Failure of Fluid Absorption in the Endolymphatic Sac Initiates Cochlear Enlargement that Leads to Deafness in Mice Lacking Pendrin Expression

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Abstract

Mutations of SLC26A4 are among the most prevalent causes of hereditary deafness. Deafness in the corresponding mouse model, Slc26a4−/−, results from an abnormally enlarged cochlear lumen. The goal of this study was to determine whether the cochlear enlargement originates with defective cochlear fluid transport or with a malfunction of fluid transport in the connected compartments, which are the vestibular labyrinth and the endolymphatic sac. Embryonic inner ears from Slc26a4−/− and Slc26a4+/− mice were examined by confocal microscopy ex vivo or after 2 days of organ culture. Culture allowed observations of intact, ligated or partially resected inner ears. Cochlear lumen formation was found to begin at the base of the cochlea between embryonic day (E) 13.5 and 14.5. Enlargement was immediately evident in Slc26a4−/− compared to Slc26a4+/− mice. In Slc26a4−/− and Slc26a4+/− mice, separation of the cochlea from the vestibular labyrinth by ligation at E14.5 resulted in a reduced cochlear lumen. Resection of the endolymphatic sacs at E14.5 led to an enlarged cochlear lumen in Slc26a4−/− mice but caused no further enlargement of the already enlarged cochlear lumen in Slc26a4+/− mice. Ligation or resection performed later, at E17.5, did not alter the cochlear lumen. In conclusion, the data suggest that cochlear lumen formation is initiated by fluid secretion in the vestibular labyrinth and temporarily controlled by fluid absorption in the endolymphatic sac. Failure of fluid absorption in the endolymphatic sac due to lack of Slc26a4 expression appears to initiate cochlear enlargement in mice, and possibly humans, lacking functional Slc26a4 expression.

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Introduction

Mutations of SLC26A4 are worldwide among the most prevalent causes of deafness [1,2,3,4]. Particularly in Chinese populations, mutations of SLC26A4 are found in as many as 13.7% of deaf subjects [5]. Phenotypes associated with mutations of SLC26A4 include deafness at birth as well as fluctuating hearing loss that is more common in Slc26a4−/− subjects than in the general population [5]. Phenotypes associated with mutations of SLC26A4 are the most prevalent causes of hereditary deafness. Deafness in the corresponding mouse model, Slc26a4−/−, results from an abnormally enlarged cochlear lumen [9,10]. Scala media, an endolymph-filled luminal space that is lined by the cochlear epithelium, which includes both the sensory hair cells as well as stria vascularis (Fig. 1A). Stria vascularis secretes K+ into endolymph and generates the endocochlear potential that provides the majority of the driving force for the sensory transduction, which occurs in the hair cells. Enlargement of scala media leads to hampened cell-to-cell communication and disruption of development toward hearing [10].

1) The enlargement of scala media forces stria vascularis in the postnatal cochlea to elevate rates of K+ secretion in order to maintain a normal endolymphatic K+ concentration [11]. Oxidative stress ensues from increased transport and leads to a loss of the K+ channel Kcnj10, which generates the endocochlear potential [12,13,14]. Loss of Kcnj10 leads to a loss of the endocochlear potential and thereby of the driving force necessary for hearing.

2) The enlargement of scala media forces stria vascularis in the postnatal cochlea to elevate rates of K+ secretion in order to maintain a normal endolymphatic K+ concentration [11]. Oxidative stress ensues from increased transport and leads to a loss of the K+ channel Kcnj10, which generates the endocochlear potential [12,13,14]. Loss of Kcnj10 leads to a loss of the endocochlear potential and thereby of the driving force necessary for hearing.

3) The enlargement of scala media and the associated elevated rates of K+ secretion lead to an enhanced metabolic acid production by marginal cells of stria vascularis that is partially released into endolymph. Acid release into endolymph in conjunction with a loss of pendrin-mediated HCO3− secretion lead to a luminal acidification by ∼0.3 pH-units [12]. Acid-mediated inhibition of Ca2+ absorption results in a ∼100-fold elevation in the endolymphatic Ca2+ concentration in Slc26a4−/− mice compared to Slc26a4+/− mice [12]. Excessive Ca2+ concentrations in endolymph impair sensory transduction by damaging outer hair cells through excessive Ca2+ influx via the apical transduction channel and subsequent cellular Ca2+ overload [9,15].
From these studies in the mouse model, it is evident that the enlargement of scala media is a key event in the etiology of deafness. Most likely, the enlargement is due to a dysfunction of epithelial fluid transport, since Slc26a4 codes for pendrin, which is an epithelial anion exchanger and since the enlargement occurs shortly after the onset of expression [9]. Conceptually, the enlargement could be the result of a net increase in fluid secretion or decrease in absorption. Pendrin itself does not mediate net solute transport, since it transports anions with a stoichiometry of 1:1 [16]. Whether and how pendrin contributes to fluid secretion or absorption is unknown. This question is complicated by two issues:

