MEASUREMENTS OF ENDOLYMPHATIC K^+ CONCENTRATIONS IN THE UTRICLE OF PRE- AND POSTNATAL *SLC26A4*$^{\Delta/+}$ AND *SLC26A4*$^{\Delta/\Delta}$ MICE

by

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Abstract

SLC26A4 and its murine ortholog *Slc26a4* code for pendrin, an anion-exchanger that is expressed in the inner ear. Patients with mutations in *SLC26A4* have syndromic or non-syndromic hearing loss that is associated with a prenatal enlargement of the membranous labyrinth. The mouse model *Slc26a4*$^{\Delta/\Delta}$ recapitulates the enlargement, develops an enlargement of the inner ear and fails to acquire hearing. The vestibular labyrinth secretes fluid, accounting for enlargement of the membranous labyrinth. The objective of the current study was to measure K^+ concentrations in the utricular endolymph of *Slc26a4*$^{\Delta/+}$ and *Slc26a4*$^{\Delta/\Delta}$ mice as a first step toward a mechanistic understanding of fluid secretion during perinatal development. Double-barreled K^+-selective electrodes were used to measure K^+ concentrations of the utricular endolymph *in vitro*. Potassium concentrations were ~10 mM in both genotypes at embryonic (E) day 16.5. The K^+ concentrations started to rise at E17.5 in *Slc26a4*$^{\Delta/+}$ mice. There was a 1-day delay in *Slc26a4*$^{\Delta/\Delta}$ mice. This delay may be the consequence of a larger fluid volume. K^+ concentrations rose to 150 mM and 132 mM in *Slc26a4*$^{\Delta/+}$ and *Slc26a4*$^{\Delta/\Delta}$ adult mice, respectively. Consistently, expression of KCNQ1 and the $Na^+/2Cl^-/K^+$ cotransporter SLC12A2 was found in the utricle at E19.5 in *Slc26a4*$^{\Delta/+}$ and *Slc26a4*$^{\Delta/\Delta}$ mice. In conclusion, the data suggest that K^+ secretion is not the major driving force of fluid secretion in the utricle of the developing mouse inner ear.

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Acknowledgements

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Dedication

To my parents, for their unconditional love all the time.

Chapter 1 - Introduction

Mammalian ears consist of the outer ear, the middle ear and the inner ear (Figure 1-1). The inner ear consists of the pinna, the external auditory canal and the tympanic membrane. The pinna is made of cartilage and skin. It collects and directs sound waves through and external auditory canal to the tympanic membrane and causes it to vibrate. The middle ear and the inner ear are located inside the temporal bones of the skull. The middle ear is connected to the back of the nose and throat through the Eustachian tube. The middle ear is an air-filled cavity consisting of three ossicles known as the malleus, incus and stapes, which transmit sound. Sound waves reach the tympanic membrane and induce vibration, in turn causing the movement of the three ossicles. The stapes transform sound energy into mechanical energy. The inner ear consists of two epithelial compartments, the cochlea and the vestibular labyrinth. The cochlea, which looks like a snail, is involved with hearing. The vestibular labyrinth, consisting of the saccule, utricle and semicircular canals, maintains a sense of position and balance. Both the cochlea and the vestibular labyrinth are surrounded with perilymph and filled with endolymph. The vibrations transmitted from the middle ear induce waves in the cochlear endolymph, which are interpreted as sound. Hair cells with nerve endings in the cochlea respond to sounds of different frequencies convert them into impulses and transmit them to the brainstem *via* auditory nerves.

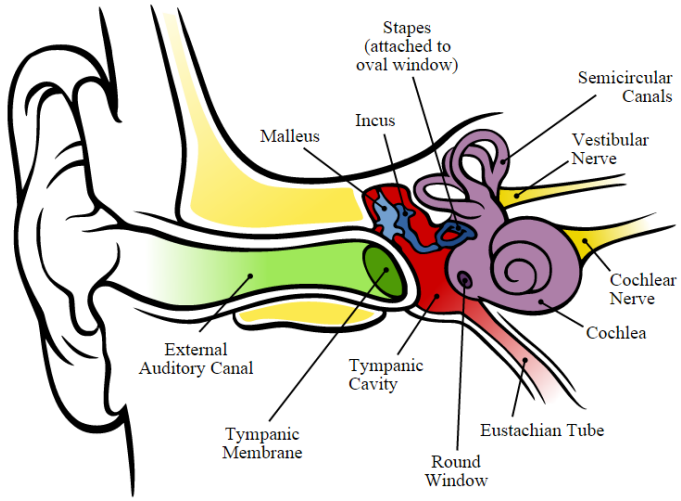

Figure 1-1: Structure of the ear.
Overview of the ear, with the outer ear, middle ear and the inner ear. Figure is reproduced from Chittka et al. 2005 using the creative commons license [1].

The development of the murine inner ear starts at E9.5 with the formation of an otic vesicle (Figure 1-2). At E10.5, from the otocyst arise two protrusions: one forms the endolymphatic sac and the other coils to form the cochlea, leaving the center of the otocyst to reorganize into the vestibular labyrinth. The lumen of the cochlea opens at E14.5. At this time, cochlear enlargement begins in *Slc26a4*$^{\Delta/\Delta}$ mice [2, 3]. Normal luminal formation is regulated by balanced fluid secretion and absorption, which likely occur in the vestibular labyrinth and the endolymphatic sac, respectively [4].

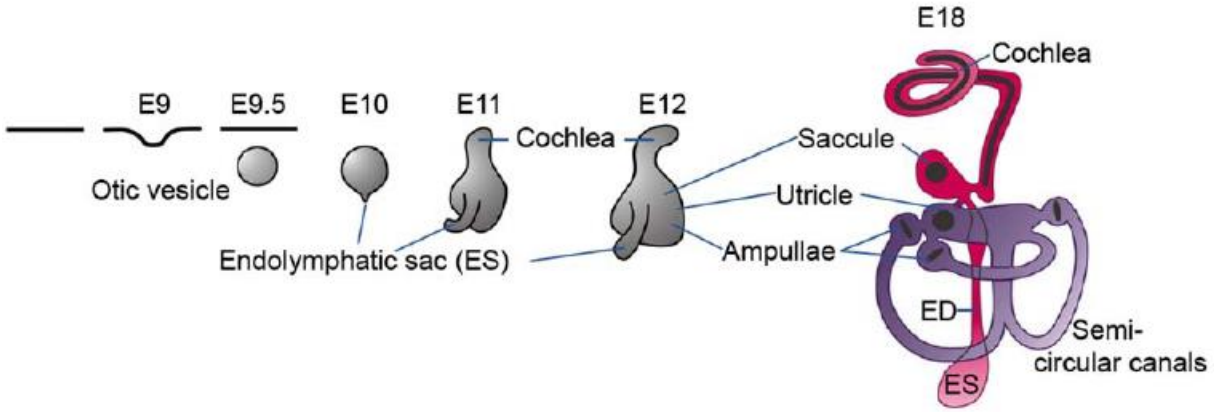

Figure 1-2: Embryonic murine inner ear development.
ES: endolymphatic sac; ED: endolymphatic duct. The inner ear develops from the auditory placode, which invaginates and forms the otic vesicle. Figure is reproduced with permission from the author and the copyright holder, Karger, from Wangemann 2011 [2].

