao, Y. and Han, Z. (2013) An in vivo functional analysis system for renal gene discovery in Drosophila pericardial nephrocytes. *J. Am. Soc. Nephrol.* 24; 191–197.

31. Zhuang, S., Shao, H., Guo, F., Trimble, R., Pearce, E. and Abmayr, S. M. (2009) Sns and Kirre, the Drosophila orthologs of Nephrin and Neph1, direct adhesion, fusion and formation of a slit diaphragm-like structure in insect nephrocytes. *Development* 136; 2335–2344.