First, virtually nothing is known about ion and fluid transport in the embryonic cochlea, which is when the enlargement develops. The adult ion composition, characterized by a high K⁺ concentration and low Na⁺ and Ca²⁺ concentrations, is established during postnatal development [12,17,18]. This means that investigations of ion transport in the postnatal cochlea cannot be extrapolated to the early embryonic stage when the cochlear lumen first develops.

Second, the epithelial enlargement in Slc26a4−/− mice is not limited to the cochlea. The vestibular labyrinth and the endolymphatic sac are prominently enlarged, too [9]. Interestingly, these compartments are connected to the cochlea and each contains pendrin-expressing epithelial cells. The role of pendrin in fluid transport may be different in the different compartments and it is conceivable that one compartment ‘pumps up’ or ‘drains’ the others. Thus, the goal of the present study was to determine the onset of cochlear enlargement and to investigate whether the enlargement of the cochlea in Slc26a4−/− mice is due to a local cochlear dysfunction or whether the cochlear enlargement is caused by dysfunction of the adjacent vestibular labyrinth or endolymphatic sac.

Methods

Animals

A colony of Slc26a4−/− and Slc26a4+/− mice was maintained at Kansas State University. Pairs of Slc26a4−/− dams and Slc26a4+/− sires were housed together. Litter sizes averaged 5.1 pups with a range of 4 to 7. Gestational age was counted from the day, when a vaginal plug was detected. This day was set to embryonic (E) day 0.5 (E0.5). Gestational age, however, was verified, and in rare cases corrected, by evaluating gross morphological features including limbs, digits, and appearance of the pinna and auditory meatus [19,20]. Neonatal mice (P0-P3) were anesthetized by a combination of i.p. injection of 0.013 ml/g body weight of 4% tri-bromo-ethanol and rapid cooling on an ice slush. Older mice (P5-P7) were anesthetized solely by i.p. injection of 0.015 ml/g body weight of 4% tri-bromo-ethanol. Embryos and postnatal mice were sacrificed by decapitation. All procedures involving animals were approved by the Institutional Animal Care and Use Committee of Kansas State University (IACUC #: 2613).

Organ culture

Procedures for organ culture were adapted with modifications from methods developed by Dr. Thomas van de Water [21]. Embryos were harvested at E14.5 and E17.5. Otocysts were isolated in sterile NaCl solution maintained at 4°C. NaCl solution contained (in mM) 150 NaCl, 5 HEPES, 3.6 KCl, 1 CaCl₂, 1 MgCl₂, and 5 glucose, pH 7.4. The cranium was opened medially and otocysts were isolated after removal of the cerebrum. Otocysts were left intact or altered either by ligation of the vestibular labyrinth or by resection of the endolymphatic sac. One otocyst from each embryo was left intact and the other was altered to facilitate paired comparisons. In one set of experiments, the cochlea was isolated from the vestibular labyrinth by ligation. Otocysts were isolated and tied at the base of the cochlea with a braided silk surgical suture (size 10-0, Deknatel, Queens Village, NY). After tying off the cochlea, the vestibular part of the otocyst,
including the endolymphatic sac, was cut away from the isolated cochlear preparation. In another set of experiments, the endolymphatic sac was removed by resection. The endolymphatic sac was carefully peeled off the otocyst. Great care was taken in this set of experiments to not injure the endolymphatic sac, particularly the distal end, which is located in the posterior cranial fossa within the dura.

Otocysts, altered or intact, were maintained in organ culture for two days. Otocysts were placed into 12-well plates (Cat# 353503, Fisher, St. Louis, MO) submersed in DMEM/F-12 media (Cat# 12500-062, Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Cat# 30-2020, ATCC, Waldorf, MD) and 1% penicillin-streptomycin (Cat# 15140-122, Invitrogen) and incubated at 37°C in a humidified atmosphere enriched with 5% CO₂ (HeraCell 240, Heraeus, Germany). Media was changed every 24 hrs.

