DNAJC14 Ameliorates Inner Ear Degeneration in the DFNB4 Mouse Model

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The His723Arg (H723R) mutation in SLC26A4, encoding pendrin, is the most prevalent mutation in DFNB4, an autosomal recessive type of genetic hearing loss. Although the main pathological mechanism of H723R was identified as a protein-folding defect in pendrin, there is still no curative treatment for associated hearing loss. Here, we show that H723R-pendrin expression and activity are rescued by activation of the chaperonin DNAJC14. In vitro, DNAJC14 was activated via Japanese encephalitis virus (JEV) inoculation, and toxin-attenuated JEV rescued the surface expression and anion exchange activity of H723R-pendrin. Human H723R-pendrin transgenic mice (hH723R Tg) were established in a mouse slc26a4 knockout background, in which only hH723R-pendrin was expressed in the inner ear (Pax2-Cre dependent) to mimic human DFNB4 pathology. Crossing hH723R Tg with DNAJC14-overexpressing mice resulted in reduced cochlear hydrops and more preserved outer hair cells in the cochlea compared to those in hH723R Tg mice. Furthermore, the stria vasularis and spiral ligament were thicker and KCNJ10 expression was increased with DNAJC14 overexpression; however, hearing function and enlarged endolymphatic hydrops were not recovered. These results indicate that DNAJC14 overexpression ameliorates the cochlear degenerations caused by misfolded pendrin and might be a potential therapeutic target for DFNB4.

INTRODUCTION

SLC26A4 encodes pendrin protein, which is an anion exchanger in the inner ear.1–3 Mutations in SLC26A4 cause non-syndromic, autosomal recessive hearing loss (DFNB4), which is the most common cause of congenital deafness in East Asia,4–9 In Korea, the allele frequency of the major variants of SLC26A4 that cause DFNB4 is very high, specifically as much as approximately 0.7%, upon referring to the gnomAD database (http://gnomad.broadinstitute.org/). DFNB4 results in congenital, fluctuating, progressive, and severe-to-profound sensorineural hearing loss.10,11 Vestibular functions are also impaired and linked to hearing loss in patients with DFNB4.11 Most patients with DFNB4 have congenital and severe hearing loss and, thus, undergo cochlear implantation and require hearing rehabilitation. Although residual hearing is often observed at an early age, there is a limited window of several years to remedy or prevent hearing loss during the early stage.12 Unfortunately, no curable treatment options are currently available for the sensorineural hearing loss that occurs with DFNB4.

Among the pathogenic mutations in the protein encoded by SLC26A4, p.H723R (His723Arg) in pendrin is the most common among Korean and Japanese populations.6,13,14 The pathological mechanism of p.H723R is related to protein misfolding, retention in the endoplasmic reticulum (ER), and degradation by ER-associated degradation (ERAD) pathway.15,16 Recent studies have shown that misfolded proteins can be expressed on the cellular surface via an unconventional secretion pathway comprising Golgi-bypassing trafficking under ER stress.16–18 Thus, p.H723R-pendrin (H723R-pendrin) can be rescued on the cell surface via this unconventional trafficking pathway, which is mediated by Hsp70 and its co-chaperone DNAJC14. Hsp70 and DNAJC14 then interact with H723R-pendrin and retrieve the misfolded pendrin from the ERAD pathway to deliver it to the cell surface.16