Enlargement of the vestibular aqueduct (EVA) is an inner ear malformation that is associated with syndromic and non-syndromic hearing loss in children [5]. *SLC26A4* encodes a multipass transmembrane protein pendrin, which is a Cl^-/HCO_3^- exchanger expressed in the apical membrane of epithelial cells in the inner ear, kidney and thyroid [6-15]. Mutations in *SLC26A4* that compromise or reduce pendrin expression or function are among the most common causes for sensorineural hearing loss associated with EVA. Pendred syndrome, an autosomal recessive disease caused by *SLC26A4* mutations, includes thyroid goiter and sensorineural hearing loss, which is also present in many cases of nonsyndromic EVA [16]. Mutations of *SLC26A4* have been observed in as many as 13.7% of deaf cases in some populations [17].

The human gene *SLC26A4* and its murine ortholog *Slc26a4* have substantial structural similarity and analogue mutations result in similar phenotypes [18]. The mouse models *Slc26a4*$^{\Delta/+}$ and *Slc26a4*$^{\Delta/\Delta}$ were developed to analyze the role of pendrin in the physiology of hearing and balance. In *Slc26a4*$^{\Delta/\Delta}$ mice, exon 8 is replaced with a neomycin cassette, which results in a frameshift. No functional pendrin is produced. *Slc26a4*$^{\Delta/\Delta}$ mice develop an enlarged membranous labyrinth (Figure 1-3) in the inner ear and fail to acquire normal hearing and balance [16, 19].

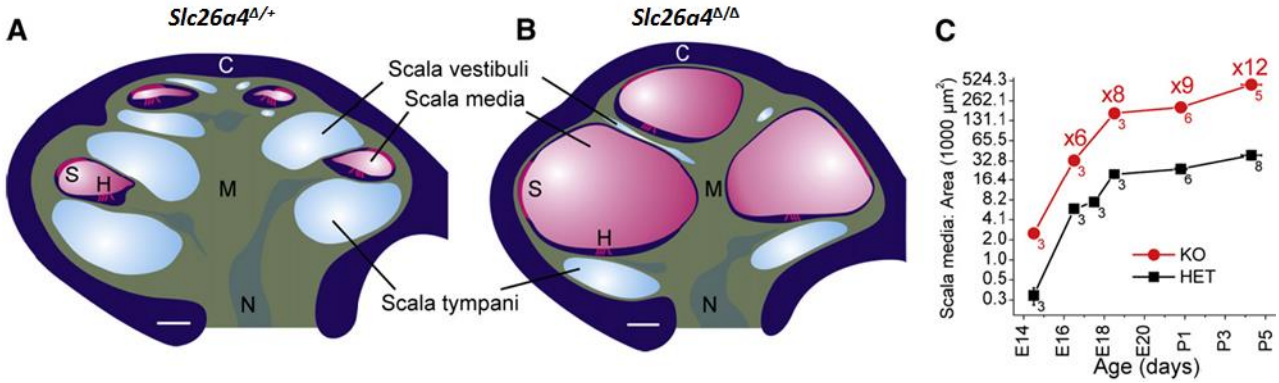

Figure 1-3: Cochlear enlargement in *Slc26a4*$^{\Delta/\Delta}$ mice.
C: otic capsule; S: stria vascularis; H: hair cells; M: modiolus; N: cochlear nerve; KO: *Slc26a4*$^{\Delta/\Delta}$; HET: *Slc26a4*$^{\Delta/+}$. (A) Cross-section of E18.5 *Slc26a4*$^{\Delta/+}$ otocyst. (B) Cross-section of E18.5 *Slc26a4*$^{\Delta/\Delta}$ otocyst. (C) Measurements of the scala media area from the basal turn of *Slc26a4*$^{\Delta/+}$ and *Slc26a4*$^{\Delta/\Delta}$ otocysts. Figure is reproduced with permission from the author and the copyright holder, Elsevier, from Griffith & Wangemann 2011 [5].

In the inner ear, pendrin is expressed in the epithelial cells of outer sulcus, spiral prominence and spindle cells of stria vascularis in the cochlea, transitional cells in the ampullae, utricle and saccule and mitochondria-rich cells in the endolymphatic sac [2] (Figure 1-4). Pendrin first expresses in the endolymphatic sac in the murine inner ear at E11.5, followed by the cochlea (E14.5) and the vestibular labyrinth (E16.5), which includes the utricle, saccule and semicircular canals. There is a dramatic surge in pendrin expression in the endolymphatic sac between E13.5 and E14.5. The expression of pendrin in the cochlea starts from the hook region at E14.5 and expands to include the basal and upper turn of the cochlea by E17.5 [20].

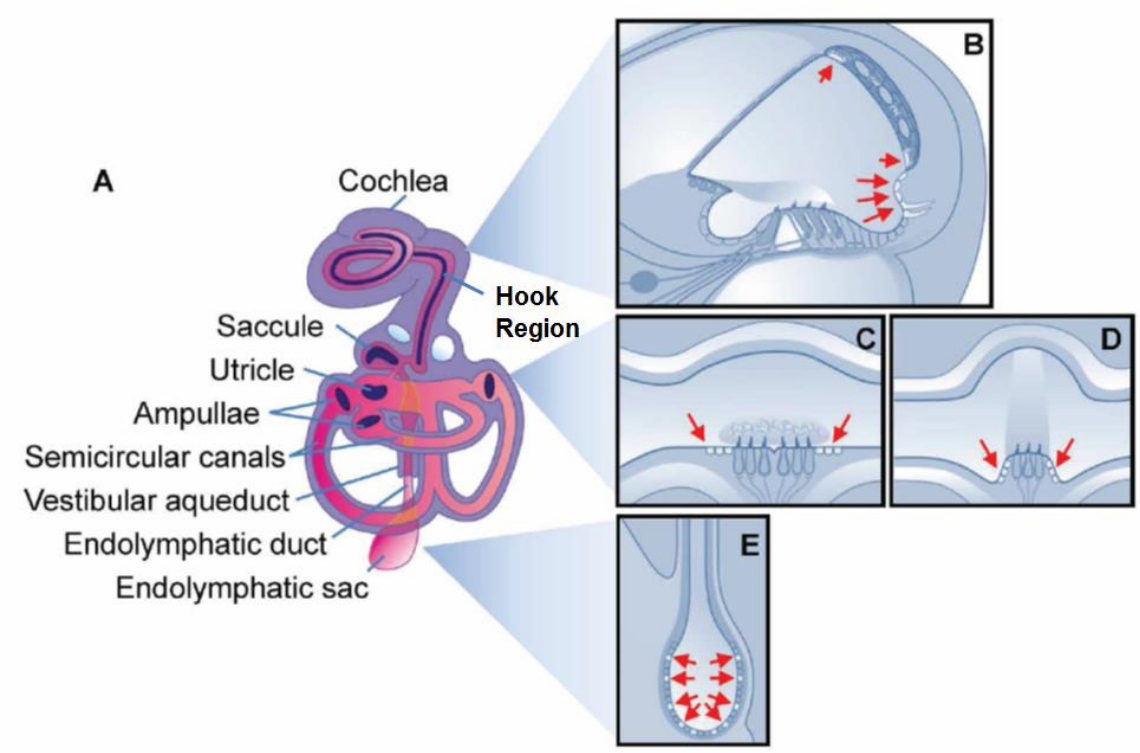

Figure 1-4: Pendrin expression in the inner ear.
(A) Structure of the inner ear. (B) Pendrin expression sites in the cochlea: epithelial cells of the spiral prominence (long arrows) and spindle cells of stria vascularis (arrowhead). Pendrin expression sites in the saccule or utricle (C) and the ampullae (D): transitional cells (arrows). Pendrin expression sites in the endolymphatic sac (E): mitochondria-rich cells (arrows). Figure is reproduced with permission from the author and the copyright holder, Karger, from Wangemann 2013 [20].