Confocal immunocytochemistry
Freshly isolated or cultured otocysts were fixed at 4°C for 2 hrs in a solution containing (in mM) 150 NaCl, 3.6 KCl, 5 HEPES, 1 CaCl₂, 1 MgCl₂, and 5 glucose, pH 7.4 and 4% paraformaldehyde. Fixed otocysts were processed through a sucrose gradient (10% and 20%, each 20 min, followed by 30% overnight, all at 4°C), infiltrated with polyethylene glycol (Cat# 72592-B, Electron Microscopy Sciences, Hatfield, PA) and cryo-sectioned (12 μm, CM3050S, Leica, Germany). Serial sections throughout the entire cochlea were obtained from embryos between ages E11.5 and E14.5 and mid-modiolar sections of the cochlea were obtained from embryos between ages E15.5 and E18.5 and from neonates aged P3.

Sections mounted on charged slides (Cat# 22-230-900, Fisher) were blocked for 1 hr with 5% bovine serum albumin (BSA) in PBS-TX containing (in mM) 137 NaCl, 2.7 KCl, 10.1 Na₂HPO₄, 1.8 KH₂PO₄, pH 7.4, and 0.2% Triton-X-100. For morphological observations, sections were stained for actin filaments (Alexa488 conjugated phalloidin, Invitrogen) and for nuclei (DAPI, Invitrogen). Slides were incubated for 1 hr at room temperature with phalloidin and DAPI diluted with PBS-TX at 1:40 and 1:1,000, respectively. For immunocytochemistry, slides were incubated overnight at 4°C with primary antibody (rabbit anti-Na⁺/K⁺ ATPase 1 alpha subunit, Novus Biologicals, Littleton, CO) diluted 1:200 with PBS-TX containing 1–3% BSA. Slides were washed three times in PBS-TX and incubated for 1 hr at room temperature with secondary antibody (Alexa594 conjugated goat-anti-rabbit (Invitrogen) diluted 1:1,000 with PBS-TX containing 1–3% BSA. Stains for actin filaments (Alexa488 conjugat-

Figure 2. Cochlear development from E12.5 to P3 in Slc26a4⁻/⁻ mice. Na⁺/K⁺ ATPase (red) was visualized by immunocytochemistry. F-actin (green) and nuclei (blue) were labeled. A-F: Six different stages of development ranging from E12.5 to P3 are shown. Abbreviations: SV, stria vascularis; OS, outer sulcus; K, Köllicker’s organ; RM, Reissner’s membrane; Ca, otic capsule; SL, spiral ligament. The thickness of the otic capsule is marked by dashed lines.

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ed phalloidin (Invitrogen) and for nuclei (DAPI) were added to the secondary antibody at dilutions 1:40 and 1:1,000, respectively. After staining, slides were washed three times with PBS-TX, cover-slipped with FluorSave (Calbiochem, La Jolla, CA) and observed by confocal laser scanning microscopy (LSM 510 Meta, Carl Zeiss, Göttingen, Germany). The size of the cochlear lumen was evaluated by digital area measurements using image analysis software (Carl Zeiss).

Statistics

Data are generally presented as average ± sem with n being the number of animals. Data acquired in paired experiments using littermates were evaluated by paired t-test. Significance was assumed when p<0.05.

Results

Cochlear lumen formation

In a first set of experiments, we determined the onset of lumen formation in the cochlea. Inner ears ranging from embryonic (E) day E12.5 to postnatal (P) day P3 were sectioned and examined by confocal microscopy. At E12.5 and E13.5, the cochlear duct consisted of a closed epithelial tube (Fig. 2). Kolliker’s organ, the precursor of the organ of Corti, was evident on the basal side of...
Cochlear enlargement occurs prior to cochlear Slc26a4 expression

In a second set of experiments, we compared the onset of cochlear lumen formation in Slc26a4+/+ and Slc26a4−/− mice and monitored the progression of the enlargement between E14.5 and ~P4.5. Lumen formation at E14.5 was advanced in Slc26a4−/− mice compared to Slc26a4+/− littersmates (Fig. 4). The cochlear lumen increased with development in Slc26a4+/+ and Slc26a4−/− mice (Fig. 5). The ratio between the luminal size in Slc26a4+/+ and Slc26a4−/− increase from factor 6 at E16.5 to factor 12 at E18.5.

