Enlargement of the vestibular aqueduct (EVA; OMIM 600791) is commonly observed in temporal bones of children with sensorineural hearing loss (1). The corresponding soft tissue abnormality is enlargement of the endolymphatic duct and sac (2), nonsensory epithelial organs whose primary function is thought to be ionic and osmotic regulation of endolymph. Hearing loss associated with EVA is predominantly sensorineural, variable in severity, and asymmetric or unilateral, with an onset in the first few years of life (3). EVA may be the sole radiologic abnormality or it may be associated with abnormalities of other organs as part of a syndrome. The most common syndrome associated with EVA is Pendred syndrome (OMIM 274600), an autosomal recessive disorder originally reported to comprise thyroid goiter and sensorineural hearing loss (5). Pendred syndrome is caused by mutations in the SLC26A4 gene (formerly called PDS; OMIM 605646) (6), which can also be identified in many cases of nonsyndromic EVA (7–11). Although goiter is incompletely penetrant, EVA is a highly penetrant manifestation of 2 mutant alleles of SLC26A4 (2, 10, 12).

SLC26A4 encodes a multipass transmembrane protein called pendrin (6). In the mouse inner ear, pendrin functions as an exchanger of Cl\(^-\) and HCO\(_3^-\) (13). It is expressed in nonsensory epithelial cells of the outer sulcus and spiral prominence of the cochlear duct, transitional cells surrounding the vestibular neuroepithelia, and mitochondria-rich cells of the endolymphatic sac (refs. 14–16 and Figure 1). These cells are thought to contribute to pH and ionic homeostasis of endolymph, which is the potassium-rich fluid filling the scala media of the cochlear duct. Endolymph bathes the apical mechanosensory stereocilia bundles of hair cells, and maintenance of its distinctive ionic composition and pH is necessary for auditory function (17). Pendrin is first expressed at E11.5 in the mouse endolymphatic sac and at E13.5 to E16.5 in the cochlea, utricle, and saccule (18).

An Slc26a4\(^{-}\) knockout mouse allele was generated by targeted deletion of exon 8 of Slc26a4 (19). Slc26a4\(^{-}\) mice have vestibular dysfunction, profound hearing loss, and massively enlarged endolymphatic spaces throughout the entire inner ear (19). Enlargement of the endolymphatic sac and cochlea begins at E14.5, with cochlear endolymphatic acidification evident at E15.5, developmental...
To define the transitional timing of 

dNA concentrations in the cochlear duct that is filled with endolymph. This duct is surrounded by 2 open fluid compartments that allow for 


the level of staining in the endolymphatic sac was less than in Slc26aΔΔ control (Figure 3). There was no detectable immunoreactivity at 1 month of age when dox was discontinued at E17.5 (DE17.5; Figure 3). These results demonstrate inducible expression of pendrin protein from Tg[E] and Tg[R] and low or no pendrin expression in the absence of dox.

Temporal requirements of auditory function for Slc26aΔ expression. To define the temporal requirements of Slc26aΔ expression for cochlear function, the time point of dox initiation (I) was varied between E0 and P6. The auditory brainstem response (ABR) thresholds were measured at P25–P35, and the EP and endolymphatic perilymphatic pH were measured at P25–P56. Since Slc26aΔ expression is strongly and rapidly induced within the first few hours of initiation of dox (not shown), the onset of dox administration approximates the onset of Slc26aΔ expression.

When dox was initiated before E16.5, Tg[E];Tg[R];Slc26aΔ/Δ mice had ABR thresholds within the range of normal-hearing Slc26aΔ/Δ controls (Figure 4A). When dox was initiated at E16.5, ABR thresholds were still within the range of normal-hearing Slc26aΔ/Δ controls (Figure 4A), but the EP and the endolymphatic pH were reduced compared with controls (Figures 5, A and B). When dox initiation was delayed until E18.5, there were large elevations of hearing thresholds with mean ABR thresholds of approximately 110 dB SPL in worse-hearing ears and 95 dB SPL in better-hearing ears, which are indicative of severe hearing loss (Figure 4A). These were associated with severe reductions in the EP from approximately 90 mV to 10 mV (Figure 5A). The endolymphatic pH was reduced from approximately 7.5 to 7.1 (Figure 5B). These values are similar to those reported in Slc26aΔ/Δ mice (13). Further delay of initiation of dox until P2 led to even higher ABR thresholds that were indistinguishable from those of Slc26aΔ/Δ controls (Figure 4A).

