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Original Article

Tricellulin Expression and its Deletion Effects in the Endolymphatic Sac

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OBJECTIVES: Tricellulin is a tight junction (TJ)-forming protein that participates in the sealing function of tricellular TJs. *Tricellulin*-knockout *(Tric–/–)* mice show progressive hearing loss with degeneration of hair cells in the cochlea without physiological or physical disorders. In the present study, we investigated the tricellulin expression and its deletion effects in the endolymphatic sac (ES) using *Tric–/–* mice.

MATERIALS and METHODS: The ES epithelia from wild-type (WT) mice were laser-microdissected, and RT-PCR was performed. The ES sections from *Tric–/–* and WT mice were immunostained with an anti-tricellulin antibody. Hematoxylin and eosin staining was performed for morphological examination. The inner ear of *Tric–/–* mice was perfused with biotinylation reagents, and the ES sections were observed for tracer permeability assay after applying streptavidin–Alexa Fluor 488 conjugate.

RESULTS: The tricellulin expression was confirmed by RT-PCR and by immunohistochemistry in the WT ES. The ES in *Tric–/–* mice showed normal morphology and revealed no biotin leakage from the lumen.

CONCLUSION: The ES in *Tric–/–* mice showed no changes in morphology or disruption in macromolecular barrier function. The effects of solute leakages in the ES of *Tric–/–* mice may be very limited and compensatable, or that the ES epithelia may have other sealing system covering the lack of tricellulin.

KEYWORDS: Tricellulin, endolymphatic sac, paracellular transport, tight junction, knockout

INTRODUCTION

The endolymphatic sac (ES) in the inner ear comprises a part of the membranous labyrinth. The vestibule, semicircular canals, and cochlea also contain membranous labyrinth that is filled with the endolymph. It is thought that the ES absorbs the endolymph and maintains balance in specific ionic components (low K^+ and high Na^+) to regulate the volume and hydrostatic pressure of the endolymph [1, 2]. Dysfunction of the ES is considered to induce excess of endolymph, leading to endolymphatic hydrops typically observed as bulging of the Reissner's membrane in the cochlea, and causes vertigo and hearing loss [3].

Tight junctions (TJs) are a junctional complex between epithelial cells sealing the paracellular space across the epithelia. TJs have been observed by the freeze-fracture electron microscopy as the network of intramembranous strands (TJ strands) [4], formed by the assembly of claudins and TJ-associated myelin and lymphocyte protein and related proteins for vesicle trafficking and membrane link (MARVEL) proteins. Bicellular TJs (bTJs) are composed of TJ strands laterally connecting two neighboring cells, whereas tricellular TJs (tTJs) are formed by vertically extending TJ strands at the contact point of three cells [4].

The ES epithelia have also been revealed to have the TJs in an electron microscopic study [5]. We previously reported the mRNA expression of several claudins in the ES and suggested that claudins participate in paracellular ion transport to maintain the unique ionic concentration and electrical charge of the endolymph [6].

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Tricellulin (MARVELD2), a member of the TJ-associated MARVEL protein group, is mainly located at the tTJs, although it also localizes sparsely to the bTJs. The epithelial cells (mouse Eph4 cells) with the *tricellulin* (*Tric*) gene knocked down show disorganized formation and increased ion permeability in both tTJs and bTJs [7]. Therefore, tricellulin is thought to play an important role in the formation and barrier function of tTJs and bTJs. Moreover, permeability to macromolecules in tTJs decreased when *Tric* was overexpressed in Madin–Darby canine kidney (MDCK) II cells independently to the ion permeability [8]. *Tric* is also known as a causative gene for recessive nonsyndromic familial deafness (DFNB49) in humans and mice [9]. We recently investigated *Tric*-knockout (KO; *Tric*$^{-/-}$) mice, which suffered from early-onset progressive hearing loss associated with the degeneration of hair cells in the cochlea [10].