Fluid secretion in the vestibular labyrinth ‘pumps up’ the cochlea during lumen formation

In a third series of experiments, we determined whether the vestibular labyrinth contributes to cochlear lumen formation. Embryonic inner ears were harvested at E14.5, which is at the onset of lumen formation, and later, at E17.5. The endolymphatic sac was removed and the cochlea was separated from the vestibular labyrinth by ligation (Fig. 6). To ensure complete isolation of the cochlea from the vestibular labyrinth, the latter was removed micro-surgically after ligation. Ligated and non-ligated inner ears were maintained in organ culture for two days, then dissected and examined by confocal microscopy. Scala media of inner ears harvested at E14.5 from Slc26a4−/− mice was found to be significantly smaller in ligated compared to non-ligated preparations (Fig. 7). Similar observations were made in inner ears harvested at E14.5 from Slc26a4+/− mice. In contrast, no difference in the size of scala media was found between ligated and non-ligated inner ears harvested at E17.5. These observations suggest that the onset of cochlear lumen formation depends in Slc26a4+/− and Slc26a4−/− mice on fluid secretion by the vestibular labyrinth. This dependency is transient and was found to be lost at E17.5.

Ligation of the vestibular labyrinth can be expected to isolate the cochlea from the vestibular labyrinth and the endolymphatic sac. The endolymphatic sac was removed in this series of experiments to avoid variations in the preparations due to injury of this extremely fragile structure. The question, whether the endolymphatic sac affects the cochlear lumen was addressed in the next series of experiments.

Fluid absorption in the endolymphatic sac ‘drains’ the cochlea during lumen formation

In a fourth series of experiments, we determined whether the endolymphatic sac contributes to cochlear lumen formation. Embryonic inner ears were again harvested at E14.5 and E17.5. Great care was taken to not injure the endolymphatic sac during dissection. The endolymphatic sac was then removed by manual resection (Fig. 8). Resected and non-resected inner ears were maintained in organ culture for two days, then sectioned and examined by confocal microscopy. The cochlear lumen of inner ears harvested at E14.5 from Slc26a4−/− mice was found to be

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significantly larger in resected compared to non-resected inner ears (Fig. 9). In contrast, the cochlear lumen of inner ears harvested at E14.5 from \(\text{Slc26a4}^{+/+}\) mice was enlarged and no difference was apparent between resected and non-resected inner ears. Further, no difference in the cochlear lumina were found between resected and non-resected inner ears harvested at E17.5.

These observations suggest that the onset of cochlear lumen formation is controlled in \(\text{Slc26a4}^{+/+}\) mice by fluid absorption in the endolymphatic sac. This control is transient and found to be lost at E17.5. The observed enlargement in \(\text{Slc26a4}^{-/-}\) suggests that loss of \text{Slc26a4} expression impairs fluid absorption in the endolymphatic sac.

**Discussion**

The most salient findings of this study are 1) that lumen formation begins between E13.5 and E14.5 at the base of the cochlea, 2) that the cochlear lumen is transiently, prior to E17.5, controlled by fluid secretion in the vestibular labyrinth and absorption in the endolymphatic sac and 3) that loss of pendrin expression leads to an enlargement of the cochlear lumen that is similar to the enlargement caused by resection of the endolymphatic sac.

The inner ear develops from an invagination of the ectoderm that forms a vesicular otocyst [22]. At ~E10.5, two protrusions begin to extend from the otocyst: one forms the cochlea and the other forms the endolymphatic sac. While the protrusions elongate and coil, in case of the cochlea, the center of the otocyst reorganizes into the vestibular labyrinth. Pendrin is an anion exchanger that is prominently expressed in the cochlea, the vestibular labyrinth and the endolymphatic sac. In the postnatal cochlea, pendrin is expressed in a spiraling sheet of outer sulcus and spindle cells located in the lateral wall [14]. In the vestibular labyrinth, pendrin is expressed in sheets of transitional cells that surround sensory cell patches, and in the endolymphatic sac, pendrin is expressed in mitochondrial-rich cells that are
interspersed among the principal ribosomal-rich cells [11,14,23]. The onset of pendrin expression differs among the different compartments of the inner ear. In the endolymphatic sac the onset is at E13 and in the vestibular labyrinth and the cochlea at E15 [24]. A dysfunction of fluid transport due to a lack of pendrin expression can be expected to occur after but is not likely to occur before the onset of expression. The enlargement of the cochlear lumen at E14.5 is therefore unlikely due to local cochlear fluid transport. The relationship been the onset of expression and the onset of cochlear enlargement suggests an involvement of the endolymphatic sac. A reduction in fluid absorption in the endolymphatic sac generates a lumen-positive transepithelial potential that is sensitive to inhibition of Na\(^+\)/H\(^+\) exchangers, Na\(^+\)/H\(^+\) exchanger pendrin would inhibit H\(^+\) production by carbonic anhydrase through product-inhibition and a reduce the current that drives Na\(^+\) reabsorption.