DNAJC14 is an ER chaperon that modulates the cell-surface trafficking of dopamine D1 receptor and SNARE complex-mediated lysosomal trafficking.19,20 Interestingly, DNAJC14 also modulates RNA replication for viruses of the Flaviviridae family after infection in mammalian cells.21 This protein is activated by Flavivirus infection and is recruited to the virus replication complex, where it mediates viral
replication on the ER membranes by affecting viral polyprotein processing through the promotion of protein folding.21–23 This phenomenon is highly reminiscent of the unconventional trafficking of H723R-pendrin via the Hsp70/DNAJC14 machinery in terms of protein folding and trafficking from the ER to the cell surface.16 Therefore, we hypothesized that the administration of a Flavivirus such as yellow fever virus or Japanese encephalitis virus (JEV) would activate the Hsp70/DNAJC14 machinery to enhance the unconventional trafficking of H723R-pendrin and subsequently rescue the function of pendrin. We tested this hypothesis using an in vitro model of JEV-infected human pancreatic cancer cell line (PANC-1) cells expressing H723R-pendrin. Furthermore, we investigated the effect of enhanced DNAJC14 on changes in inner ear histology and auditory function in human p.H723R-pendrin (hH723R) transgenic (Tg) mice and DNAJC14-overexpressing mice, mimicking the pathology of hearing loss associated with human DFNB4.

RESULTS
JEV Rescues the Expression and Function of hH723R-Pendrin
We previously reported that H723R-pendrin has a folding defect and is rescued by DNAJC14 overexpression.16 Here, we also observed that the surface expression of hH723R-pendrin was significantly increased by JEV treatment (106 pfu) (n = 8 in each group). **p < 0.01. (D) OâHCO3− exchange activity measured by recording the pH-sensitive fluorescent probe 2′,7′-bis(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) in PANC-1 mock cells and in WT- and hH723R-pendrin stably expressing cells, as detailed in the Materials and Methods. (E) The quantitation of multiple experiments showed that the OâHCO3− exchange activity was significantly increased in the cells expressing hH723R-pendrin with JEV (3 × 106 pfu) treatment (n = 6 in each group). **p < 0.01.
hH723R-pendrin and DNAJC14 in the lysate were not changed, indicating improved trafficking efficiency to the membrane surface (Figures 1B and 1C).

We also investigated the anion-exchange activity of pendrin with the treatment of JEV according to changes in the fluorescence curve of the pH-sensitive fluorescent probe 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF), as described previously. The pH increased when the extracellular solution was changed from 129 mM Cl\(^-\) to 0 mM Cl\(^-\), owing to the influx of extracellular HCO\(_3\)\(^-\). Strong Cl\(^-\)/HCO\(_3\)\(^-\) exchange activity was also elicited by transfection with human wild-type (WT)-pendrin but not with hH723R-pendrin. Notably, the compromised anion exchange activity (Cl\(^-\)/HCO\(_3\)\(^-\)) of hH723R-pendrin was significantly increased by JEV treatment (Figures 1D and 1E). The effect of the JEV treatment was also consistent when we measured formate and bicarbonate (HCO\(_3\)\(^-\)/HCO\(_2\)\(^-\)) exchange activity (Figures S1A and S1B). Immunostaining showed that hH723R-pendrin was restricted to the ER but that WT-pendrin was well expressed on the cellular surface (Figure 2). With variable amounts of JEV (10 or 50 μL of a 10^6 pfu/mL suspension), the membrane surface fraction of hH723R-pendrin remarkably increased (Figures 2A, lower panel, and 2B). Taken together, these results suggest that JEV treatment, which activates DNAJC14, increases the cell-surface expression and anion-exchange activity of hH723R-pendrin.