Despite the reported barrier dysfunction in the *Tric*-deficient epithelial cell lines and the severe cochlear dysfunction in *Tric*$^{-/-}$ mice, we were unable to detect vestibular dysfunction in *Tric*$^{-/-}$ mice [10], suggesting that the function of the endolymphatic system can be maintained even without tricellulin. However, the details of the tricellulin expression and the effects of the tricellulin deletion in the ES have not yet been studied. In the current study, we first confirmed the tricellulin expression in the ES of wild-type (WT) mice and tested whether the morphology and barrier function to macromolecules in the ES are maintained in the absence of the tricellulin expression using *Tric*$^{-/-}$ mice.

MATERIALS and METHODS

Animals and Tissue Preparation

Tric$^{-/-}$ mice (accession no. CDB0806K, http://www2.clst.riken.jp/arg/mutant%20mice%20list.html) were used, and its generation was previously described by Kamitani et al. [10]. The ethics committee of the Animal Care and Use Committees of our institutions approved the study.

For hematoxylin and eosin (H&E) staining and immunohistochemistry (IHC), deeply anesthetized mice were perfused with a fixative solution (4% paraformaldehyde (PFA) in PBS) for approximately 5 min and decapitated. The temporal bones were removed and fixed in 4% PFA in PBS for 6 h at 4°C, followed by decalcification in 0.12 M ethylenediaminetetraacetic acid (EDTA) at 4°C for 7 days. Decalcified temporal bones including the ES were substituted with a graded sucrose series and embedded in optimal cutting temperature (OCT) tissue compound for cryosectioning.

For laser capture microdissection (LCM), deeply anesthetized mice were perfused with a fixative solution of 70% ethanol/RNase-free water and decapitated. The temporal bones were removed, immersed in 70% ethanol/RNase-free water for 6 h at 4°C, and decalcified in 0.12 M EDTA including RNA later (Thermo Fisher Scientific, Waltham, MA, USA) for approximately 5 days at 4°C and then embedded in OCT tissue compound for LCM.

Laser Capture Microdissection

LCM was performed as previously described [11]. In brief, the entire ES was sliced (10–15 µm thick) using a cryostat at −20°C, refixed, and dehydrated. The whole ES epithelia were microdissected using the Arcturus Pixcell II system (MDS Analytical Technologies, Sunnyvale, CA, USA). The LCM samples were proceeded to extract RNA using the PicoPure RNA Isolation Kit (Thermo Fisher Scientific). One RNA sample (LCM–ES) was extracted from the bilateral ES epithelia samples.

RT-PCR and Sequencing

RNA of the ES epithelia isolated from the LCM samples was reverse-transcribed and amplified using the SuperScript® III One-Step RT-PCR System with Platinum® Taq (Thermo Fisher Scientific). The specific primer pairs were the following: *Tric* (accession no. NM_001038602.4) forward: 5′-CGCGCAGGTGTGAAAATCA-3′ and reverse: 5′-GGTACCATCTGGACAAGACGTG-3′, amplicon size: 106 bp and *glyceraldehyde-3-phosphate dehydrogenase* (*Gapdh*; accession no. NM_001289726.1) forward: 5′-GAGAGTGTTTCCTCGTCCC-3′ and reverse: 5′-TGGCAACAATCTCCACTTTGC-3′, amplicon size: 119 bp. The primers were designed using Primer-BLAST (NCBI, Bethesda, MD, USA). PCR products were separated and visualized on 1.0% agarose gels by electrophoresis. Positive and negative controls were used, with RNA sample from the kidney of the WT mouse and PCR template replaced with pure water, respectively. Purified PCR products were checked for nucleotide sequences by an Applied Biosystems ABI 3130 Genetic Analyzer (Thermo Fisher Scientific) using BigDye Terminator ver. 3.1 (Thermo Fisher Scientific).

H&E Staining

After washing with distilled water, the ES sections (7–10 µm thick) from *Tric*$^{-/-}$ mice were placed in hematoxylin without stirring for 10 min, washed with water at 40°C for 15–20 min, and stained with 0.1%–0.5% eosin for 1–3 min. Samples were dehydrated, permeabilized with xylene, mounted, and observed under a light microscope (Olympus BX51; Olympus, Tokyo, Japan). Cochlea samples were made from *Tric*$^{-/-}$ mice temporal bones on postnatal day 21, paraffin-embedded, and stained with H&E [10].

