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Prox1 expression in the endolymphatic sac revealed by whole-mount fluorescent imaging of *Prox1*-GFP transgenic mice

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ABSTRACT

This study describes a technical breakthrough in endolymphatic sac research, made possible by the use of the recently generated *Prox1*-GFP transgenic mouse model. Whole-mount imaging techniques through the decalcified temporal bone and three-dimensional observations of *Prox1*-GFP mouse tissue revealed the positive labeling of the endolymphatic sac in adult stage, and allowed, for the first time, the GFP-based identification of endolymphatic sac epithelial cells. Prox1 expression was observed in all parts of the endolymphatic sac epithelia. In intermediate portion of the endolymphatic sac, mitochondria-rich cells did not express Prox1, although ribosome-rich cells showed strong GFP labeling. The anatomical relationship between the endolymphatic sac and the surrounding vasculature was directly observed. In the endolymphatic sac, expression of Prox1 may suggest progenitor cell-like pluripotency or developmental similarity to systemic lymphatic vessels in other organs. This whole-mount imaging technique of the endolymphatic sac can be combined with other conventional histological, sectioning, and labeling techniques and will be very useful for future endolymphatic sac research.

1. Introduction

The transcription factor Prox1 belongs to the family of homeobox transcription factors. Prox1 is critical for organ development during embryogenesis and is involved in neurogenesis [1]. *Prox1* knockout mice die before birth at approximately E15 due to multiple developmental defects [2,3]. Prox1 regulates differentiation of progenitor cells and initiation of neurogenesis [1]. In the hippocampus, dentate gyrus, lymphatic vessels, and lens, Prox1 expression continues until post natal and adult stages [1]. The hippocampus and dentate gyrus are essential areas for adult neurogenesis. In the inner ear, Prox1 is expressed in vestibular hair cells, endolymphatic duct, and supporting cells (Deiters and Pillar cells) in the embryonic stage but no longer detectable in the adult stage except in the spiral ganglion [4]. The function of Prox1 in the cochlea and vestibule is thought to relate to the state of differentiation of the stem/progenitor cells [4]. In the endolymphatic sac, which is a part of the inner ear, the expression of Prox1 has not been reported.

The lumen of the endolymphatic sac is connected to the cochlea, vestibular organs, and semicircular canals. Previous studies have suggested that the endolymphatic sac controls endolymph. In fact, endolymphatic sac dysfunction causes an excess in endolymph called endolymphatic hydrops [5,6]. Endolymphatic hydrops induces hearing loss and vestibular dysfunction. Thus, endolymph regulation by the endolymphatic sac is important for hearing and equilibrium sensing. Thus, the clarification of the mechanism of endolymph regulation could lead to treatments for endolymphatic hydrops. The endolymphatic sac is divided into three parts: the proximal portion, the intermediate portion, and the distal portion. In the intermediate portion of the endolymphatic sac, two types of epithelial cells have been reported: in the mouse and the guinea pig, mitochondria-rich cells (also named cytoorganelle-rich, type 1 or light cells) and filament-rich cells (type 2 or dark cells) were identified [7–11]. Our recent study suggests that endolymphatic sac epithelial cells in the intermediate portion have sufficient Na^+, K^+-ATPase activity to provide the driving force for the Na^+ transport required to absorb the endolymph [12]. This active ion transport needs sufficient blood supply. In fact, several earlier reports have found many vessels in the

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endolymphatic sac, using light microscopy, stereomicroscopy or electron microscopy studies, and observation of tissue sections or vascular corrosion casts [13–17]. To further investigate the endolymphatic sac and associated blood vessels in more detail, three dimensional imaging of endolymphatic sac epithelial cells and vessels using confocal laser scanning microscopy or multiphoton fluorescence microscopy may be feasible, however, technical difficulties so far have prevented the optical sectioning through the temporal bone and the three dimension imaging of the endolymphatic sac.

In this study, we established a whole-mount imaging approach to directly visualize the endolymphatic sac, and examined Prox1 expression in the endolymphatic sac epithelia in adult mice.