DNAJC14 Ameliorates Structural Degeneration in hH723R Tg(+) Mice

To evaluate the pathology of DFNB4 with DNAJC14 activation for potential therapeutic development, the DFN4 mouse model expressing H723R-pendrin was used. To establish an H723R mouse model with deafness, we first generated H723R knockin in mice (Figure S2A–S2C). However, hearing function in these mice remained normal, and there were no abnormalities in the inner ear (Figures S2D and S2E), consistent with results of a previous study. Thus, we established hH723R Tg mice (strain name, B6-Tg(CMV-SLC26A4tm1Egr)1JJ, allele1: H723R (c.2139G >A)) in a mouse sk26a4 homozygous knockout (mPDS/−/−) (strain name, 129S-Slc26a4tm1Egr/Ajg) background. The hH723R-flanked transgene (Figure S3A) was designed and inoculated into the embryos of B6 mice, which were then crossed with Pax2-Cre mice to achieve inner ear- and kidney-specific expression of hH723R-pendrin. The hH723R Tg mice were subsequently crossed with mPDS/−/− mice to only express hH723R in the inner ear and kidney, without the expression of mouse pendrin. As a positive control, we also established human WT-pendrin Tg (hPDS Tg; strain name, B6-Tg(CMV-SLC26A4tm1Egr)1JJ, allele1: H723R (c.2139G >A)) mice. Western blotting confirmed ectopic expression of WT-pendrin in the kidney and cochlea but not in the liver and intestine (Figure S3B, left panel). In hH723R Tg mice, hH723R-pendrin was identified in the cochlea (Figure S3B, right panel). hPDS Tg mice were underweight, and their lifespan was limited to only 2 months, which might be attributable to the ectopic overexpression of pendrin in the kidneys causing kidney dysfunctions such as electrolyte imbalance.

The auditory brainstem response (ABR) test demonstrated deafness in the hH723R Tg mice, mimicking human DFNB4, whereas the hPDS Tg mice showed normal hearing (Figure S3C). Cochlear histology clearly identified cochlear hydrops in both mPDS/−/− and hH723R Tg(+)mPDS/−/− mice, but not in hPDS Tg(+) mice ubiquitously expressing ectopic WT-pendrin in the cochlea (Figure S3D). Based on whole-mount images and surface

**Figure 2. Japanese Encephalitis Virus (JEV) Restores the Expression of Cell-Surface hH723R-Pendrin**

Cell-surface pendrin expression was examined in PANC-1 cells with immunostaining. (A) The cells were stained for pendrin (anti-Flag, green) and co-stained for DNAJC14 (red). Nuclei were counterstained with DAPI. DIC images were overlapped in the second lane to clarify the boundary of cells (dashed line). Yellow arrow line indicates the axis for the quantification of fluorescence. In the lower panel, fluorescence intensities for pendrin (green) and DAPI (blue) were quantified along the long axis of each cell, where the yellow box refers to the position of the plasma membrane. Scale bar, 20 μm. (B) The plasma membrane fraction of pendrin was calculated as fluorescence signals between a distance of 5 μm from the cell membrane-to-total fluorescence signal in the cell. Compared to that in lane 2 (hH723R-pendrin), both groups treated with JEV at different titers (1 × 10^4 and 5 × 10^4 pfu) showed increased expression of pendrin in the cell membrane (n = 4). ***p < 0.001.
electron microscopy of cochlear hair cells, the outer and inner hair cells were more preserved in hH723R Tg(+); mPDS/C0/C0 mice than in mPDS/C0/C0 mice, indicating that the ectopic expression of hH723R in the inner ear was minimally helpful to maintain cochlear structure and function (Figure S3E). Finally, hH723R Tg(+); mPDS/C0/C0 mice showed enlarged endolymphatic sacs and ducts compared to those in hPDS Tg(+); mPDS/C0/C0 animals based on computed tomogram sagittal images (Figure S3F). Taken together, we considered that hH723R Tg(+); mPDS/C0/C0 mice effectively mimic human pathologies associated with DFNB4, such as deafness.