The observation that resection of the endolymphatic sac caused an enlargement at E14.5, but not at E17.5, is intriguing. The loss of control of cochlea fluid homeostasis by the endolymphatic sac may be the result of the maturation of local cochlear fluid homeostasis. Further, the capacity for Na\(^+\) reabsorption in the endolymphatic sac may be higher at E14.5 than at E17. Support for this hypothesis comes from the finding that numbers of mRNA copies coding for the three subunits of the Na\(^+\) channel ENaC decline from P2 to P6 of early postnatal development, which is ~4 days after E17 [29]. It is conceivable that this decline can be extrapolated to the embryonic phase of development.

Lumen formation was observed in the cochlea to progress from base to apex. A base-to-apex gradient in developmental maturation is consistent with the cochlea developing from an elongating protrusion of the otocyst, which implies that cells near the base are developmentally more mature than cells at the apex. Cochlear lumen formation could thus be due to a base-to-apex progressing onset of local fluid transport. Alternatively, lumen formation could be aided by the vestibular labyrinth, which matures earlier than the cochlea. For example, the onset of mechanotransduction in vestibular hair cells is at E17, which is ~4 days earlier than in the cochlea [38,39]. The observation that ligation of the vestibular labyrinth caused a reduction of the cochlear lumen at E14.5 but not at E17.5 suggests that cochlear lumen formation is transiently aided by fluid secretion in the vestibular labyrinth. Fluid secretion in the embryonic cochlea or vestibular labyrinth is poorly understood. Mechanism of ion transport, that are well established in the postnatal cochlea, cannot be extrapolated to the embryonic stage since the adult ion composition of the luminal fluid, which is characterized by a high K\(^+\) concentration and low Na\(^+\) and Ca\(^{2+}\) concentrations, is established only during postnatal development [12,17,18]. Nevertheless, the observation that ligation of the vestibular labyrinth led to a reduction in the cochlear lumen in Slc26a4\(^{-/-}\) mice suggests that pendrin does not play a major role in fluid secretion in the embryonic vestibular labyrinth.

Studies in Slc26a4\(^{-/-}\) mice have provided a basis to understand the spectrum of phenotypes seen in human patients that range from deafness at birth to deafness during childhood. Mice lacking pendrin have been shown to develop a ~10-fold enlargement of the cochlear lumen. This enlargement forms during embryonic development and persists throughout postnatal life [9,10,14].

During the developmental phase, the enlargement may disrupt cell-to-cell signaling that is necessary for the development of hearing. Gap junction mediated signaling between epithelial cells, for example, may be compromised by increased diffusion distances that are inherent to stretched epithelial cells enclosing an enlarged lumen [Fig. 1B]. The importance of gap junction mediated cell signaling is underlined by the fact that loss-of-function mutations of the gap junction protein connexin 26 cause deafness at birth. Coincidentally, mutations of connexin 26 are the most frequent cause for non-syndromic hearing loss in children [40,41]. Further, the enlargement may disrupt signaling between mesenchymal and epithelial cells as shown in the early postnatal cochlea where mesenchymal cells generate thyroid hormone by
conversion of the prohormone to the hormone and epithelial cells bear the thyroid hormone receptors that control proper maturation of the cochlea [42,43,44,45]. The enlargement of the cochlea lumen in Slc26a4+/− mice displaces mesenchymal cells and thereby disrupts cell signaling, which leads to local cochlear hypothyroidism during the early postnatal phase of development [10]. Taken together, cell-to-cell signaling is critically important during development of the cochlea. The enlargement appears to disrupt critical cell-to-cell signaling mechanisms at multiple stages, thereby disrupt proper development and account for deafness at birth in mice and possibly human patients.