Results

Transgenic mice with doxycycline-inducible expression of Slc26aΔ. A binary transgenic mouse line was generated in which all Slc26aΔ expression is inducible with doxycycline (dox) (reviewed in ref. 21). The effector transgene (Tg[E]) expresses reverse tetracycline-controlled transactivator (rtTA) under the control of the mouse Slc26aΔ promoter and cis-regulatory elements (Figure 2). The responder transgene (Tg[R]) expresses a full-length Slc26aΔ cDNA under bidirectional control of the tetracycline operator. In the presence of dox, within the temporal and spatial expression domain of Slc26aΔ regulatory elements, rtTA binds to the tetracycline operator to activate expression of Slc26aΔ cDNA. The transgenic lines were crossed onto the Slc26aΔ/Δ background so that the responder transgene is the only source of functional pendrin. We generated and characterized 2 founder lines for Tg[E] and 5 lines for Tg[R] to identify combinations of Tg[E] and Tg[R] lines with Slc26aΔ expression that were inducible only in the presence of dox.

We identified a single combination of Tg[E] and Tg[R] lines with inducible and tight expression of Slc26aΔ. Figure 3 shows the results of anti-pendrin immunostaining of the cochlea and endolymphatic sac of Tg[E];Tg[R];Slc26aΔ/Δ mice at 1 month of age. We were able to detect strong pendrin immunoreactivity in Tg[E];Tg[R];Slc26aΔ/Δ ears when dox was initiated at E0 (IE0: dox initiated at E0 and administered for entire lifespan), although the level of staining in the endolymphatic sac was less than in Slc26aΔ/Δ controls (Figure 3). We therefore generated a mouse line in which we could reversibly activate and deactivate Slc26aΔ expression to define the temporal requirements for pendrin in the auditory system and to model human EVA phenotypes.

Given the severe developmental abnormalities of Slc26aΔ/Δ ears, it remains unknown whether pendrin is also required for homeostatic function of the mature inner ear. This question is relevant to strategies to prevent or retard progressive hearing loss in EVA. Moreover, the profound hearing loss and inner ear malformations in the Slc26aΔ/Δ mouse do not model the less severe phenotypes frequently observed in human EVA patients (3, 4, 9, 10). We thus generated a mouse line in which we could reversibly activate and deactivate Slc26aΔ expression to define the temporal requirements for pendrin in the auditory system and to model human EVA phenotypes.

Figure 1

Pendrin expression in the inner ear. (A) Overview of the inner ear. The mammalian inner ear consists of the cochlea, which is the organ for hearing, the vestibular labyrinth, which is the organ for detecting linear acceleration in the saccule and utricle, and angular acceleration along 3 spatial axes in the ampullae. The membranous labyrinth (shown) is encapsulated in bone (not shown). (B) Cross-section of 1 turn of the cochlea. The cochlea consists of an epithelia-lined duct that is filled with endolymph. This duct is surrounded by 2 open fluid compartments that are filled with perilymph. The main structures of the cochlear duct are the stria vascularis (SV), Reissner membrane (RM), and the organ of Corti (OC). The stria vascularis generates the EP and secretes K+ into endolymph. The organ of Corti contains the sensory hair cells. Pendrin is expressed in epithelial cells of the spiral prominence (SP), in root cells (R), and in spindle cells of the stria vascularis. Cross sections are shown for the saccule or utricle (C), an ampulla (D), and the endolymphatic duct and sac (E). The saccule, utricle, ampu lae, and endolymphatic duct are epithelia-lined structures that are filled with endolymph and surrounded by perilymph. The endolymphatic sac is surrounded by cerebrospinal fluid. Pendrin is expressed in nonsensory epithelial cells surrounding the sensory hair-cell patches in the saccule, utricle, and ampulla. In addition, pendrin is prominently expressed in the endolymphatic sac.

retardation of the stria vascularis at P3, loss of the endocochlear potential (EP) at P10, degeneration of hair cells as early as P15, and failure to acquire hearing between P12 and P15 (13, 16, 18–20).