For the activation of DNAJC14, we crossed hH723R Tg(+); mPDS/C0/C0 mice with Tg mice overexpressing DNAJC14 (DNAJC14 Tg(+); strain name, B6-Tg(CMV-DNAJC14)2JJ) (Figure 3A). Overexpression of DNAJC14 was confirmed in the cochlea, and the expression of hH723R-pendrin was also increased in the cochlea (Figures 3B and 3C). Because the hH723R Tg mouse model is dependent on Pax2-Cre expression, most of the cochlear tissue was positive for ectopic pendrin expression (Figure 3D). With respect to histological changes, the cochlear hydrops was significantly reduced when DNAJC14 was overexpressed in the cochlea (Figures 4A and 4B), and the stria vascularis and spiral ligament in DNAJC14 Tg(+); hH723R Tg(+); mPDS/C0/C0 mice were more preserved and thicker when compared to those of hH723R Tg mice (Figures 4C–4E). In addition, degeneration of the outer hair cells in hH723R Tg mice was mitigated by the expression of DNAJC14 (Figures 4F and 4G) and KCNJ10, which is important for the generation of endocochlear potential, was more preserved in the stria vascularis with DNAJC14 overexpression (Figures 4H and 4I). Collectively, these results indicate that DNAJC14 ameliorates cochlear degeneration in hH723R Tg mice.

Meanwhile, when we measured the hearing thresholds based on the ABR, DNAJC14 overexpression did not ameliorate hearing function with respect to auditory thresholds in click and frequency-specific stimuli (Figures 5A and 5B). Furthermore, we observed that the ectopic expression of hH723R in the endolymphatic duct, endolymphatic sac, and ampulla of the semi-circular canal increased with DNAJC14 overexpression (Figure 5C). However, the enlarged endolymphatic duct and sac were not restored with DNAJC14 overexpression. In addition, the giant otoconia identified in hH723R Tg(+); mPDS/C0/C0 mice was also observed in DNAJC14

Figure 3. Overexpression of DNAJC14 in hH723R-Pendrin Transgenic Mouse
(A) For activation of DNAJC14 in the established hH723R-pendrin transgenic (hH723R Tg) mouse model, hH723R Tg mice were crossed with Tg mice overexpressing DNAJC14. (B) Based on western blotting of the cochlea, expression of pendrin was increased by overexpressing DNAJC14. (C) Quantification of the density of pendrin is depicted in (AU, arbitrary unit) (n = 4 in each group). *p < 0.05. (D) DNAJC14 expression (red) was identified in the DNAJC14 Tg(+); hH723R Tg(+); mPDS/C0/C0 mice, and the expression of pendrin was increased in most of the cochlear tissue (Pax2-Cre dependent), as shown by immunostaining using the Alexa 488 secondary antibody (green). DAPI (blue) was used for nuclear staining. Scale bar, 100 μm; sm, scala media; st, scala tympani; sv, scala vestibule.
These data indicate that DNAJC14 overexpression was not sufficient to ameliorate hearing loss and vestibular malformation, including enlarged vestibular aqueduct and abnormal otoconia formation.

**DISCUSSION**

Genetic hearing loss is highly prevalent, with an incidence of 1 in 500–1,000 children. More than half of all cases of congenital hearing loss are caused by genetic variants. However, there is still no clinically curative treatment option for patients with genetic hearing loss. Because mutations in SLC26A4, encoding pendrin, are the most common cause of deafness in East Asia, it is important to develop effective therapeutics for patients with DFNB4 or Pendred syndrome.

We previously demonstrated that DNAJC14 is the key modulator that rescues the misfolded pendrin proteins from the ERAD and consequently enhances its expression and function.

Although the specific functional interaction underlying the mechanism through which DNAJC14 mediates the RNA replication of *Flavivirus* species such as yellow fever virus and JEV remains unknown, it seems clear that DNAJC14 is activated and rearranged on the ER membrane in response to *Flavivirus* infection. The present study demonstrated that DNAJC14 assists with the proper folding of misfolded pendrin, thereby facilitating its escape from the ER to the membrane surface. Thus, we reasoned that activated DNAJC14 due to *Flavivirus* infection might also promote the protein folding of H723R-pendrin. As expected, we demonstrated that JEV infection increased the cellular surface expression of H723R-pendrin, which was likely related to the promotion of protein folding and rescue from ERAD. This finding suggests the possible application of attenuated *Flavivirus*, as with the
strategy used in vaccines, as a therapeutic modality for patients with DFNB4 or Pendred syndrome.