Many human patients bearing loss-of-function mutations of SLC26A4 are born hearing, which suggests that cochlear development in humans may to some extent escape the detrimental hampered cell-to-cell signaling during early development. The majority of patients bearing mutations of SLC26A4 lose hearing during early childhood - often after a period of fluctuating hearing loss that progresses toward deafness [8,46]. Based on studies in the Slc26a4−/− mouse model, fluctuating hearing loss may be due to oscillations of a negative feedback system that oscillates and generates fluctuations in the endocochlear potential and thereby in the driving force on which hearing depends. This feedback loop is comprised of reactive oxygen species (ROS) generated by marginal cells of stria vascularis as a byproduct of elevated rates of K⁺ secretion and the ROS-sensitive K⁺ channel KCNJ10 that generates the endocochlear potential and supplies K⁺ to the marginal cells [12,13]. The ROS-induced loss of KCNJ10 abolishes the endocochlear potential and hearing and the associated reduction in K⁺ flux to marginal cells would limit K⁺ secretion, reduce ROS production, allow restoration of KCNJ10 expression, restoration of the endocochlear potential and restoration of hearing. This feedback loop may oscillate and thereby generate fluctuating hearing loss. Irreversible deafness would ensue, when endolymphatic Ca²⁺ concentrations rise and hair cells succumb to Ca²⁺ overload [9,12].
Figure 10. Diagram of ion transport in the endolymphatic sac. The endolymphatic sac epithelium consists mainly of ribosomal-rich cells that are interspersed by mitochondrial-rich cells. Mitochondrial-rich cells express H^+ATPase and the Cl^-/HCO_3^- exchanger pendrin in their apical membrane. Ribosomal-rich cells express Na^+ channels including ENaC. A current generated by the H^+ATPase drives Na^+ reabsorption via Na^+ channels. The role of the Cl^-/HCO_3^- exchanger pendrin is to export HCO_3^- that is generated by carbonic anhydrase (CA) in the reaction that leads to the generation of H^+.

In summary, the present study demonstrates that a lack of functional Slc26a4 expression leads to a failure of fluid absorption in the endolympathic sac that initiate cochlear enlargement in mice, and possibly humans. It is conceivable that deafness in mice, and possible humans, is a direct consequence of the cochlear enlargement and inherent disruption of cell signaling and cochlear ion homeostasis.

Author Contributions
Conceived and designed the experiments: HMK PW. Performed the experiments: HMK. Analyzed the data: HMK PW. Contributed reagents/materials/analysis tools: PW. Wrote the paper: PW.