Immunohistochemistry

Immunostaining was performed as previously described [12]. Briefly, sections (7–10 µm thick) were refixed with 4% PFA in PBS and washed, and a blocking reagent (Protein Block Serum-Free; Dako Japan, Tokyo, Japan) was applied. Primary antibodies against tricellulin (diluted at 1:100 in PBS; ABfinity™ Recombinant Rabbit Monoclonal Antibody-Purified; Thermo Fisher Scientific) and zonula occludens-1 (ZO-1; diluted at 1:200; TJP1/ZO-1 rat anti-mouse monoclonal antibody; LSBio, Seattle, WA, USA) were reacted to the sections overnight at 4°C. After repeated washes, the sections were incubated with secondary antibody Alexa Fluor 488 donkey anti-rabbit IgG or Alexa Fluor 555 donkey anti-rat IgG (diluted at 1:200 in PBS; Thermo Fisher Scientific) for 2 h at 23°C and washed. 4′,6-Diamidino-2-phenylindole (DAPI) nucleic acid stain (diluted at 1:5000 in distilled water; Thermo Fisher Scientific) was applied to the sections and washed. A light microscope (Olympus BX51; Olympus, Tokyo, Japan) was used to observe the sections. The intermediate portion of the ES was analyzed in IHC. Cultured MDCK cells were used as a positive control for tricellulin and ZO-1 detection. Sections that omitted the primary antibodies were prepared for negative control. Samples from 15 mice were used to confirm the accuracy and reproducibility of the procedure.

Tracer Permeability Assay

Animals (5-week-old *Tric*$^{-/-}$ mice) were anesthetized using 2.0% isoflurane, and the cochlear ducts were perfused through the stria vascularis with 10 µl of EZ-Link Sulfo-NHS-LC-Biotin (10 mg/ml, molecular weight: 556.59; Thermo Fisher Scientific) in PBS for 10 min. After 30 min, mice were perfused with a fixative solution of 4% PFA in PBS for approximately 5 min, and the temporal bones were removed. After overnight fixation at 4°C, the temporal bones were decalcified with 0.12 M EDTA

in PBS for 7 days at 4°C. Samples were embedded, and frozen sections (9 µm thick) were made. Streptavidin–Alexa Fluor 488 conjugate (Thermo Fisher Scientific) was used for detection of biotin after refixing and blocking of the sections. The nuclei were counterstained with DAPI.

RESULTS

RT-PCR and Sequencing

PCR products from the WT LCM–ES showed a DNA band of *Tric* (106 bp) by electrophoresis (Figure 1). RT-PCR from the WT mouse kidney detected *Tric* expression as a positive control, and no detection from the template replaced with water. Each sequence of PCR products was found to be similar as the initial design.

Immunohistochemistry

The tricellulin expression in the WT mouse ES was then verified by IHC with counterstaining by ZO-1 (Figure 2). ZO-1 was observed uniformly in the epithelial cell junctions of both *Tric*$^{-/-}$ and WT ES. Staining for tricellulin was observed at the tricellular corners of the epithelial cells in the ES from the WT mouse (Figure 2, arrows). In contrast, no staining for tricellulin was observed from the *Tric*$^{-/-}$ mouse. Intense staining of tricellulin was confirmed at tTJ in cultured MDCK cells as a positive control.

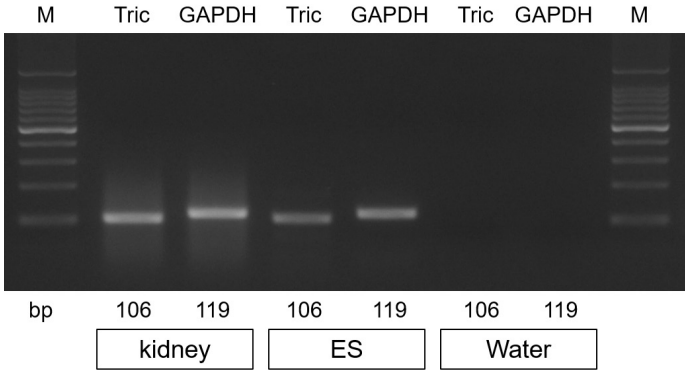

Figure 1. Agarose gel electrophoresis of PCR-amplified products from WT mice. A band for Tric was detected at the expected size in RNA isolated from the WT ES epithelia. The WT kidney template was used as a positive control.
ES: endolymphatic sac; Tric: tricellulin; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; M: measure; WT: wild-type.