Another important finding of this study is the establishment of a mouse model of H723R mutation-related DFNB4. Although several animal models with mutated pendrin have been reported,\textsuperscript{26,27} it has thus far been a challenge to establish a suitable H723R-pendrin mouse model with the human phenotype including deafness.\textsuperscript{24} In this study, we successfully established an hH723R-pendrin mouse model with deafness. This model can

Figure 5. DNAJC14 Overexpression Results in the Insufficient Rescue of Hearing Function and Vestibular Abnormal Morphologies in hH723R Transgenic Mice

(A and B) Auditory brainstem responses by click and tone-burst stimuli were measured in wild-type (WT), hH723R Tg(+);mPDS\textsuperscript{-/-} and DNAJC14 Tg(+);hH723R Tg(+) mPDS\textsuperscript{-/-} mice. Auditory threshold was not recovered even if DNAJC14 was overexpressed in hH723R Tg(+);mPDS\textsuperscript{-/-} mice based on click (A) and tone-burst (B) stimuli (n = 8 mice in each group). (C) Immunostaining of endolymphatic duct, endolymphatic sac, and ampulla in P0 mice of each strain. hH723R-pendrin (green) was predominantly identified in the epithelial lines (endolymphatic duct, endolymphatic sac, and ampulla) as well as supporting cells, around the endolymphatic duct and common crus (possibly ectopic expression of pendrin depending on CMV promoter) of DNAJC14 Tg(+);hH723R Tg(+) mPDS\textsuperscript{-/-} mice. DNAJC14, red; DAPI, blue; ed, endolymphatic duct; cc, common crus; es, endolymphatic sac; ca, crista ampullaris; cu, cupula; white arrow, ed in wild-type; scale bar, 100 μm. (D) Light microscopy and surface electron microscopy were used to observe otoconia morphology (red arrow). Giant otoconia were identified in the macula of H723R Tg(+) mPDS\textsuperscript{-/-} mice (middle), whereas normalized otoconia were observed in the macula of WT mice (upper). Giant otoconia were also observed in the macula of DNAJC14 Tg(+);hH723R Tg(+) mPDS\textsuperscript{-/-} mice (lower). Scale bar, 100 μm.
thus serve as a useful research tool for drug screening and validation, as well as preclinical trials for hearing preservation drugs that facilitate protein folding.

We also demonstrated that the overexpression of DNAJC14 in the inner ear could ameliorate the histological degeneration caused by the pendrin H723R mutation. Because slc26a4 knockout results in an enlarged vestibular aqueduct and endolymphatic hydrops in mice, bulging Reissner’s membrane in the cochlea due to dilatation of the scala media is typical. This finding was also observed in hH723R Tg(+) mice, which led us to speculate that patients with DFNB4 caused by the H723R mutation might also have cochlear hydrops. Importantly, the size of the cochlear hydrops in hH723R Tg(+) mice was significantly reduced by DNAJC14 overexpression in the inner ear. This indicates that rescued hH723R-pendrin can restore anion exchange (Cl⁻/HCO₃⁻) activity in the epithelia lining the membranous labyrinth, including the scala media, endolympathic duct, and sac. We speculated that the partial expression and functional rescue of hH723R-pendrin makes a difference in the milieu of the endolymph in terms of pH and endocochlear potential. Consequently, outer hair cells, as well as cells in the stria vasculaaris, might become more stable, and cell survival could increase. However, the protective effect of DNAJC14 on inner ear histology appears to be limited, as hearing loss and hydrops remained and hair cell degeneration occurred over time. Given that DNAJC14 expression was not sufficient to overcome hydrops caused by embryological defects in the hH723R Tg mouse model, hearing function could not be recovered by DNAJC14 in these animals. In addition, the vestibular phenotype, including circling and head tilting, cannot be rescued by the overexpression of DNAJC14 in the inner ear, and it is possible that the mixture of various background strains might affect histological changes in the inner ear. Nevertheless, the hH723R Tg mouse model holds promise for utilization in strains might affect histological changes in the inner ear. Neverthe-