Currently, the mechanisms for the enlargement of the membranous inner ear in *Slc26a4*$^{\Delta/\Delta}$ mice are not clear. It has been shown that the cochlear endolymphatic K^+ concentration increases from 10 mM during embryonic stages (E14.5) to approximately 140 mM at adult stages [21]. Further evidence suggests that fluid secretion takes place in the vestibular labyrinth [4]. If K^+ secretion is the source of fluid secretion that results in formation of the lumen, there should be no obvious difference in utricular endolymphatic K^+ concentration between *Slc26a4*$^{\Delta/+}$ and *Slc26a4*$^{\Delta/\Delta}$ mice during embryonic stages and further, it would be expected that endolymphatic K^+ concentration rises earlier in the utricle than in the cochlea. In order to address this hypothesis, it was necessary to measure the K^+ concentrations in the utricle.

Chapter 2 - Materials and Methods

Ethics Statement

All animal experiments and procedures were approved by the Institutional Animal Care and Use Committee at Kansas State University (IACUC#: 3245.3).

Animals

A colony of *Slc26a4*$^{\Delta/+}$ and *Slc26a4*$^{\Delta/\Delta}$ mice, free of known and suspected murine pathogens, was maintained at Kansas State University College of Veterinary Medicine. *Slc26a4*$^{\Delta/+}$ dams and *Slc26a4*$^{\Delta/\Delta}$ sires were paired and the average litter size was 4.6 with *Slc26a4*$^{\Delta/+}$ and *Slc26a4*$^{\Delta/\Delta}$ offspring occurring at a near-Mendelian ratio (50.1 to 49.9). The average gestational period was 21.4 (±0.6, n=29) days. 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). Pregnancies were tested by ultrasound (Terason t3000, Universal Medical Systems, Bedford Hills, NY). In addition, gestational age was verified by evaluating gross morphological features including limbs, digits, and appearance of the pinna and auditory meatus [22]. *Slc26a4*$^{\Delta/+}$ and *Slc26a4*$^{\Delta/\Delta}$ mice ranging from E14.5 to P64 were used in the present study. Time-pregnant dams were deeply anesthetized with 4% tri-bromo-ethanol and sacrificed by decapitation after harvesting embryos by sterile laparotomy. Embryos were sacrificed by decapitation. Neonatal mice (P0-P2) were anesthetized by a combination of intraperitoneal (i.p.) injection of 0.013 mL/g body weight of 4% tri-bromo-ethanol and rapid cooling on a slush of ice. Older mice (>P3) were anesthetized solely by i.p. injection of 0.020 mL/g body weight of 4% tri-bromo-ethanol. Neonatal and older mice were sacrificed by decapitation after being deeply anesthetized.

Organ culture

Procedures for organ culture were adapted with modifications from methods developed earlier in this laboratory [21]. *Slc26a4*$^{\Delta/+}$ and *Slc26a4*$^{\Delta/\Delta}$ embryos were harvested at E15.5. The cranium was cut medially in halves and intact otocysts were isolated from the temporal bone in sterile NaCl solution maintained at 4 ^{o}C. The NaCl solution for dissection contained (in mM)

150 NaCl, 1.6 K_2HPO_4, 0.4 KH_2PO_4, 1 $MgCl_2$, 0.7 $CaCl_2$, 5 glucose, pH 7.4. Preparations of the vestibular labyrinth and the endolymphatic sac were obtained by micro-dissection from E15.5 *Slc26a4*$^{\Delta/+}$ and *Slc26a4*$^{\Delta/\Delta}$ mice and submersed in DMEM medium (Cat#12800-017, Invitrogen, Carlsbad, CA) supplemented with 2.3 g/L $NaHCO_3$, 10% fetal bovine serum (Cat# 10082, Invitrogen, Carlsbad, CA), and 1% penicillin-streptomycin (Cat# P7539, Sigma) (Figure 2-1). Tissues were incubated at 37°C in a humidified atmosphere enriched with 5% CO_2. Preparations of utricles with attached ampullae of the anterior and lateral semicircular canals were isolated by micro-dissection from otocysts obtained from E15.5 *Slc26a4*$^{\Delta/+}$ and *Slc26a4*$^{\Delta/\Delta}$ mice. Tissues were maintained in organ culture and periodically imaged by laser-scanning microscopy. Endolymph was stained by injection of dye (BCECF acid + 5 mg/mL Fast Green) into the cochlea. Endolymphatic sacs were freed by microdissection and cut off from the inner ear with scissors. The cutting force from the scissors sealed the epithelia at the cutting edge. Preparations were maintained in organ culture and compartment volumes were periodically monitored by confocal microscopy.

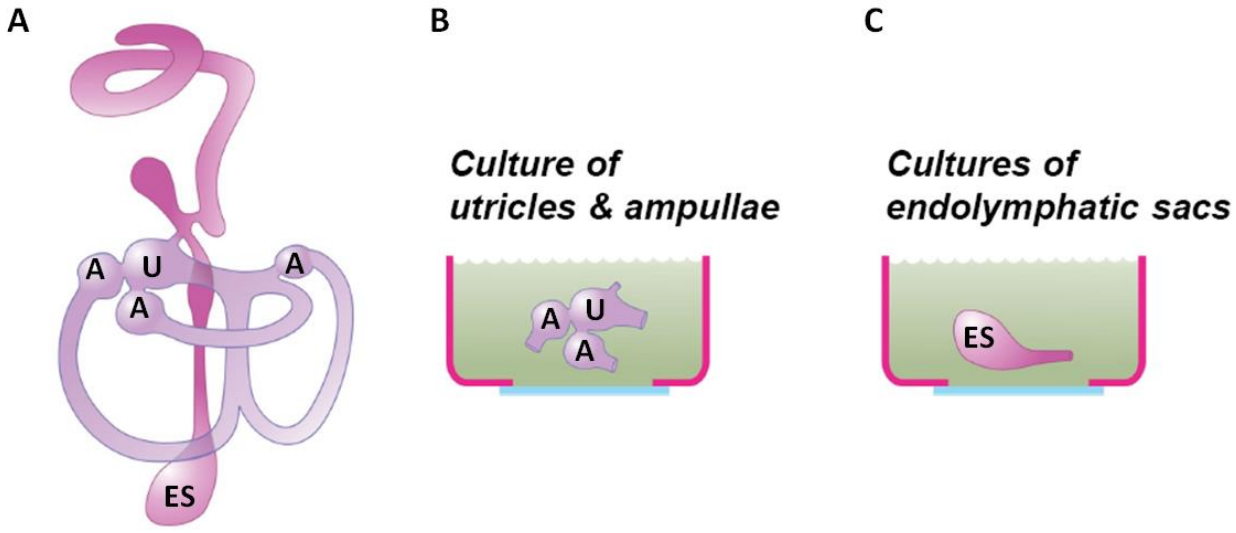

Figure 2-1: Organ culture of inner ear tissues.
A) Structure of the inner ear inside the cartilage. B) Utricles (U) with two ampullae (A) attached were isolated and cultured in medium in glass-bottom dishes. C) Isolated endolymphatic sacs (ES) were isolated and cultured in medium in glass-bottom dishes. Figure drawn by Dr. Philine Wangemann and used with permission.