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We performed a converse analysis by varying the time point of
dox discontinuation (D) for Tg[E];Tg[R];Slc26a4Δ/Δ mice that had
been administered dox since conception. Western blot analysis
showed the half-life of pendrin to be approximately 18 hours after
discontinuation of dox, with less than 1% of pendrin present in
DP6 tissues at P25–P35 (not shown). When dox was discontinued
at or before E15.5, the Tg[E];Tg[R];Slc26a4Δ/Δ mice were deaf, with
ABR thresholds indistinguishable from those of Slc26a4Δ/Δ controls
(Figure 4B). Delaying the discontinuation of dox until E16.5 pro-
duced a significant improvement in ABR thresholds (Figure 4B),
although the EP and endolymphatic pH were severely reduced even
when dox had been discontinued 1 day later at E17.5 (Figure 5, A
and B). In contrast, perilymphatic pH remained normal (Figure 5C).
A further delay of dox discontinuation until P2 led to thresholds
that were within the range of normal-hearing Slc26a4Δ/+ controls
(Figure 4B), and a delay until P6 led to endolymphatic pH values
that were also within normal limits of Slc26a4Δ/+ controls (Figure 5B).
Delaying dox discontinuation until P6 improved the EP, but did
not restore it to normal levels (Figure 5A).

Although several dox initiation and discontinuation time
points resulted in differences between better- and worse-hear-
ing ear thresholds, the differences appeared to be larger for dis-
continuation paradigms (Figure 4). The mean ABR thresholds
for DE19.5, DP0, or DP1 mice collectively reflect a high preva-
lence of unilateral hearing loss, since the mean ABR thresholds
of better-hearing ears were within the range of normal-hearing
Slc26a4Δ/+ controls (Figure 4B).

Morphology of OE18.5 and DE17.5 inner ears. The effect of time
of Slc26a4 expression on morphogenesis of the membranous lay-
birnhth was evaluated by varying the time point of dox initiation
to E16.5 or E18.5 and paint filling a portion of the membranous
labyrinth of Tg[E];Tg[R];Slc26a4Δ/Δ mice at P3 (Figure 6, A–D) to
visualize their gross morphology. The cochlear duct appeared nor-
mal. The endolymphatic sac and duct appeared slightly enlarged
when dox was initiated at E16.5, but were very enlarged when dox
initiation was delayed until E18.5 (Figure 6, B and C). In contrast,
these structures appeared normal when dox was initiated at E13.5
(data not shown). We also evaluated the effect of time of Slc26a4
expression on the size of the ves-
tibular aqueduct in adult (P28 to
P109) Tg[E];Tg[R];Slc26a4Δ/+ mice (Figure 6, E–H). Initiation of dox
at E16.5 and E18.5 led to an EVA with a 3- and 25-fold increase in
cross-sectional area, respectively (Figure 6, F and G). The cross-
sectional area in Slc26a4Δ/Δ mice was 106-fold larger compared with
that in Slc26a4Δ/+ control mice (Figure 6, E and H). These results
indicate a strong effect of expression of Slc26a4 on morpho-
genesis of the vestibular aqueduct and endolymphatic sac and duct
during the late embryonic period beginning at or near E16.5. In
contrast, Tg[E];Tg[R];Slc26a4Δ/- DE17.5, and even DE16.5, mice
had no endolymphatic enlarge-
ment (not shown).
levels does not underlie the reduced EP in Tg[E];Tg[R];Slc26a4Δ/Δ IE18.5 mice. We also stained the cochlea (Figure 8I) and endolymphatic sac (Figure 8J) of Tg[E];Tg[R];Slc26a4Δ/Δ IE18.5 and genotype control mice at P25 to P35 with anti-pendrin antibodies. Tg[E];Tg[R];Slc26a4Δ/Δ IE18.5 cochlear pendrin immunoreactivity was undetectable or negligible in comparison with that in controls. This indicates that the absence of pendrin through E18.5 results in irreversible loss of inducibility of Slc26a4 expression in Tg[E];Tg[R];Slc26a4Δ/Δ cochlea.