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**Surface Biotinylation Assay**

The surface proteins were biotinylated with sulfo-NHS-SS-biotin (Pierce) for 30 min before cell lysis. The cells were washed with quenching buffer containing 1% bovine serum albumin (BSA) and then washed three times with phosphate-buffered saline (PBS). After cell lysis with lysis buffer containing 50 mM tris-HCl (pH 8.0), 150 mM NaCl, 1% (v/v) Triton X-100, and complete protease inhibitor mixture (Roche Applied Science, Mannheim, Germany), the lysates were incubated overnight at 4°C with 10% NeutraAvidin beads (Pierce). NeutraAvidin-bound biotinylated proteins were centrifuged and washed three times with PBS and then eluted in 2× sodium dodecyl sulfate (SDS) sample buffer. Protein samples were separated by SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred to a nitrocellulose membrane and blotted with appropriate primary and secondary antibodies. Protein bands were detected by enhanced chemiluminescence.

**Measurement of pH₄ and Cl⁻/HCO₃⁻ exchange activity**

The pH₄ in PANC-1 cells, which have minimal intrinsic anion exchange activity, was measured with a pH-sensitive fluorescent probe, BCECF, as described previously. In brief, the pH-sensitive fluorescent probe BCECF was detected in PANC-1 cells transiently transfected WT-pendrin or H723R-pendrin. After JEV treatment for 24 h, PANC-1 cells were incubated with 2 mM BCECF acetoxy-methyl ester for 15 min and then perfused with an HCO₃⁻-buffered solution (120 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 10 mM d-glucose, 5 mM HEPES, and 25 mM NaHCO₃ [pH 7.4]). BCECF fluorescence was recorded at the excitation wavelengths of 490 nm and 440 nm at a resolution of 2/s on a recording setup (Carl-Zeiss AxioObserver with Ex-ET436/ET495 and Em-ET540 filters and optics MOS CCD camera). Cl⁻/HCO₃⁻ exchange activities were estimated from the initial rate of pH increase as a result of Cl⁻ removal in the HCO₃⁻-containing buffer (25 mM HCO₃⁻ with 5% CO₂). To formate/HCO₃⁻ exchange activity, Cl⁻ was replaced with HCO₃⁻ using a 3% CO₂/97% O₂ atmosphere.
formate\textsuperscript{−}. The signal of BCECF in cells perforated with nigericin (5 mM) was calibrated with solutions of various pH levels (pH 6.2, 7.2, and 8.2). The buffer capacity of each cell was measured as described previously.\textsuperscript{14} Data were analyzed with the Metallfluor system (Molecular Devices).

**Immunocytochemistry**

PANCl-1 cells were cultured on coverslips. The cells were permeabilized by incubation in cold ethanol and acetone (1:1) at 20°C for 8 min and blocked by incubation with blocking medium (0.2 mL PBS containing 5% normal donkey serum, 1% BSA, 0.1% gelatin, and 0.001% NaAzide) at 20°C for 1 h to block non-specific binding sites. Subsequently, the cells were stained through incubation with target-specific primary and secondary antibodies (Table S1). Cells were viewed under an LSM780 confocal microscope (Zeiss Laboratories, Jena, Germany).

**Animal Experiments**

All experimental procedures were approved by Yonsei University Health System Institutional Animal Care and Use Committee (2016-0254). All mice were housed under specific pathogen-free conditions at the animal facility of Yonsei University. All mice were raised at a maximum density of 15 mice per cage. All mice were provided with nesting materials. All mice were provided a 12 h lighting cycle (ambient temperature 18°C, humidity 40%–60%, 12/12 h light/dark cycle, lights on at 8:00 a.m.). Breeding mice were supplied with nesting materials. All mice were provided with radicidation feed and reverse osmosis water. The cages were ventilated and managed individually with an automatic watering system. All experiments were performed with mice under general anesthesia with tiletamine and zolazepam (100 mg/kg) and xylazine (23 mg/kg) via intraperitoneal injection.