Measurement of voltage and K^+ concentrations

K^+ concentrations of the utricular endolymph and transepithelial voltages were measured with double-barreled ion-selective electrodes *in vitro* using established methods [21, 23] (Figure 2-2). Temporal bones were isolated from pre- and postnatal mice and placed in a bath chamber superfused at 37°C with HCO_3^--containing physiological solutions (In-line heater SHM-8 and controller TC-344B, Warner instruments, Hamden, CT). The physiological solution contained (in mM): 135 NaCl, 25 $NaHCO_3$, 4 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$ and 5 glucose and was bubbled with a humidified gas mixture consisting of 5% CO_2 and 20% O_2 to set the pH to pH 7.3 - pH 7.4. The bath chamber was grounded *via* a Ag/AgCl electrode that was bathed in a vial filled with 1 M KCl solution and connected to the bath chamber *via* an agar-bridge made from 150 mM NaCl solution with 2% agar. The endolymphatic K^+ concentrations and the transepithelial voltages were measured with double-barreled K^+-selective electrodes within 1 hour after sacrifice. The double-barreled electrode approached the utricle *via* the oval window. One barrel of the double-barreled electrode was used to measure the K^+ concentration and the other barrel was used to measure the transepithelial voltage.

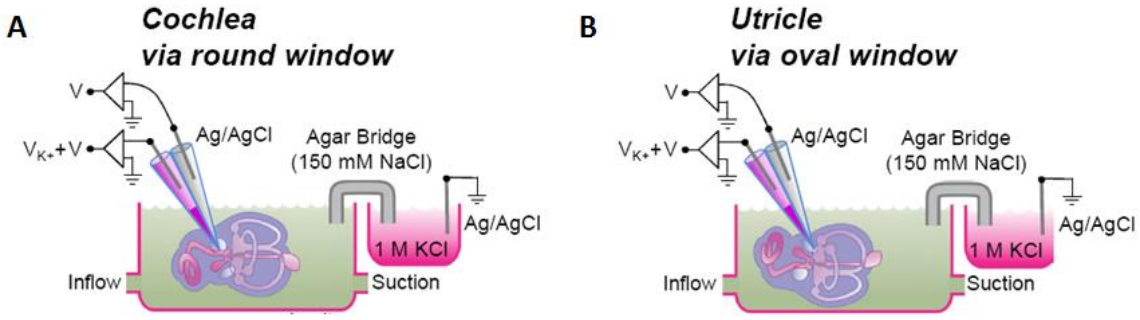

Figure 2-2: Configuration for endolymphatic K^+ measurements.
Measurements of voltage and K^+ concentrations in cochlear and utricular. The voltage barrel of the electrode was used to measure the transepithelial voltage (V) and the ion barrel, with the tip filled with ion exchanger, was used to record the signal which was the sum of the ion-related voltage and the transepithelial voltage (V_{K+}+V). Temporal bones were dissected and placed in a chamber superfused with warm artificial perilymph. The stapes covering the oval window was removed to expose the utricle. Both figures were drawn by Dr. Philine Wangemann and are used with permission. Part A is reproduced with permission from the author and copyright holder, Dr. Philine Wangemann, from Li et al 2013 [21].

Electrodes were calibrated at 37°C immediately after measurement in the utricle using solutions containing 3, 10, 50 and 150 mM KCl with the sum of NaCl and KCl being constant at 150 mM. Data were recorded analog via a flat-bed chart recorder (BD12E, Kipp & Zonen, Delft, The Netherlands) for easy annotation. Parallel, data were recorded digitally (Digidata 1322A and AxoScope 9, Molecular Devices, San Francisco, CA) for convenient analysis.

Confocal immunohistochemistry

Otocysts were isolated from embryonic *Slc26a4*$^{\Delta/+}$ mice and fixed overnight at 4°C in Phosphate Buffered Saline (PBS contained (mM): 137 NaCl, 2.7 KCl, 10.1 Na_2HPO_4, and 1.8 KH_2PO_4, pH 7.4) with 4% paraformaldehyde (Cat# 15714, Electron Microscopy Sciences, Hatfield, PA). Fixed otocysts were processed through a sucrose gradient (10% and 20%, each 30 min, followed by 30% overnight, all solutions in PBS at 4 °C), infiltrated with polyethylene glycol (Cat# 72592-B, Electron Microscopy Sciences) and cryosectioned (12 μm, CM3050S, Leica, Wetzlar, Germany). Sections of the otocyst were mounted on charged slides (Cat# 22-230-900, Fisher, Pittsburgh, PA) and blocked for 1 hour with 5% bovine serum albumin (BSA) in PBS containing 0.2% TritonX-100 (PBS-TX solution). Sections were incubated at 4°C overnight with primary antibodies containing goat anti-KCNQ1 (1:200, Cat# SC-10646, Santa Cruz, Dallas, TX) or rabbit anti-SLC12A2 (1:200, Cat# AB3560P, Chemicon, Temecula, CA) and 2.5% BSA in PBS solution. Sections were washed 3 times for 2 min each with PBS-TX solution and incubated at room temperature for 1 hour with secondary antibodies containing Alexa Fluor 594 chicken-anti-goat (1:1000, Cat# A21468, Invitrogen, Grand Island, NY) or Alexa Fluor 594 goat-anti-rabbit (1:1000, Cat# A11037, Invitrogen) phalloidin 488 (1:40, Cat# A-12379, Invitrogen), DAPI (1:1000; Cat# D-3571, Invitrogen) and 2.5% BSA in PBS solution. After labeling, sections were washed another 3 times in PBS-TX solution with 2 min each, immersed with mounting medium (Cat# H-1400, Vector laboratories, Burlingame, CA), covered with a cover slip and examined by confocal laser scanning microscopy (LSM 510 Meta, Carl Zeiss, Göttingen, Germany).

Statistical analysis and presentation

Data were presented as average ± SD with n being the number of replicates. Statistical significance was determined based on unpaired t-tests. Significance was assumed at $p<0.05$.

Chapter 3 - Results

Vestibular labyrinth secretes fluid *in vitro*

We first tested for fluid secretion by cultured embryonic vestibular labyrinth. Embryonic inner ears were harvested from both *Slc26a4*$^{\Delta/+}$ and *Slc26a4*$^{\Delta/\Delta}$ mice at E15.5, which is near the onset of cochlear lumen formation. Preparations of utricles with anterior and posterior ampullae attached were isolated by micro-dissection. Tissues were maintained in organ culture and imaged periodically with confocal microscopy. Freshly isolated tissues were imaged and recorded as 0 hours (hrs) after dissection. For tissues from both genotypes, there was a healing process in the first few hours after microdissection. Openings of the ampullae and utricle at the semicircular canals and the opening of the utricle at the saccular duct healed and closed automatically during organ culture. The tissues shrank and got condensed during the healing process. 30 hours after dissection, there was an obvious swelling in the tissues of both genotypes. All three compartments were blown up by fluids and the epithelia appeared stretched, thus making it easier for the laser to penetrate and therefore appearing lighter. At ~50 hours, tissues were substantially bigger, especially for the ones originally from E15.5 *Slc26a4*$^{\Delta/\Delta}$ mice (Figure 3-1).

The ion secretion rate in a cultured E15.5 *Slc26a4*$^{\Delta/\Delta}$ utricle was estimated based on images taken at 9 hours and 52 hours after dissection. At 9 hours the tissue was relatively flat and the volume was estimated based on three luminal areas, representing two ampullae and one utricle, and a common height (Figure 3-2). The sum of the three luminal areas was 91,272 μm^2. The average height of the lumen estimated to be 30 µm. The volume of the lumen at 9 hours was estimated to be 2.7 nL, calculated from [2.7 nL=91,272×30 $\mu m^3 \cdot nL/10^6$ μm^3]. At 52 hours the tissue appeared swollen and the volume was estimated based on three spheres (Figure 3-2). Thereby their luminal volumes can be calculated with the equation: $V = (4/3)\ \pi \cdot r^3$. The volume of the swelled tissue at 52 hours was estimated to be 71.9 nL. The major ions in the cytosol are KCl and NaCl, the total concentration of which stays constant at 150 mM. With the increased luminal volume calculated from 9 hours to 52 hours, the ion secretion rate was 241.4 pmol/h, calculated from [241.4 pmol/h=(71.9-2.7) nL×150 mM/(52-9) hrs].