**Discussion**

Here we describe a mouse line in which all pendrin is derived from dox-induced expression of an Slc26a4 cDNA transgene. Our data reveal a developmental period, E16.5 to P2, during which expression of Slc26a4 is required for acquisition of normal hearing (Figure 4), although the EP did not reach normal levels (Figure 5A). This may suggest that pendrin is required over a longer period for the development of a normal EP. It could also indicate that Tg[E];Tg[R];Slc26a4Δ/Δ mice are unable to acquire a normal EP even in the presence of continuous dox exposure. Continued expression of pendrin through P6 increased the EP, but not to normal levels. The onsets of the critical intervals for dox initiation (E16.5) and discontinuation (E15.5) differ by 1 day. This likely reflects the slower kinetics associated with discontinuation of dox and pendrin turnover. The results of the discontinuation and initiation models are thus consistent with each other and collectively suggest that pendrin is required during a limited period at or shortly after E16.5 for development of normal hearing.

The critical time interval for pendrin expression corresponds to the period during which the cochlea grows rapidly (20, 24). The transience of this critical time period for pendrin expression could reflect a temporary period of sensitivity of inner ear development to disruptions of endolymph pH or ionic homeostasis. It is also possible that pendrin mediates exchange of an anion, base, or both that is only temporarily required for normal inner ear physiology and development. I is a known in vitro exchange substrate of pendrin (25), and its concentration probably rises soon following the onset of type 2 deiodinase activity in the late embryonic cochlea (26). Cochlear concentrations and mechanism of I− homeostasis are unknown but, if there were not a mechanism to disperse I−, an excess could conceivably exert a toxic effect on cells within the cochlea, inhibit deiodinase reaction(s) leading to cochlear hypothyroidism (27), or produce a combination of these effects. Finally, the dependence of renal Na+ reabsorption on luminal HCO3− secretion and alkalinization by pendrin (28) raises the possibility of an analogous role in embryonic endolymph. Although K+ is the major osmolyte in mature endolymph, the composition of embryonic endolymph is unknown and Na+ may be an important developmental osmolyte. A decrease in Na+ reabsorption thus might be poorly tolerated, leading to increased osmotic pressure, scala media acidification and expansion, and EVA.

Pendrin is not required, or is required only at undetectable low levels, for acquisition of normal hearing in a normally formed mature inner ear. This raises the possibility that replacement or upregulation of pendrin in a mature ear might not reverse or prevent hearing loss caused by Slc26a4 mutations in human subjects. However, we have not yet determined whether the low EP associated with normal hearing in Tg[E];Tg[R];Slc26a4Δ/Δ DP6 ears leads to later-onset hearing loss. If so, upregulation of pendrin may still be a logical therapeutic objective.

One advantage of inducible Slc26a4 expression is the opportunity to distinguish early primary pathogenetic events from later secondary effects. We were able to distinguish functional consequences associated with loss of pendrin expression in a mature cochlea versus secondary effects of the severe structural malformations associated with complete pendrin deficiency. Both Tg[E];Tg[R];Slc26a4Δ/Δ
IE18.5 (dox initiated at E18.5) and DE17.5 (dox discontinued at E17.5) mice had severe to profound hearing loss, yet scala media enlargement was present in IE18.5 mice only during embryonic development (Figure 7C and ref. 20) and was not observed in DE17.5 mice (Figure 7B). Therefore scala media expansion is unlikely to be a sole direct cause of loss of EP and hearing.