**Generation of the hH723R-Pendrin Mouse Model**

The mouse pendrin H723R knockin mouse model was generated by Macrogen (Seoul, Korea). The mice were generated using a homologous recombination approach as previously reported.\textsuperscript{24} The targeting scheme is described in Figure S2A. The c.2168A>G mutation in exon 19 was targeted for the generation of H723R-PDS mice. Genotype sequencing was performed with the sense primer 5’-AGAACCTGAGATGGGATTCATG-3’ and antisense primer 5’-AGCACAATGCCTCATGCTCATGAG-3’ (size of amplicons, WT, 842 bp; knockin, 2,126 bp) (Figure S2B).

The human pendrin H723R Tg mouse model was established with a Cre-inducible system, in which the LoxP-STOP-LoxP sequence from pBGI, terminating transcription, was subcloned into the EcoRI site of a pCA GGS vector, and the cDNA fragment encoding human SLC26A4 (WT or c.2139G>A) was subsequently ligated using XhoI and NotI sites. After confirmation of transgene expression in HEK293T cells with CMV-Cre plasmid expression, the linearized transgene construct was microinjected into pronuclear-stage mouse embryos (C57BL/6N) and transferred to pseudopregnant mice. One Tg founder mouse harboring human SLC26A4 (WT or c.2139G>A) was identified by PCR amplification of tail genomic DNA and established through germline transmission. Passage-3 hPDS Tg mice were mated with slc26a4 knockout mice (kindly gifted by Prof. Sung Huhn Kim) to eliminate mouse pendrin. hPDS Tg(+);mPDS\textsuperscript{−/−} mice were then mated with Pax2-Cre(+) mice (strain name, B6-Tg(Pax2-mcre)1Akg/Mmnc [stock, MMRRC no. 010569], kindly gifted by Prof. Jinwoong Bok) to generate mice with Pax2-specific expression of human pendrin without mouse pendrin. The final genotype of mice used in the experiments was hPDS Tg(+)Pax2-Cre(+)mPDS\textsuperscript{−/−}, in which only human pendrin (WT or hH723R) was expressed, but no mouse pendrin was expressed in the inner ear. The genotyping for hPDS Tg, hH723R Tg, Pax2-Cre Tg, and slc26a4 knockout mice was performed with the following specific primers: human pendrin Tg, fwd 5’-AACCATGTTCATGCTTCTTC-3’ and rev 5’-CTAAAGGCAATGTCCTCAGA-3’ (amplicon size: 226 bp); Pax2-Cre, fwd 5’-GCTGCTGATTACGGTGATGCAAC-3’ and rev 5’-GTGCGCATGCGCGCAGAAAC-3’ (amplicon size, 700 bp); slc26a4 KO, fwd 5’-TGCGATTTCATCGCCTCAGA-3’ and rev 5’-GCATGTTGATTCTTTCACCAGTGG-3’ and Rev 5’-GGTGCGGAGAAAGGATAGTGG-3’ (WT, 287 bp; KO, 243 bp).

**Inner Ear Western Blotting**

Mice at approximately 4–5 weeks of age were anesthetized and then perfused to remove all blood from the entire body. The inner ears of the mice of each genotype were dissected, and then the tissues were chopped and mashed in lysis buffer. After centrifugation of the lysates, the supernatant was used for immunoblotting as described previously herein.