References
1. Fraser GR (1965) Association of congenital deafness with goitre (Pendred’s syndrome) a study of 207 families. Ann Hum Genet 29: 201–249.
2. Reardon W, Coffey R, Phelps PD, Luxon LM, Stephens D, et al. (1997) Pendred syndrome–100 years of underascertainment? QJM 90: 443–447.
3. Park HJ, Shaukat S, Liu NZ, Han S, Naz S, et al. (2003) Origins and frequencies of SLC26A4 (PDS) mutations in east and south Asian: global implications for the epidemiology of deafness. J Med Genet 40: 242–248.
4. Albert S, Blons H, Jonard L, Feldmann D, Chauvin P, et al. (2006) SLC26A4 gene is frequently involved in nonsyndromic hearing impairment with enlarged vestibular aqueduct in Caucasian populations. Eur J Hum Genet 14: 773–779.
5. Yuan Y, You Y, Huang D, Cui J, Wang Y, et al. (2009) Comprehensive molecular etiology analysis of nonsyndromic hearing impairment from typical areas in China. J Transl Med 7: 79–(epub).
6. Luxon LM, Cohen M, Coffey RA, Phelps PD, Britton KE, et al. (2003) Neuro-otological findings in Pendred syndrome. Int J Audiol 42: 82–88.
7. Colvin IB, Beale T, Harrop-Griffiths K (2006) Long-term follow-up of hearing loss in children and young adults with enlarged vestibular aqueducts: relationship to radiologic findings and Pendred syndrome diagnosis. Laryngoscope 116: 2027–2036.
8. Choi BY, Stewart AK, Madro AC, Pyor SP, Lenhard S, et al. (2009) Hypofunctional SLC26A4 variants associated with nonsyndromic hearing loss and enlargement of the vestibular aqueduct: Genotype-phenotype correlation or coincidental polymorphisms? Hum Mutat 30: 599–608.
9. Everett LA, Belyantseva IA, Noben-Trauth K, Santos R, Chen A, et al. (2001) Targeted disruption of mouse Pds provides insight about the inner-ear defects encountered in Pendred syndrome. Hum Mol Genet 10: 153–161.
10. Wangemann P, Kim HM, Billings S, Nakaya K, Li X, et al. (2009) Developmental delays consistent with cochlear hypothyroidism contribute to failure to develop hearing in mice lacking Slc26a4/pendrin expression. Am J Physiol Renal Physiol 297(5): F1435–47.
11. Royaux IE, Belyantseva IA, Wu T, Kachar B, Everett LA, et al. (2003) Localization and functional studies of pendrin in the mouse inner ear provide insight about the etiology of deafness in pendred syndrome. J Assoc Res Otolaryngol 4: 394–404.
12. Wangemann P, Nakaya K, Wu T, Maganti R, Itza EM, et al. (2007) Loss of cochlear HCO_3^- secretion causes deafness via endolymphatic acidification and inhibition of Ca^{2+} reabsorption in a Pendred syndrome mouse model. Am J Physiol Renal Physiol 292: 1345–1353.
13. Singh R, Wangemann P (2008) Free radical stress mediated loss of Kcnj10 protein expression in stria vascularis contributes to deafness in Pendred syndrome mouse model. Am J Physiol Renal Physiol 294: F139–F148.
14. Wangemann P, Itza EM, Albrecht B, Wu T, Jabbah SV, et al. (2004) Loss of KCNJ10 protein expression abolishes endocochlear potential and causes deafness in Pendred syndrome mouse model. BMC Medicine 2: 30.
15. Wangemann P (2006) Supporting sensory transduction: cochlear fluid homeostasis and the endocochlear potential. J Physiol 576: 11–21.
16. Shcheynikov N, Yang D, Wang Y, Zeng W, Karniski LP, et al. (2008) The Slc26a4 transporter functions as an electroneutral Cl^-/HCO_3^- exchanger: role of Slc26a4 and Slc26a6 in I^- and HCO_3^- secretion and in regulation of CFTR in the parotid duct. J Physiol 586: 3813–3824.
17. Nakaya K, Harbridge DG, Wangemann P, Schultz BD, Green K, et al. (2007) Lack of pendrin HCO_3^- transport elevates vestibular endolymphatic [Ca^{2+}] by inhibition of acid-sensitive TRPV5 and TRPV6. Am J Physiol Renal Physiol 292: 1314–1321.
18. Yamasaki M, Komune S, Shimozono M, Matsuda K, Haruta A (2000) Development of monovalent ions in the endolymph in mouse cochlea. ORL J Otorhinolaryngol Relat Spec 62: 241–246.
19. Thélier K (1972) The house mouse. Springer Verlag.
20. Rugh R (1968) The mouse: Its reproduction and development. Burgess Publishing Company.
21. Van De Water TR, Rubin RJ (1973) Quantification of the "in vitro" development of the mouse embryo inner ear. Ann Otol Rhinol Laryngol 82: Suppl-21.
22. Mansour SL, Schoenwolf GC (2005) Morphogenesis of the inner ear. In: Kelley MW, Wu D, Popper AN, Fay RR, eds. Springer Handbook of Auditory Research: Development of the inner ear Springer, pp 43–84.