2. Materials and methods

2.1. Prox1-GFP BAC transgenic mouse

Prox1-GFP BAC transgenic mice (Tg[*Prox1*-EGFP]221Gsat/Mmcd, cryoarchived) were purchased from the Mutant Mouse Regional Resource Center. The founder mouse (FVB/N) was crossed with Crl:CD1(ICR) mice before cryopreservation and recovered litters present the FVB/N-Crl:CD1(ICR) outbred background, as described previously [18]. Overall, the morphology of the *Prox1*-GFP BAC transgenic mice appeared normal.

2.2. Whole-mount imaging of the endolymphatic sac

All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and all experiments were performed using protocols approved by the Institutional Animal Care and Use Committee of the University of Southern California (USC) and Kagawa University. *Prox1*-GFP BAC transgenic mice (4–5 weeks old, weight, 10–20 g; both sexes) were deeply anesthetized using ketamine (80–100 mg/kg) and xylazine (10 mg/kg), and were injected with 10 μL of tetramethylrhodamine labeled dextran (70,000 MW, Neutral, 25 mg/mL, Sigma, St. Louis, MO) intravenously. After 20 min, the mouse was perfused via the left ventricle with a fixative solution (4% paraformaldehyde in phosphate-buffered saline [PBS]) for approximately 5 min. The temporal bones on both sides were carefully removed, including the surrounding bone tissues, under a stereomicroscope. The brain and cochlea were also collected. The samples were fixed in 4% paraformaldehyde in PBS for 6 h at 4 °C. The temporal bone and the cochlea were then decalcified in 0.12 M ethylene-diaminetetraacetic acid (EDTA; pH 6.5) at 4 °C for 7 days. The temporal bone and the cochlea were examined using a 2-photon laser scanning fluorescence microscope (TCS SP5 AOBS MP confocal microscope system; Leica-Microsystems, Germany). Images were collected as a z-series file and analyzed with Leica LCS imaging software [19].

2.3. Immunostaining of endolymphatic sac epithelial cells

The decalcified temporal bones and brain were embedded in optimal cutting temperature (OCT) tissue compound (Sakura Fintechnical, Japan). Sections (7–10 μm thick) were cut on a cryostat at −20 °C. Immunostaining was performed as described in our previous study with minor modifications [20]. To reduce nonspecific binding, sections were washed in PBS for 10 min and incubated for 15 min with 20% normal goat serum.

After being washed with PBS, the sections were incubated with an anti-Oxphos antibody (mitochondria-specific protein, 1:200, Invitrogen, Carlsbad, CA) in PBS overnight at 4 °C. After three washes with PBS, the sections were incubated with a fluorophore-labeled donkey anti-mouse IgG (1:200) secondary antibody (Alexa Fluor 564, Molecular Probes, Carlsbad, CA) in PBS at room temperature for 30 min. The slides were observed using the same laser scanning fluorescence microscope system described above.

3. Results

3.1. Prox1 expression in the endolymphatic epithelial cells of the adult mice

Strong Prox1-GFP fluorescence signal was observed in epithelial cells of the endolymphatic sac in adult mice (Fig. 1A). In the intermediate portion of the endolymphatic sac, mitochondria rich cells did not express Prox1, although ribosome-rich cells showed strong GFP signal (Fig. 1A). As a control, Prox1-GFP signal was determined in the hippocampus of the brain (Fig. 1B).

3.2. Whole-mount imaging of the endolymphatic sac

To observe the endolymphatic sac in the temporal bone, we used whole-mount imaging techniques in *Prox1*-GFP BAC transgenic mice. As a control, the Prox1-GFP signal was determined in the spiral ganglion in the cochlea and lymphatic vessels after decalcification, using the same whole-mount imaging technique (Fig. 2A and B). Strong GFP fluorescence was observed in the endolymphatic sac epithelia by optical sectioning through the temporal bone after decalcification. The Prox1 GFP signal was detected in all