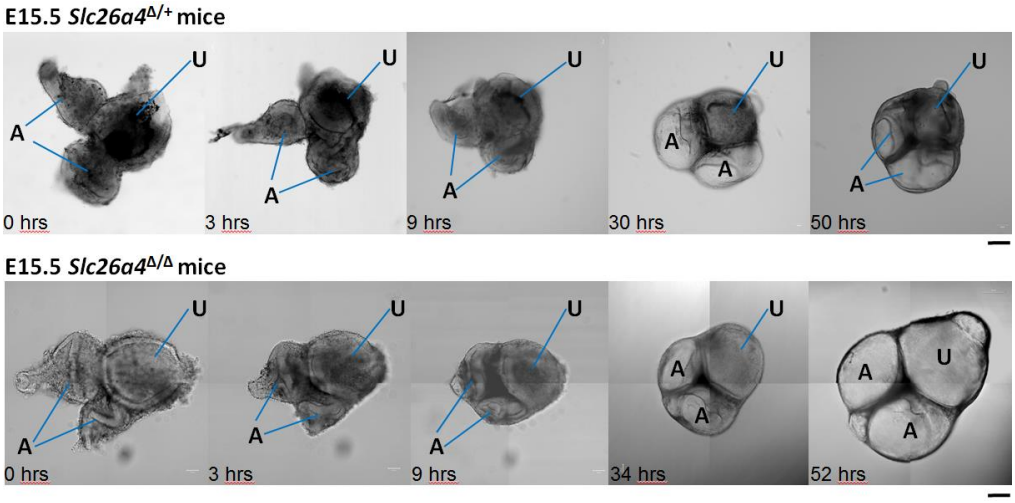

Figure 3-1: Organ culture of the vestibular labyrinth.
Utricles (U) with attached ampullae (A) from E15.5 *Slc26a4*$^{\Delta/+}$ and *Slc26a4*$^{\Delta/\Delta}$ mice both sealed and formed cysts during ~50 hours (hrs) of organ culture. At 0 hrs, freshly isolated tissues were transferred to glass-bottom dishes with medium and periodically imaged over ~50 hours. Relative compartments of the tissues were distinguishable during organ culture (blue arrows). Recordings of these organ cultures of tissues collected from E15.5 *Slc26a4*$^{\Delta/+}$ and *Slc26a4*$^{\Delta/\Delta}$ mice are representatives of experiments yielding similar results. The scale bar, 100 µm, pertains to all images.

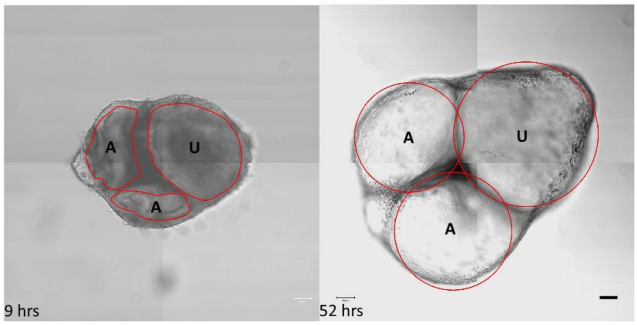

Figure 3-2: Estimations of the luminal volume of a cultured *Slc26a4*$^{\Delta/\Delta}$ utricle.
At 9 hrs, the luminal volume was estimated based on three areas, representing a utricle (U) and two attached ampullae (A) and a common height. At 52 hrs when the tissue appeared swollen, the volume was estimated based on three spheres, representing a utricle (U) and two attached ampullae (A). The areas circled by red lines are considered as lumen. Same tissue as the E15.5 *Slc26a4*$^{\Delta/\Delta}$ utricle in Figure 3-1. Scale bar, 50 µm, pertains to both images.

Endolymphatic sacs absorb fluid *in vitro*

Next, we determined if the embryonic endolymphatic sac spontaneously absorbs fluid when placed in culture. Embryonic inner ears were harvested at E15.5 from *Slc26a4*$^{\Delta/+}$ mice. The endolymphatic sacs were filled with 5 mg/mL fast green and carefully isolated from the inner ears without injury during dissection. Preparations of endolymphatic sacs were cut off from the endolymphatic duct and were manually sealed by the cutting force of the scissors. Tissues were transferred to glass-bottom dishes and maintained in organ culture. Injection of dye (fast green + BCECF acid) into the endolymphatic sacs helped visualize the tissue. Image taking in time series started to count when the endolymphatic sac was freshly isolated from the inner ear. At 0 hours (hrs), the sealed endolymphatic sac was nicely filled with dye and lay flat on bottom of culture dishes. Shrinkage of the lumen and folding of the epithelium were observed within a few hours, which indicated there was fluid absorption (Figure 3-3). The epithelium of the endolymphatic sac folded as the lumen got smaller. There was almost no dye observed, implying there was no lumen inside the tissue.

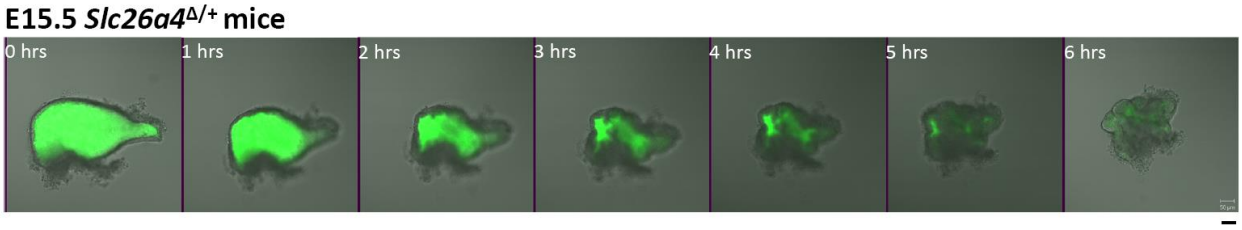

Figure 3-3: Organ culture of the endolymphatic sac.
Preparations of endolymphatic sacs from E15.5 *Slc26a4*$^{\Delta/+}$ mice shrank during 6 hours of culture (Experiments conducted by Dr. Philine Wangemann). The recording of organ culture is representative of three similar experiments. Scale bar, 50 µm, pertains to all images.

Endolymphatic K^+ concentrations in the utricle rises after E16.5

To determine if K^+ secretion is the driving force for fluid secretion in the utricle, endolymphatic K^+ concentrations and transepithelial voltages in the utricles prepared from pre- and postnatal mice were measured with double-barreled K^+ selective electrodes. Representative measurement traces were shown in Figure 3-4. K^+-selective electrodes were calibrated against standard K^+ solutions with known concentrations. K^+ concentrations and voltages were fitted to the Nernst equation:

$$V = V_i + S_{K+} \times log_{10}([K^+])$$

where V represents the measured voltage, V_i is an offset term, S_{K+} is the slope representing the sensitivity for K^+, $[K^+]$ represents the K^+ concentrations. K^+ electrodes were found to be highly sensitive with S_{K+} near the theoretical value of 60 (52 ± 1 mV, n=72).