H&E Staining

The ES of *Tric*$^{-/-}$ mice was stained with H&E and showed no anatomical and morphological anomalies compared with that of WT mice (Figure 3a and b). From the endolymphatic duct, proximal to the distal part of the ES, the epithelia were maintained in the regular one-layer sac structure. The scala media in the cochlea of *Tric*$^{-/-}$ mice did not show findings of endolymphatic hydrops, such as bulging of the Reissner's membrane (Figure 3c).

Tracer Permeability Assay

For examination of the barrier function to macromolecules in the ES of *Tric*$^{-/-}$ mice, biotin (molecular weight: 556.59) was infused into the endolymphatic space. In the ES of *Tric*$^{-/-}$ mice, biotin was confined in the endolymphatic space, and no apparent biotin leakage through the epithelia was found (Figure 4). Biotin was also detected in the lumen of the semicircular canal. These findings suggested that the barrier function against biotin in the *Tric* KO ES epithelia was maintained.

DISCUSSION

Tricellulin is characterized as a predominant protein in the tTJs and is also present in the bTJs, although to a lesser extent. It is considered to exist ubiquitously on a variety of tissues and organs [7,13,14], including the vestibule and cochlea [9], for maintaining paracellular barrier function. As expected, the tricellulin expression in the WT mouse ES was confirmed in both RT-PCR (Figure 1) and IHC (Figure 2). It has been reported that horseradish peroxidase (molecular weight: 40,000) injected in the ES lumen of guinea pigs is impermeable at the paracellular space, suggesting that there is a tight barrier to the