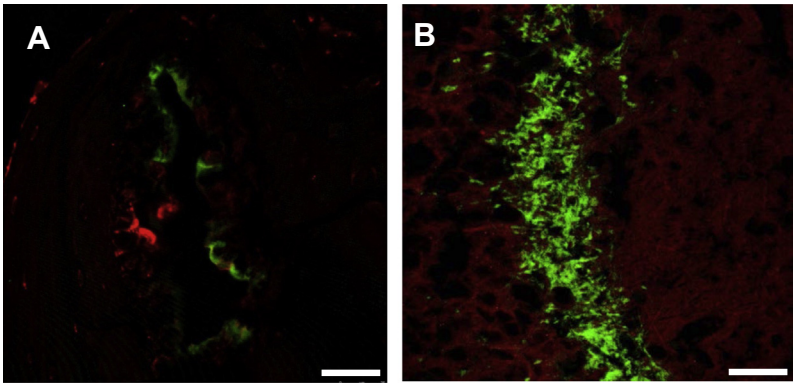

Fig. 1. Prox1 expression in endolymphatic sac epithelial cells of adult mice. Section of *Prox1*-GFP mice temporal bones showing Prox1 expression (EGFP, green) and mitochondrial staining (red). Intermediate portion of the endolymphatic sac (A). A strong GFP signal is observed in the endolymphatic sac epithelial cells in the intermediate portion. Mitochondrial rich cells did not express Prox1, although ribosome-rich cells expressed Prox1. (B) Positive control: Prox1 is expressed in the hippocampus in the brain. Scale bar = 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