We observed scala media acidification in both Tg[E];Tg[R];Slc26a4Δ/Δ IE18.5 and DE17.5 mice, confirming that cochlear endolymph acidification may indeed be an early pathogenetic event ultimately leading to reduction of the EP and hearing in Slc26a4 deficient ears. The reduction of EP might be mediated by functional degradation of the normally tight permeability barrier of the basal stria vascularis that generate the EP. The loss of the EP likely reflects a developmental disruption or retardation of nonsensory epithelial cell function in the outer sulcus or spiral prominence (i.e., the cells that express pendrin).

The results of this study and others (16) suggest that preserving or supporting strial function and thereby preventing hair cell loss could be a primary target of strategies to prevent or retard hearing loss caused by SLC26A4 mutations. Prevention of scala media acidification could be another goal.

One difference between Slc26a4Δ/Δ mice and Tg[E];Tg[R];Slc26a4Δ/Δ IE18.5 or DE17.5 mice is the lack of endolymphatic hydrops (scala media expansion with distention of Reissner membrane) in the adult cochleae of the inducible expression models. The reported time line of endolymphatic enlargement in Slc26a4Δ/Δ mice (20) suggests that Tg[E];Tg[R];Slc26a4Δ/Δ IE18.5 cochleae also develop endolymphatic hydrops with distention of Reissner membrane until E18.5. The distended Reissner membrane subsequently collapses onto the tectorial membrane by 1 month of age (Figure 7) upon reversal of endolymphatic hydrops with induction of pendrin expression at E18.5. In contrast, Tg[E];Tg[R];Slc26a4Δ/Δ DE17.5 mice probably never develop endolymphatic hydrops, since DE16.5 inner ears show no endolymphatic enlargement.

Induction of Slc26a4 expression at E18.5 did not rescue the ability to express pendrin expression in the outer sulcus of Tg[E];Tg[R];Slc26a4Δ/Δ IE18.5 cochleae (Figure 8). This finding likely reflects a developmental disruption or retardation of nonsensory epithelial cell function in the outer sulcus or spiral prominence (i.e., the cells that express pendrin).

The etiopathogenesis of incremental and progressive hearing loss in humans with SLC26A4 mutations was not addressed by our study. Elucidation of the factors underlying hearing loss stability is important, since stabilization of hearing in humans with EVA is an important therapeutic goal. Tg[E];Tg[R];Slc26a4Δ/Δ mice may be used to explore these issues, since the IE18.5 and DE17.5 paradigms result in residual hearing. The hearing loss observed with these paradigms, particularly the asymmetry and unilaterality associated with the DE19.5, DP0, and DP1 paradigms, is remarkable for its similarity to some human EVA phenotypes. Characterization of the natural history of hearing loss associated with these dox administration paradigms in Tg[E];Tg[R];Slc26a4Δ/Δ mice will be needed to fully explore and exploit this model of human hearing loss associated with EVA.


**Figure 5**

EP, endolymphatic pH, and perilymphatic pH values measured at P25 to P56. Mean ± SEM values of EP (A) and endolymphatic pH (B) are shown for Tg[E];Tg[R];Slc26a4<sup>-/-</sup> and Tg[E];Tg[R];Slc26a4<sup>-/-</sup> littermate controls. Numerals next to data points in all panels indicate numbers of tested mice. The EP and endolymphatic pH control values do not vary among dox administration or genotype groups. Shaded regions represent published mean ± SEM values for Tg[E];Tg[R];Slc26a4<sup>-/-</sup> and Tg[E];Tg[R];Slc26a4<sup>-/-</sup> controls (13) are shown as shaded regions. The EP and endolymphatic pH in Tg[E];Tg[R];Slc26a4<sup>-/-</sup> and Tg[E];Tg[R];Slc26a4<sup>-/-</sup> littermate controls were similar to published values (13) and therefore used the D12Mit270 short tandem repeat (STR) marker 46 kb downstream of Slc26a4 to distinguish WT endogenous Slc26a4<sup>-/-</sup> from the same sequence within Tg[E]. We and other groups identified and genotyped a novel STR marker 233 kb downstream with primer pair 127-2 and 128-2 (Supplemental Table 1) to differentiate Slc26a4<sup>-/-</sup> from slc26a4<sup>-/-</sup> mice and Tg[E];Tg[R];Slc26a4<sup>-/-</sup> mice were crossed to generate experimental animals.