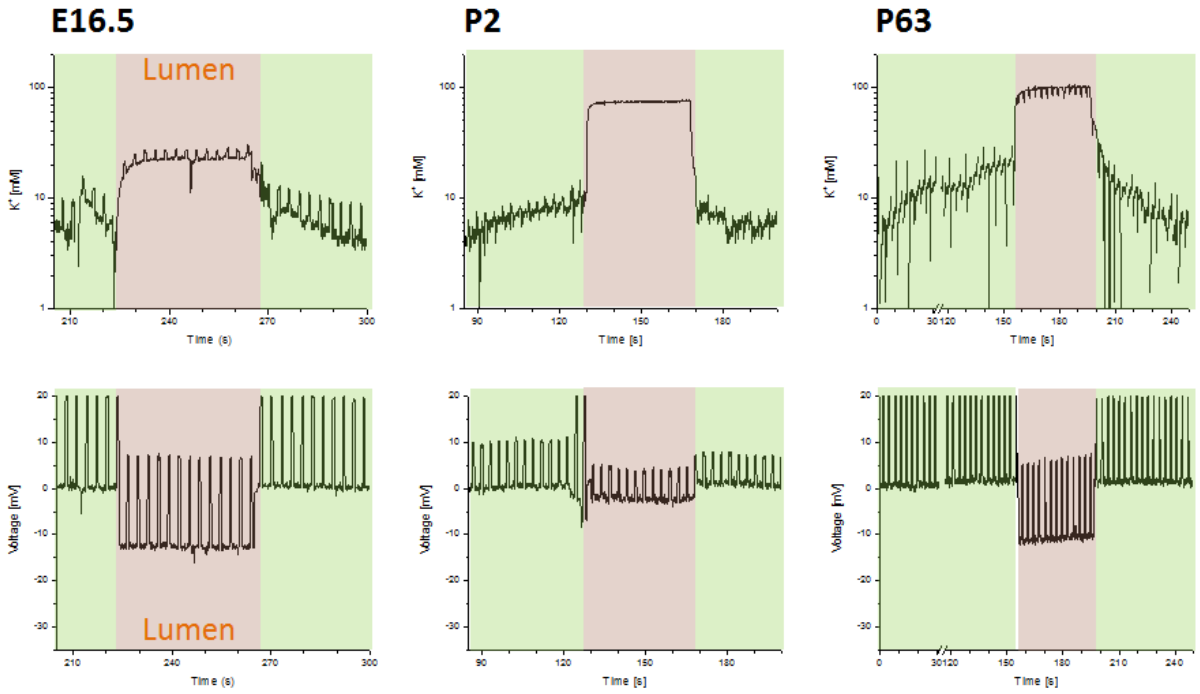

Figure 3-4: Representative measurements with K^+-selective electrodes.
Recordings of measurement on K^+ concentrations (top row) and transepithelial voltages (bottom row) in utricles isolated from *Slc26a4*$^{\Delta/+}$ mice at age E16.5, P2 and P63. Traces with a purple background were recorded while the electrodes were in the lumen of utricle.

At E16.5, endolymphatic K^+ concentrations in the utricle were about ~10 mM in both genotypes and transepithelial voltage was about -10 mV in *Slc26a4*$^{\Delta/+}$ mice and about -2 mV in *Slc26a4*$^{\Delta/\Delta}$ mice (Figure 3-5). The K^+ concentration rose to about 30 mM in *Slc26a4*$^{\Delta/+}$ mice at E17.5. There appeared to be a delay of the rise in K^+ concentration in *Slc26a4*$^{\Delta/\Delta}$ mice during prenatal development. In adult mice, the K^+ concentration and the transepithelial voltage were 150 mM and -22 mV in *Slc26a4*$^{\Delta/+}$ and 132 mM and -5 mV in *Slc26a4*$^{\Delta/\Delta}$ mice.

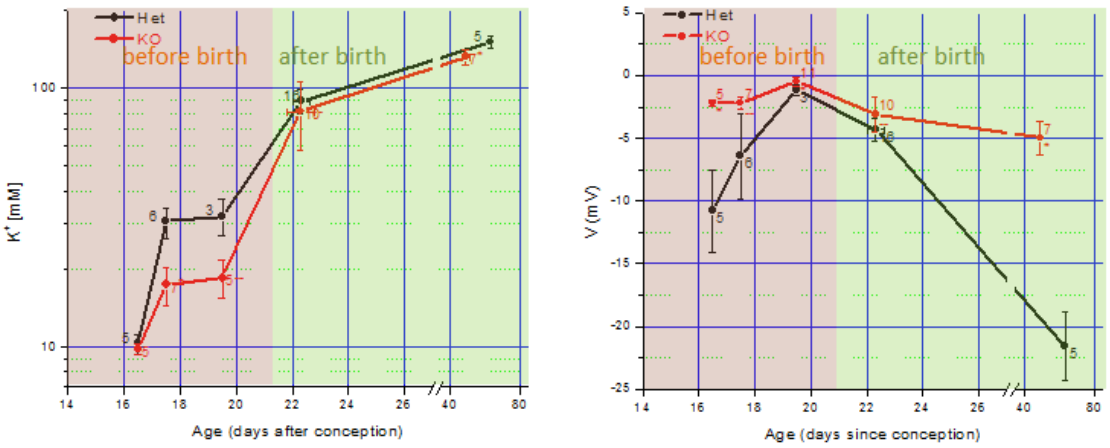

Figure 3-5: K^+ concentrations and transepithelial voltages.

Measurements of endolymphatic K^+ concentrations and transepithelial voltages in isolated utricles. Traces with a purple and green backgrounds indicated data collected from utricles isolated before and after birth, respectively. 17 out of the 72 reported measurements were collected by Xiangming Li.

KCNQ1 and SLC12A2 expression in the utricle and ampullae is evident at E19.5

We determined the expression of the K^+ channel KCNQ1 and the $Na^+/2Cl^-/K^+$ cotransporter SLC12A2 in *Slc26a4*$^{\Delta/+}$ mice by immunohistochemistry. Expression of KCNQ1 was found in the apical membranes and SLC12A2 was found in the basolateral membranes of the vestibular dark cells of the ampullae and utricle at E19.5 (Figure 3-6).

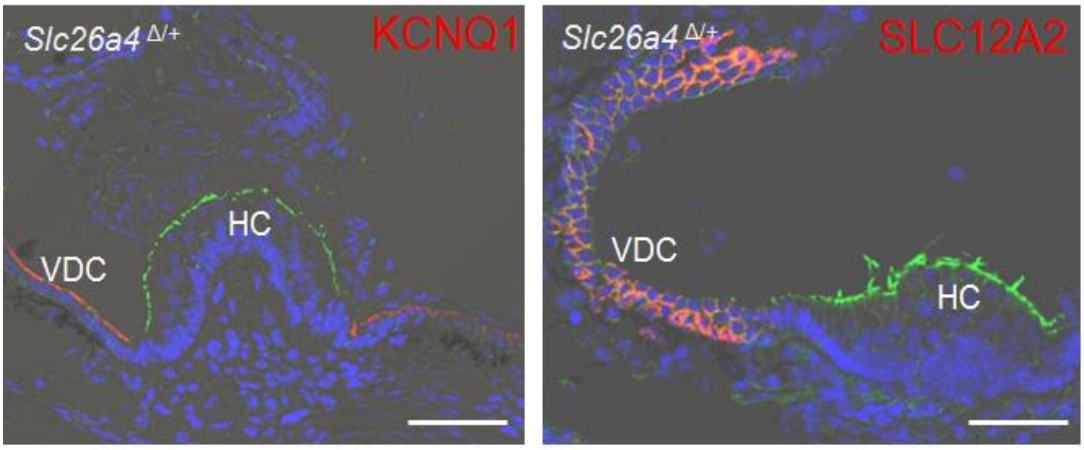

Figure 3-6: Protein expression of KCNQ1 and SLC12A2 in the utricle.
Immunohistochemistry of KCNQ1 (*red*, left image) and SLC12A2 (*red*, right image) in the vestibular labyrinth of E19.5 *Slc26a4*$^{\Delta/+}$ mice. F-actin was stained with phalloidin (*green*) and nuclei were stained with DAPI (*blue*). (Data were collected by Xiangming Li). VDC: vestibular dark cells; HC: hair cells. Scale bar: 50 µm.