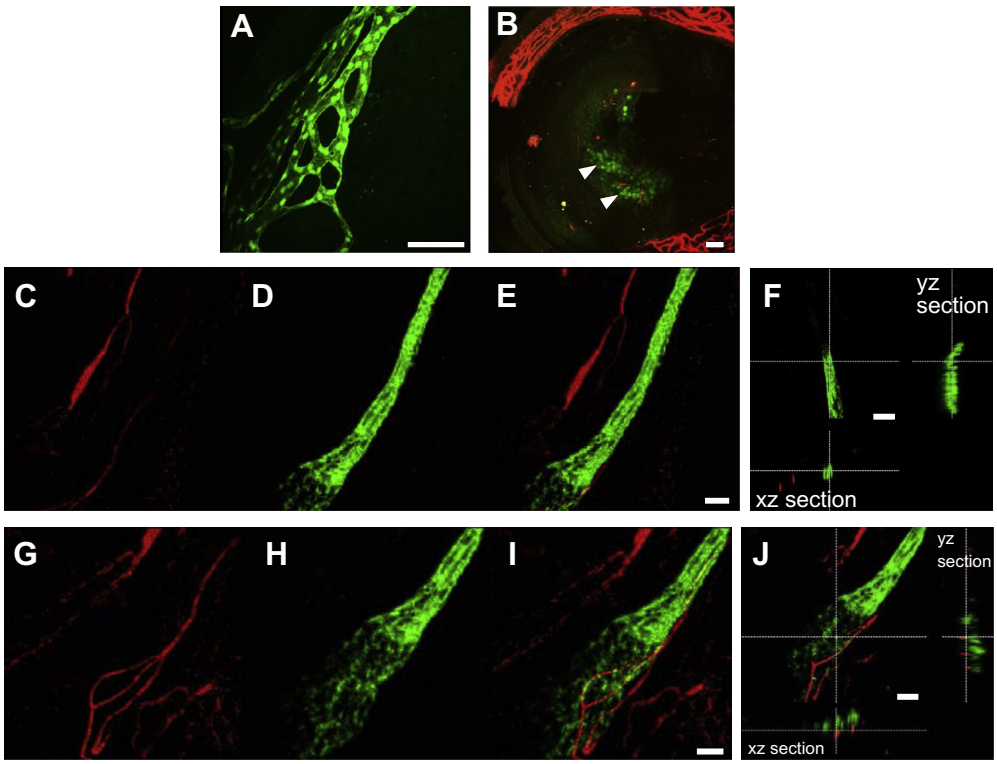

Fig. 2. Whole-mount projection images and section (*xz* and *yz*) images of the endolymphatic sac Prox1 (EGFP: green), blood vessels (70 kDa dextran-rhodamine: red), and merged images of the endolymphatic sac. (A) Lymphatic vessels (control), (B) spiral ganglion (arrow head) in the cochlea (control). (C) Blood vessels, (D) Prox1, (E) merge image of C and D. Whole-mount projection images of proximal and intermediate portion of the endolymphatic sac. (F) Section (*xz* and *yz*) images of the proximal and intermediate portions of the endolymphatic sac. (G) Blood vessels, (H) Prox1, (I) Merge image of G and H. Whole-mount projection images of intermediate portion of the endolymphatic sac. (J) Section (*xz* and *yz*) images of intermediate portion of the endolymphatic sac. Images were acquired as Z stacks of 1 μm slices. Scale bar = 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

parts of the endolymphatic sac epithelia, including the proximal, intermediate, and distal portions (Fig. 2C–J). All parts of endolymphatic epithelial cells were clearly observed through the decalcified temporal bone and could be evaluated in three dimensions. Several branched blood vessels were observed in the intermediate and distal portion of the endolymphatic sac and the branched vessels were close to endolymphatic sac epithelial cells (Fig. 2G–J).

4. Discussion

The present study described the expression of Prox1 in the endolymphatic epithelial cells in adult mice. Prox1 expression was found in all parts of the endolymphatic sac epithelia. In the intermediate portion of the endolymphatic sac, mitochondria-rich cells did not express Prox1, while ribosome-rich cells expressed Prox1. Mitochondria-rich cells have many mitochondria and higher Na transporting ability, and ribosome-rich cells are thought to support the mitochondria-rich cells [12]. A population of supporting cells in the inner ear of chickens have been identified as progenitor cells [21,22]. Progenitor cells in the inner ear express cProx1 [23]. Strong expression of cProx1 was observed in mature supporting cells during differentiation into hair cells [23]. Therefore, cProx1 may regulate the proliferative or differentiation functions of progenitor cells [23]. The expression of Prox1 in the endolymphatic sac observed in the present study may suggest progenitor cell-like pluripotency. Also, the expression of Prox1 in the endolymphatic sac may suggest the presence of similar developmental or differentiation mechanisms in the endolymphatic sac and in systemic lymphatic vessels throughout the body, where Prox1 expression was recently identified [18]. Further studies are required to reveal the function of Prox1 in the endolymphatic sac.

The present study is a technical breakthrough in endolymphatic sac research which was made possible by the use of the recently generated *Prox1*-GFP transgenic mouse model [18]. Whole-mount imaging techniques and three-dimensional observations of *Prox1*-GFP mouse tissue revealed the positive labeling of the endolymphatic sac and allowed, for the first time, the GFP-based identification of endolymphatic sac epithelial cells. *Prox1*-GFP mice showed a strong GFP signal in the entire length of endolymphatic sac epithelia. Using the combination of this whole-mount imaging technique of the endolymphatic sac and vascular staining with dextran-rhodamine, we directly observed the anatomical relationship between the endolymphatic sac and the surrounding vasculature. In the proximal portion, the endolymphatic sac was narrow and surrounded by few blood vessels. In the intermediate portion, which is the most active ion transporting part, the endolymphatic sac was extended and surrounded by many branched blood vessels. In the distal portion, the endolymphatic sac was close to the sigmoid sinus. These results are in agreement with previous studies in rats [10].

In conclusion, we demonstrated the expression of Prox1 in all parts of the endolymphatic sac epithelia in adult mice. Expression of Prox1 may suggest the potency as progenitor cells in the endolymphatic sac. The use of *Prox1*-GFP mice allowed the positive labeling and identification of endolymphatic sac epithelia for the first time, using whole-mount imaging and three-dimensional observations. The same tissue preparations could be further studied by the combination with other conventional histological, sectioning, and labeling techniques, e.g. immunohistochemistry. This

whole-mount imaging technique of the endolymphatic sac will be a very useful method for future endolymphatic sac research.

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