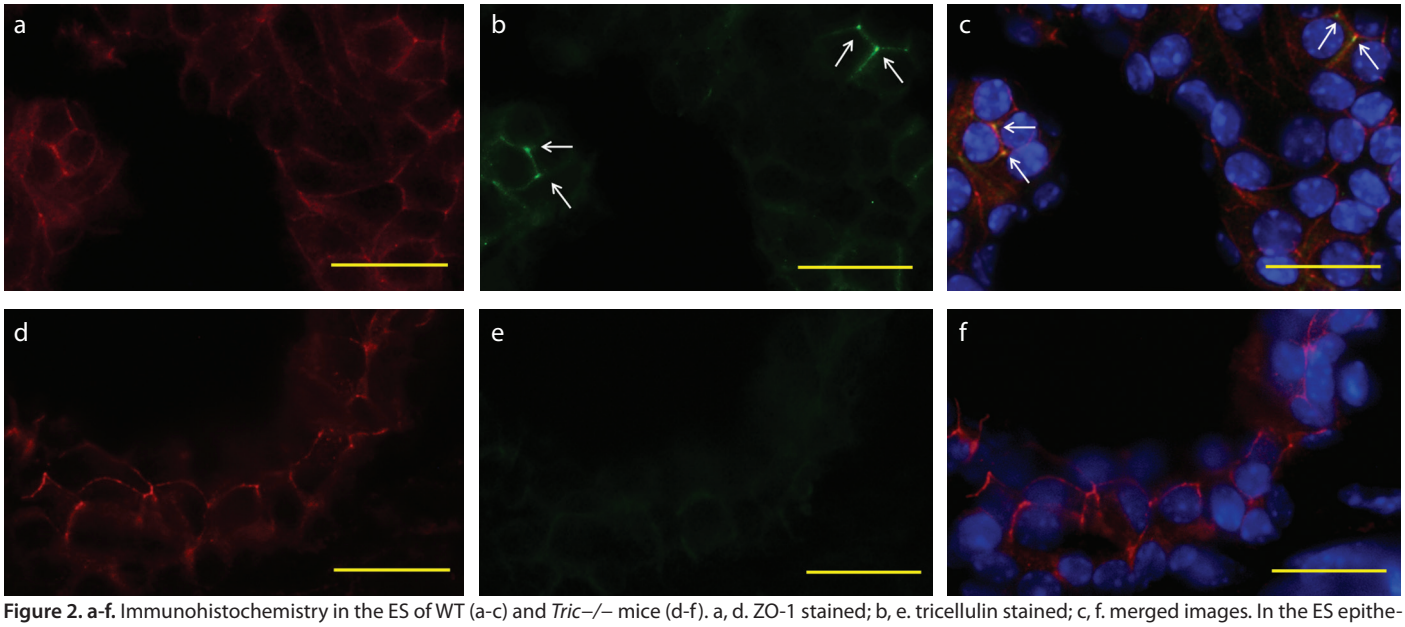

Figure 2. a-f. Immunohistochemistry in the ES of WT (a-c) and *Tric*–/– mice (d-f). a, d. ZO-1 stained; b, e. tricellulin stained; c, f. merged images. In the ES epithelium from the WT mouse, tricellulin was stained at the corners of three-cell contacts (arrows in b and c). Tricellulin was not detected in the ES epithelium from the *Tric*–/– mouse (e). ZO-1 was double-stained red, showing linear expression around the cells (a and d).
ES: endolymphatic sac; WT: wild-type; *Tric*–/–: *tricellulin*-knockout; ZO-1: zonula occludens-1. Scale bar: 20 µm.