Chapter 4 - Discussion

The observations presented in this report are consistent with, and supportive of, previous assumptions and hypotheses that state that enlargement of the membranous labyrinth and failure to acquire normal hearing in *Slc26a4*$^{\Delta/\Delta}$ mice result from of imbalanced fluid secretion and absorption [4]. The finding of a recent study, that involved the truncation of the vestibular labyrinth and the endolymphatic sac, implicated that these compartments participated in fluid secretion and absorption respectively [4]. If true, it would be expected that isolated sealed segments of the embryonic vestibular labyrinth would swell and that sealed embryonic endolymphatic sacs would shrink. Our organ culture experiments addressed this hypothesis. Utricles from both embryonic *Slc26a4*$^{\Delta/+}$ and *Slc26a4*$^{\Delta/\Delta}$ mice sealed and swelled during organ culture and ion secretion rates during the swelling process of utricles from *Slc26a4*$^{\Delta/\Delta}$ mice can be estimated. However, the mechanism of fluid secretion is yet to be figured out.

It is known that the K^+ concentration increases significantly in cochlear endolymph during pre- and postnatal stages [21], lumen formation, however, precedes the rise in the K^+ concentration, which indicates the lumen formation is driven by K^+ secretion. The mechanisms for lumen formation during embryonic development are not clear, although the molecular mechanisms of K^+ secretion into cochlear endolymph by strial marginal cells [24] and mechanisms of K^+ secretion into the vestibular labyrinth by vestibular dark cells are well understood [25, 26]. Vestibular dark cells take up K^+ across the basolateral membrane *via* the Na^+/K^+-ATPase and the $Na^+/2Cl^-/K^+$-cotransporter SLC12A2 [27, 28] and secrete K^+ into endolymph across the apical membrane *via* the K^+ channel KCNQ1/KCNE1 (previously known as KvLQT1and IsK or MinK). The presence of KCNQ1 and SLC12A2 expression in the utricle and ampullae at E19.5 correlates with the elevated K+ concentration in utricular endolymph at this stage of development. The rise of the K^+ concentration between E16.5 and E19.5 may be mediated by Na^+/K^+-ATPase and a non-selective cation channel, such as $P2X_2$ or ENaC. Expression of Na^+/K^+-ATPase has been observed [29], however, expression and function of a putative non-selective cation channel needs to be determined.

The negative voltages recorded in utricles isolated from adult mice suggest that tissues were deprived of oxygen during measurement. However, this does not invalidate measurements

of the endolymphatic K^+ concentrations since endolymphatic K^+ concentrations are maintained for at least 50 min under anoxic conditions [30]. There was a more negative transepithelial voltage in the utricle of *Slc26a4*$^{\Delta/+}$ mice at E16.5, which may be related to a non-selective cation channels, such as $P2X_2$ or ENaC. The growth phase of the inner ear, which includes the development of non-selective cation channels, comes to a conclusion at about E18.5 [20]. This is consistent with the no significant difference between *Slc26a4*$^{\Delta/+}$ and *Slc26a4*$^{\Delta/\Delta}$ mice in transepithelial voltage at E19.5. The negative transepithelial voltages in *Slc26a4*$^{\Delta/+}$ and *Slc26a4*$^{\Delta/\Delta}$ mice at E17.5 may at least in part originate with open transduction channels and other non-selective cation channels in hair cells and other epithelia cells [31, 32]. The smaller transepithelial voltage in adult utricles of *Slc26a4*$^{\Delta/\Delta}$ mice is consistent with non-functional hair cells and with non-functional cation absorptive mechanisms in *Slc26a4*$^{\Delta/\Delta}$ mice.

The rise in the endolymphatic K^+ concentration in the utricles of *Slc26a4*$^{\Delta/+}$ and *Slc26a4*$^{\Delta/\Delta}$ mice occurred with a time course similar to changes in cochlear endolymphatic K^+ concentrations [21], although the cochlea disconnects from the vestibular labyrinth after E16.5 [33]. There is no difference in endolymphatic K^+ concentration between the cochlea and the utricle, which indicates K^+ secretion is not the major driving force for cochlear lumen formation. According to the measurement on the cross-sectional area of scala media, the area in *Slc26a4*$^{\Delta/\Delta}$ mice after E16.5 is approximately 6-fold larger than that in *Slc26a4*$^{\Delta/+}$ mice [5] (Figure 1-3), which is most likely the reason for the delay of the rise of the K^+ concentration in utricular and cochlear endolymph of *Slc26a4*$^{\Delta/\Delta}$ mice.

The observed shrinkage of the lumen and folding of the epithelium during organ culture of endolymphatic sacs isolated from E15.5 *Slc26a4*$^{\Delta/+}$ mice is consistent with fluid absorption in the endolymphatic sac [4]. Although difficulty in endolymphatic sac volume measurement hampered further investigation, the shrinkage of the tissues was obvious in 2-D recorded images (Figure 3-3). In order to measure absorption rates in isolated endolymphatic sacs from *Slc26a4*$^{\Delta/+}$ and *Slc26a4*$^{\Delta/\Delta}$ mice, a superior method for volume measurement must be established.

In summary, this study has determined the respective roles of the epithelia of embryonic utricles and endolymphatic sacs in fluid secretion and absorption. The present result supports the concept that a balance between fluid secretion and absorption is necessary for cochlear development (Figure 4-1). We also measured the endolymphatic K^+ concentrations in the utricle of pre- and postnatal *Slc26a4*$^{\Delta/+}$ and *Slc26a4*$^{\Delta/\Delta}$ mice and found that K^+ concentrations increased

after E16.5. Expression of the K^+ channel KCNQ1 and the $Na^+/2Cl^-/K^+$-cotransporter SLC12A2 likely mediate the rise in K^+ concentration observed at E19.5. The observed delay of the rise of the K^+ concentration in utricular and cochlear endolymph of *Slc26a4*$^{\Delta/\Delta}$ mice likely results from the enlarged endolymph volume.

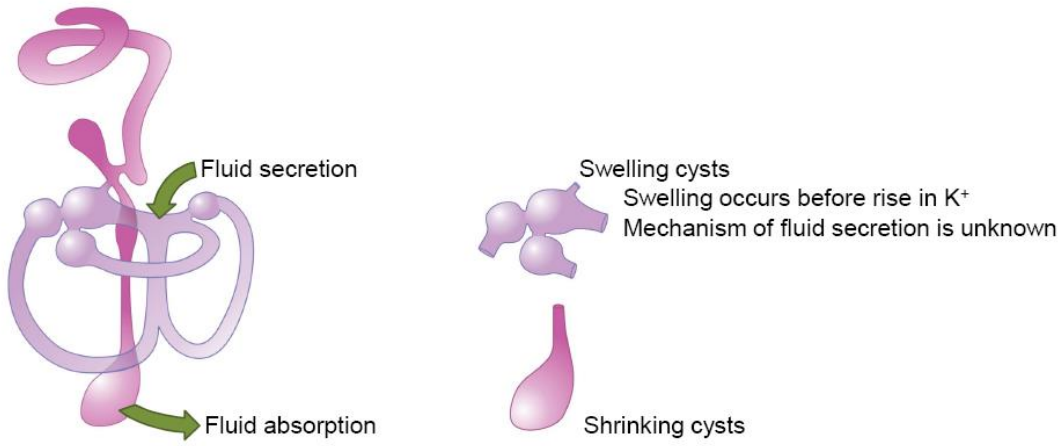

Figure 4-1: Source of fluid secretion and absorption in the inner ear.
The image on the left shows the inner ear structure and sources of fluid secretion and absorption. The isolated sealed utricles and endolymphatic sacs swelled and shrank respectively, consistent with fluid secretion and absorption. The swelling of the *Slc26a4*$^{\Delta/\Delta}$ mice inner ear starts at E14.5 [2], which is earlier than the rise in endolymphatic K^+ concentration in the utricle and cochlea, indicating the K^+ secretion is not the major driving force for fluid secretion. The mechanism of fluid secretion is still unknown. Figure drawn by Dr. Philine Wangemann and used with permission.