23. Dou H, Xu J, Wang Z, Smith AN, Soleimani M, et al. (2004) Co-expression of pendrin, vacuolar H^+-ATPase alpha4-subunit and carbonic anhydrase II in epithelial cells of the murine endolymphatic sac. J Histochem Cytochem 52: 1377–1384.
24. Everett LA, Mordoh H, Wu DK, Green ED (1999) Expression pattern of the mouse ortholog of the Pendred’s syndrome gene (Pds) suggests a key role for pendrin in the inner ear. Proc Natl Acad Sci USA 96: 9727–9732.
25. Mori N, Ninoyu O, Morgenstern C (1987) Cation transport in the ampulla of the semicircular canal and in the endolymphatic sac. Arch Otorhinolaryngol 244: 61–55.
26. Amano H, Orsulakova A, Morgenstern C (1983) Intracellular and extracellular ion current of the endolymphatic sac. Arch Otorhinolaryngol 237: 273–277.
27. Kimura RS (1967) Experimental blockage of the endolymphatic duct and sac and its effect on the inner ear of the guinea pig. A study on endolymphatic hydrops. Ann Otol Rhinol Laryngol 76: 664–687.
28. Lundquist PG, Kimura R, Wersäll J (1964) Ultrastructural organization of the epithelial lining in the endolymphatic duct and sac in the guinea pig. Acta Otolaryngol 57: 65–80.
29. Grander S, Muller A, Ruppersberg JP (2003) Developmental and cellular expression pattern of epithelial sodium channel alpha, beta and gamma subunits in the inner ear of the rat. Eur J Neurosci 13: 641–648.
30. Mori N, Wu D (1996) Low-amiloride-affinity $\text{Na}^+\text{Cl}^-$ channel in the epithelial cells isolated from the endolymphatic sac of guinea-pigs. Pflugers Arch 433: 58–64.
31. Stankovic KM, Brown D, Alper SL, Adams JC (1997) Localization of $\text{pH}$ regulating proteins $\text{H}^+\text{ATPase}$ and $\text{Cl}^-\text{HCO}_3^-$ exchanger in the guinea pig inner ear. Hear Res 114: 21–34.
32. Karet FE, Finberg KE, Nelson RD, Nayir A, Mocan H, et al. (1999) Mutations in the gene encoding B1 subunit of $\text{H}^+\text{ATPase}$ cause renal tubular acidosis with sensorineural deafness. Nat Genet 21: 84–90.
33. ten Cate WJ, Curtis LM, Rarey KE (1994) Na,K-ATPase subunit isoform expression in the guinea pig endolymphatic sac. ORL J Otorhinolaryngol Relat Spec 56: 257–262.
34. Son Ej, Moon IS, Kim SH, Kim SJ, Choi JY (2009) Interferon-gamma suppresses Na$^+$/H$^+$ exchanger in cultured human endolymphatic sac epithelial cells. J Cell Biochem 107: 965–972.
35. Wu D, Mori N (1998) Evidence for the presence of a Na$^+$/H$^+$ exchanger in the endolymphatic sac epithelium of guinea-pigs. Pflugers Arch 436: 162–168.
36. Couloigner V, Loiseau A, Sterkens O, Amiel C, Ferrary E (1998) Effect of locally applied drugs on the endolymphatic sac potential. Laryngoscope 108: 592–598.
37. Harvey BJ (1992) Energization of sodium absorption by the $\text{H}^+\text{ATPase}$ pump in mitochondria-rich cells of frog skin. J Exp Biol 172: 259–309.
38. Geleoc GS, Holt JR (2003) Developmental acquisition of sensory transduction in hair cells of the mouse inner ear. Nat Neurosci 6: 1019–1020.
39. Lelli A, Asai Y, Forge A, Holt JR, Geleoc GS (2009) Tonotopic gradient in the developmental acquisition of sensory transduction in outer hair cells of the mouse cochlea. J Neurophysiol 103: 2961–2973.
40. Kobell DP, Dunlop J, Stevens HP, Lench NJ, Liang JN, et al. (1997) Connexin 26 mutations in hereditary non-syndromic sensorineural deafness. Nature 387: 80–83.
41. Del Castillo I, Moreno-Pelayo MA, Del Castillo IJ, Brownstein Z, Marlin S, et al. (2003) Prevalence and evolutionary origins of the del(GJB6-D13S1830) mutation in the DFNB1 locus in hearing-impaired subjects: a multicenter study. Am J Hum Genet 73: 1452–1458.
42. Rueda J, Prieto JJ, Cantos R, Sala ML, Merchán JA (2003) Hypothyroidism prevents developmental neuronal loss during auditory organ development. Neurosci Res 45: 401–408.
43. Deol MS (1973) An experimental approach to the understanding and treatment of hereditary syndromes with congenital deafness and hypothyroidism. J Med Genet 10: 233–242.
44. Campos-Barros A, Amma LL, Faris JS, Shailam R, Kelley MW, et al. (2000) Type 2 iodothyronine deiodinase expression in the cochlea before the onset of hearing. Proc Natl Acad Sci USA 97: 1297–1292.
45. Ng L, Hernandez A, He W, Ren T, Srinivas M, et al. (2008) A protective role for type 3 deiodinase, a thyroid hormone-inactivating enzyme, in cochlear development and auditory function. Endocrinology 150: 1522–1560.
46. Suzuki H, Oshima A, Tsukamoto K, Abe S, Kumakawa K, et al. (2007) Clinical characteristics and genotype-phenotype correlation of hearing loss patients with SLC26A4 mutations. Acta Otolaryngol 127: 1292–1297.