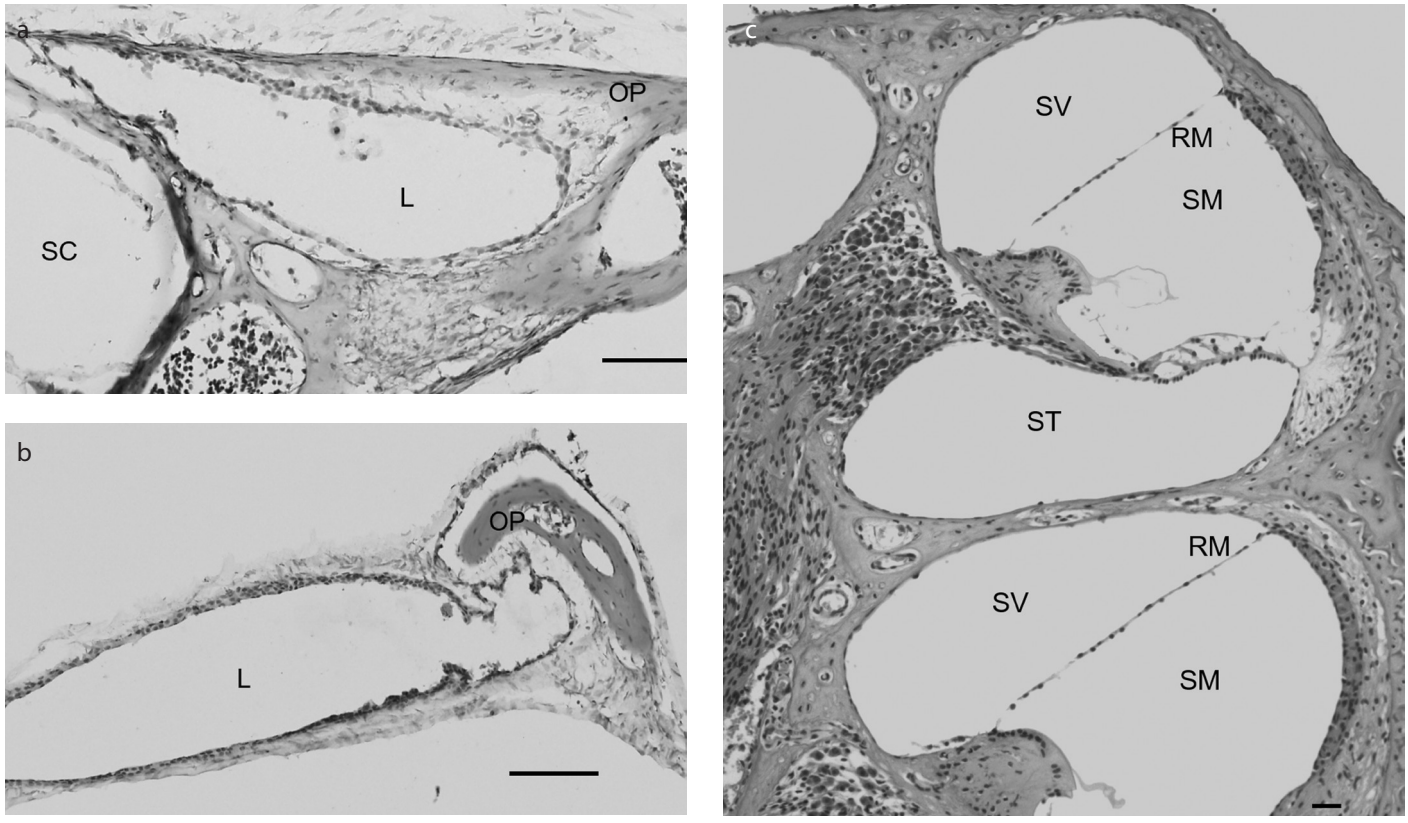

Figure 3. a-c. Hematoxylin and eosin staining in the ES and cochlea of *Tric–/–* mice. a. ES in *Tric–/–* mice; b. ES in WT mice; c. cochlea in *Tric–/–* mice. The ES in *Tric–/–* mice (a) had no anatomical and morphological anomalies compared with the ES in WT mice (b). The epithelium was maintained in the regular one-layer sac structure. The intermediate portion of the ES was partially surrounded by bone (OP) and showed no apparent ballooning of the sac. The cochlea in *Tric–/–* mice had no findings of endolymphatic hydrops (c).

ES: endolymphatic sac; *Tric–/–*: *tricellulin*-knockout; WT: wild-type; L: lumen of the ES; SC: semicircular canal; SV: scala vestibule; SM: scala media; ST: scala tympani; RM: Reissner's membrane; OP: operculum. Scale bar: 100 µm.

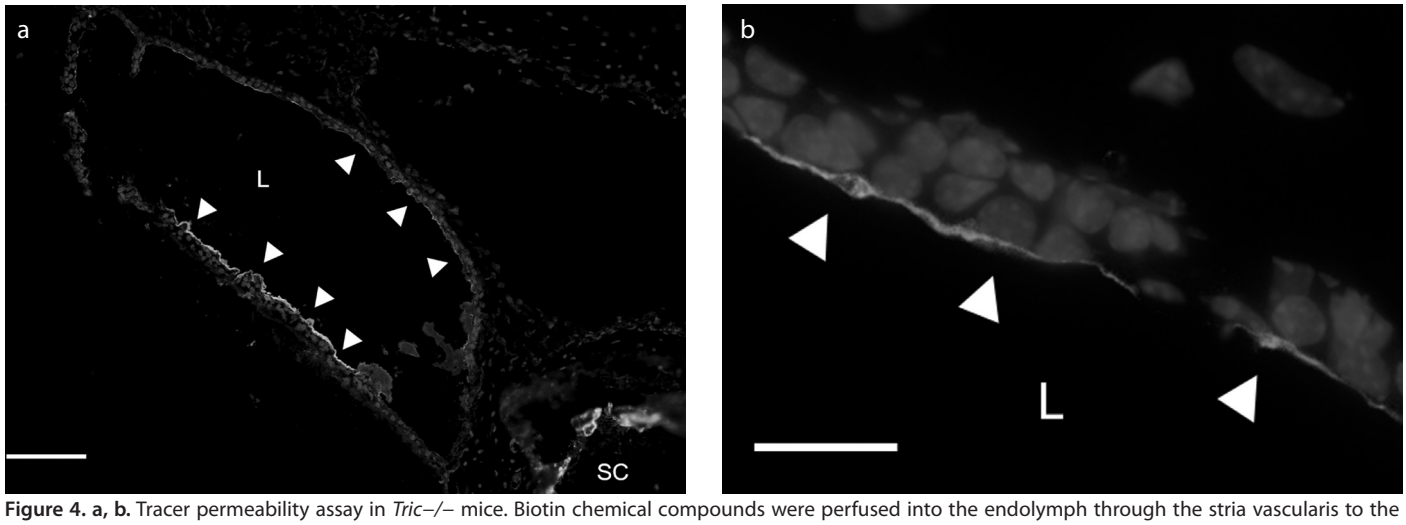

Figure 4. a, b. Tracer permeability assay in *Tric–/–* mice. Biotin chemical compounds were perfused into the endolymph through the stria vascularis to the cochlear ducts. In the ES of *Tric–/–* mice, biotin was confined in the endolymph (arrowheads), and there was no leakage through the epithelia. Biotin was also detected in the lumen of the semicircular canal (a). Biotin was detected using a streptavidin–Alexa Fluor 488 conjugate.