References

1. Chittka, L. and A. Brockmann, *Perception Space—The Final Frontier.* PLoS Biol, 2005. **3**(4): p. e137.
2. Wangemann, P., *The role of pendrin in the development of the murine inner ear.* Cell Physiol Biochem, 2011. **28**(3): p. 527-34.
3. Kelley, M.W. et al, *Development of the inner ear.* Springer handbook of auditory research, 2005. **26**.
4. Kim, H.M. and P. Wangemann, *Failure of fluid absorption in the endolymphatic sac initiates cochlear enlargement that leads to deafness in mice lacking pendrin expression.* PLoS One, 2010. **5**(11): p. e14041.
5. Griffith, A.J. and P. Wangemann, *Hearing loss associated with enlargement of the vestibular aqueduct: mechanistic insights from clinical phenotypes, genotypes, and mouse models.* Hear Res, 2011. **281**(1-2): p. 11-7.
6. Soleimani, M., et al., *Pendrin: an apical Cl-/OH-/HCO3- exchanger in the kidney cortex.* Am J Physiol Renal Physiol, 2001. **280**(2): p. F356-64.
7. Scott, D.A. and L.P. Karniski, *Human pendrin expressed in Xenopus laevis oocytes mediates chloride/formate exchange.* Am J Physiol Cell Physiol, 2000. **278**(1): p. C207-11.
8. Scott, D.A., et al., *The Pendred syndrome gene encodes a chloride-iodide transport protein.* Nat Genet, 1999. **21**(4): p. 440-3.
9. Royaux, I.E., et al., *Pendrin, encoded by the Pendred syndrome gene, resides in the apical region of renal intercalated cells and mediates bicarbonate secretion.* Proc Natl Acad Sci U S A, 2001. **98**(7): p. 4221-6.
10. Royaux, I.E., et al., *Pendrin, the protein encoded by the Pendred syndrome gene (PDS), is an apical porter of iodide in the thyroid and is regulated by thyroglobulin in FRTL-5 cells.* Endocrinology, 2000. **141**(2): p. 839-45.
11. Wangemann, P., et al., *Loss of KCNJ10 protein expression abolishes endocochlear potential and causes deafness in Pendred syndrome mouse model.* BMC Med, 2004. **2**: p. 30.
12. Nakao, I., et al., *Identification of pendrin as a common mediator for mucus production in bronchial asthma and chronic obstructive pulmonary disease.* J Immunol, 2008. **180**(9): p. 6262-9.
13. Bidart, J.M., et al., *Expression of Na+/I- symporter and Pendred syndrome genes in trophoblast cells.* J Clin Endocrinol Metab, 2000. **85**(11): p. 4367-72.
14. Rillema, J.A. and M.A. Hill, *Prolactin regulation of the pendrin-iodide transporter in the mammary gland.* Am J Physiol Endocrinol Metab, 2003. **284**(1): p. E25-8.
15. Alesutan, I., et al., *Impact of bicarbonate, ammonium chloride, and acetazolamide on hepatic and renal SLC26A4 expression.* Cell Physiol Biochem, 2011. **28**(3): p. 553-8.
16. Everett, L.A., et al., *Targeted disruption of mouse Pds provides insight about the inner-ear defects encountered in Pendred syndrome.* Hum Mol Genet, 2001. **10**(2): p. 153-61.
17. Park, H.J., et al., *Origins and frequencies of SLC26A4 (PDS) mutations in east and south Asians: global implications for the epidemiology of deafness.* J Med Genet, 2003. **40**(4): p. 242-8.

18. Dorwart, M.R., et al., *The solute carrier 26 family of proteins in epithelial ion transport.* Physiology (Bethesda), 2008. **23**: p. 104-14.
19. Wangemann, P., et al., *Loss of cochlear HCO3- secretion causes deafness via endolymphatic acidification and inhibition of Ca2+ reabsorption in a Pendred syndrome mouse model.* Am J Physiol Renal Physiol, 2007. **292**(5): p. F1345-53.
20. Wangemann, P., *Mouse models for pendrin-associated loss of cochlear and vestibular function.* Cell Physiol Biochem, 2013. **32**(7): p. 157-65.
21. Li, X., et al., *Endolymphatic Na(+) and K(+) Concentrations during Cochlear Growth and Enlargement in Mice Lacking Slc26a4/pendrin.* PLoS ONE, 2013. **8**(5): p. e65977.
22. Rugh, R., *The Mouse: Its Reproduction and Development*. 1968: Burgess Publishing Co.
23. Marcus, D.C., et al., *KCNJ10 (Kir4.1) potassium channel knockout abolishes endocochlear potential.* Am J Physiol Cell Physiol, 2002. **282**(2): p. C403-7.
24. Wangemann, P., *Supporting sensory transduction: cochlear fluid homeostasis and the endocochlear potential.* The Journal of Physiology, 2006. **576**(Pt 1): p. 11-21.
25. Wangemann, P., J. Liu, and D.C. Marcus, *Ion transport mechanisms responsible for K+ secretion and the transepithelial voltage across marginal cells of stria vascularis in vitro.* Hear Res, 1995. **84**(1-2): p. 19-29.
26. Wangemann, P., *Comparison of ion transport mechanisms between vestibular dark cells and strial marginal cells.* Hearing Research, 1995. **90**(1–2): p. 149-157.
27. Crouch, J.J., et al., *Immunohistochemical localization of the Na-K-Cl co-transporter (NKCC1) in the gerbil inner ear.* J Histochem Cytochem, 1997. **45**(6): p. 773-8.
28. Jiang, M., et al., *Sodium-Glucose Transporter-2 (SGLT2; SLC5A2) Enhances Cellular Uptake of Aminoglycosides.* PLoS ONE, 2014. **9**(9): p. e108941.
29. Kim, H.M. and P. Wangemann, *Epithelial cell stretching and luminal acidification lead to a retarded development of stria vascularis and deafness in mice lacking pendrin.* PLoS One, 2011. **6**(3): p. e17949.
30. Mori, N., O. Ninoyu, and C. Morgenstern, *Cation transport in the ampulla of the semicircular canal and in the endolymphatic sac.* Arch Otorhinolaryngol, 1987. **244**(1): p. 61-5.
31. Geleoc, G.S. and J.R. Holt, *Developmental acquisition of sensory transduction in hair cells of the mouse inner ear.* Nat Neurosci, 2003. **6**(10): p. 1019-20.
32. Lelli, A., et al., *Tonotopic gradient in the developmental acquisition of sensory transduction in outer hair cells of the mouse cochlea.* J Neurophysiol, 2009. **101**(6): p. 2961-73.
33. Cantos, R., et al., *Patterning of the mammalian cochlea.* Proceedings of the National Academy of Sciences of the United States of America, 2000. **97**(22): p. 11707-11713.

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