**Inner Ear Histology**

Tissues from the inner ears of the mice were fixed in 4% paraformaldehyde at 4°C overnight and then washed twice with PBS. Subsequently, the specimens were decalciﬁed in 25% EDTA/PBS for 24 h. Tissues were dehydrated and embedded in parafﬁn for histology and dissected into segments with the organ of Corti and permeabilized with 0.1% Triton X-100 for whole-mount immunostaining. The parafﬁn blocks were cut into 5-μm sections using a microtome (Leica Biosystems) and subjected to hematoxylin and eosin (H&E) staining and immunostaining. H&E-stained tissues were examined under a light microscope. For immunostaining, parafﬁn sections were deparafﬁnized with xylene, ethanol, and PBS. Thereafter, for antigen retrieval, the sections were incubated in sodium citrate at 95°C for 5 min and then blocked in 10% normal donkey serum for 1 h at room temperature. The tissues were incubated overnight with target-specific primary antibodies at 4°C, washed, and incubated for 1 h at room temperature with the appropriate secondary antibody (Table S1). Finally, the samples were washed and then mounted with mounting solution (Sigma-Aldrich, St. Louis, MO, USA). All images of immunostaining were obtained under a confocal microscope LSM780 (Zeiss Laboratories, Jena, Germany).

**Whole-Mount Immunofluorescence**

Whole-mount immunofluorescence of cochlea was performed as described previously.\textsuperscript{19} In brief, isolated cochleas were obtained
Auditory Function Test
ABR measurements were recorded as described previously. In brief, tests were conducted using an ABR workstation manufactured by Tucker Davis Technology (Alachua, FL, USA). Properly anesthetized mice were placed on a heating pad (37°C) in a soundproofed chamber. After electrode insertion, acoustic stimuli were applied through a receiver probe. The stimuli included 500 repeated click sounds or tone bursts with a 1-ms rise and fall time and a 5-ms plateau at frequencies of 4, 8, 16, and 32 kHz. The sound intensity began at a 90-dB sound pressure level (SPL) with 5-dB decreasing steps to the auditory threshold.

Micro-computed Tomogram
A custom-built, transmission X-ray microCT scanner of medium energy was used (Model 1076, Skyscan, Aartselaar, Belgium) to obtain images under the conditions of 100 kV of acceleration voltage. After mice were fully anesthetized and fixed, the CT scan was performed. Scanning minimized the changes in the position of the specimen during repeated processes. The 2D images were analyzed using the CTAn (Skyscan, Belgium) computer program.

Scanning Electron Microscopy
Scanning electron microscopy was performed as described previously. Decalcified and fixed cochleas were immersed in 2% glutaraldehyde and paraformaldehyde in 0.1 M PBS, pH 7.4 for 6 h. After washing twice in 0.1 M PBS for 30 min each, tissues were post-fixed with 1% OsO4 dissolved in 0.1 M PBS for 1.5 h, and dehydrated in an ascending gradual series (50%–100%) of ethanol. Tissues were then treated with isoamyl acetate and subjected to critical point drying with a Leica EM CPD300 (Vienna, Austria). Then they were coated with Platinum (5 nm) using an ion coater (Leica EM ACE600) and examined and photographed with a scanning electron microscope (FE-SEM; Merin, Zeiss).

Statistical Analysis
The results of multiple experiments are presented as the means ± SEM. Statistical comparisons were performed based on analysis of variance followed by the Bonferroni multiple comparison test, where appropriate; p < 0.05 was considered statistically significant.

SUPPLEMENTAL INFORMATION
Supplemental Information can be found online at https://doi.org/10.1016/j.omtm.2019.11.019.

AUTHOR CONTRIBUTIONS
Study conception and design, J.J., M.G.L.; Acquisition of data, H.J.C., H.J.L., I.H.J.; Analysis and interpretation of data, J.J, H.J.C., B.N., M.G.L.; Drafting of manuscript, J.J., H.J.C., H.-W.L., S.K.E.; Technical support, S.D., H.-W.L., J.Y.C., S.E.K.; Essential consulting and reagents, J.Y.C., S.K.E.

CONFLICTS OF INTEREST
The authors declare no competing interests.

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