Tric–/–: tricellulin-knockout; ES: endolymphatic sac; L: lumen of the ES; SC: semicircular canal. Scale bar: a: 100 µm, b: 20 µm.

macromolecules in the epithelial linings of the ES [15]. In the present study, the tracer assay using *Tric*$^{-/-}$ mice revealed that there was no leakage of biotin (molecular weight: 556.59) from the lumen of the ES (Figure 4). These results suggested that tricellulin in the ES participated in the formation of TJs as one of the barrier function components, but that it is not necessarily crucial for sealing macromolecules in the lumen in the ES. In addition, the morphology of the *Tric*$^{-/-}$ mouse ES remained normal (Figure 3), with no indication of endolymphatic hydrops. These findings were compatible with the observation that *Tric*$^{-/-}$ mice show no disturbance in the sense of equilibrium, [10] and the fact that *TRIC* gene (DFNB49) mutations lead to hearing loss with no obvious vestibular phenotype in humans [9, 16, 17].

Tric$^{-/-}$ mice have no functional or structural phenotype not only in the ES but also in the majority of organs, such as the colon, intestine, liver, heart, thyroid, and kidney [10]. Moreover, the paracellular permeability of the stria vascularis in the cochlea is maintained [10]. These data support the previous proposal that tTJs are essential for maintaining the function and structure of a specific part of an organ, such as the organ of Corti in the cochlea, where a strict epithelial barrier system is required [10, 18]. Nevertheless, a question still remains on the phenotypic difference between ES and cochlea, in spite of the fact that they are connected through the same isolated endolymphatic space. One possible explanation is compensation of minor ion leakage by active ion transport. At the organ of Corti in the cochlea of *Tric*$^{-/-}$ mice, the minor leakage of K^+ from endolymph to perilymph is presumed to be the trigger of eventual hair cell degeneration [10]. The cochlear endolymph has a distinctively high concentration of K^+ (150 mM) and a positive potential of 80 mV referred to as the endocochlear potential, which is meticulously maintained and indispensable to cause depolarization of hair cells. On the other hand, the ES endolymph contains high Na^+ concentration of 103.3 mM and the ES potential of 14.7 mM[3]. Although minor leak of K^+ could also exist in the *Tric*$^{-/-}$ ES, it may be unremarkable since the concentration of K^+ in the ES endolymph is smaller (11.6 mM) than that in the cochlea, and K^+ electrochemical gradient can be easily reversed even under the resting potential in the ES [19]. Similarly, active Na^+ transport by the Na^+/K^+-ATPase is capable enough to compensate the effects of small Na^+ leakage from the ES endolymph in *Tric*$^{-/-}$ mice [20].

CONCLUSION

The tricellulin expression was confirmed in the ES, although the tricellulin deletion had no adverse effects on the morphology and permeability to macromolecules in the ES. The cochlear hair cells are the only disrupted sites resulting from *Tric* KO as reported previously [10], despite the fact that the ES and scala media in the cochlea share a single endolymphatic space. These results suggest that the effects of solute leakages in the ES of *Tric*$^{-/-}$ mice may be very limited and compensatable by active ion transport of the ES, or that the ES epithelia may have other sealing system covering the lack of tricellulin in the tTJs [21]. Further studies are needed to elucidate the function of the TJs in the ES.

Ethics Committee Approval: Ethics committee approval was received for this study from the ethics committee of the Animal Care and Use Committees of Kagawa University and Kyoto Prefectural University of Medicine.

Informed Consent: N/A.

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Author contributions: Concept – T.M., R.I.; Design – T.M., A.M.; Supervision – N.M., H.H.; Data Collection and/or Processing – A.M., T.M, T.K.; Analysis and/or Interpretation – A.M., T.M., H.S.; Literature Search – A.M.; Writing – A.M., T.M.; Critical Reviews – T.M., H.S.

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Conflict of Interest: The authors have no conflict of interest to declare.

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