**Methods**

Tg[E]. BAC clone RP23-265L9 (185,766 bp; BACPAC Resources Center) encoding WT C57BL/6J mouse genomic Slc26a4 was used to construct Tg[E] using red/ET recombination technology (Gene Bridges Gmbh; Figure 2). RP23-265L9 was assumed to include all cis-regulatory elements required for inner ear expression, since we found that a similar unmodified BAC (RP23-122C13) restored normal auditory and vestibular function in mouse Slc26a4<sup>-/-</sup>. Electrocompetent E. coli containing pSC101-BAD-gbaA(tet<sup>+</sup>) and RP23-265L9 were transformed with these PCR products by electroporation (2.3 kV, 25 μF, 200 Q) and incubated overnight at 37°C. PCR analyses of colonies were confirmed with NotI (New England Biolabs) digestion and pulsed-field gel electrophoresis, as well as fingerprint analyses of digestion products of BamHI, SacII, KpnI, and NdeI.

Tg[R]. We used the pTRE-Tight-Bi-AcGFP1 vector (Clontech) to generate a bidirectional Tg[R] that also encodes SLC26A4 (penicillin) (Figure 2). Total RNA was isolated from E13.5 C57BL/6J mouse embryos using the RNaseasy Mini Kit (Qiagen), cDNA was synthesized using Superscript III First-Strand Synthesis (Invitrogen). We used primers 189 and 200 (Supplemental Table 1) to amplify a Kozak consensus sequence (GCCACC) followed by mouse Slc26a4 cDNA (32) (c.1A to c.2343G; GenBank NM_011867). The amplification product was cloned into the BamHI site of ApaLI and DdrII to liberate and purify the 4412-bp TRE-Tight-Bi-AcGFP1-Slc26a4 fragment from vector sequences.

**Transgenic mice.** Recombinant RP23-265L9 BAC and linearized TRE-Tight-Bi-AcGFP1-Slc26a4 DNA were microinjected into (C57BL/6J × SJL) F2 mouse oocytes at the Transgenic Core Facility of the University of Michigan. Two Tg[E] founder lines (numbers 928 and 946) and 5 Tg[R] founder lines (numbers 1, 35, 37, 66, 114) were maintained by crossing to C57BL/6J, and the slc26a4<sup>-/-</sup> line was intercrossed to maintain its isogenic 129Sv background. The Mouse Genome Informatics–approved locus symbols are Tg(RP23-265L9/rtTA2-M2/Neo<sup>+</sup>)<sup>Δ</sup> for Tg[E] and Tg(AcGFP/TRE/Slc26a4)<sup>Δ</sup> for Tg[R]. PCR amplifications of digestion products of BamHI, SacII, KpnI, and NdeI confirmed the expected recombination event and purified for supplemental material available online with this article; research article using 42-bp homology arms.

**Genotype analysis.** Genomic DNA was prepared from tail clips using Qiagen DNeasy (Qiagen) or the Maxwell 16 System (Promega). We performed PCR with Taq polymerase (GenScript) to amplify genomic mouse for the presence of Tg[E] (primer pairs 113/114 and 115/116; Supplemental Table 1). The published assay for (numbers 1, 35, 37, 66, 114) were maintained by crossing to C57BL/6J, and the slc26a4<sup>-/-</sup> line was intercrossed to maintain its isogenic 129Sv background. The Mouse Genome Informatics–approved locus symbols are Tg(RP23-265L9/rtTA2-M2/Neo<sup>+</sup>)<sup>Δ</sup> for Tg[E] and Tg(AcGFP/TRE/Slc26a4)<sup>Δ</sup> for Tg[R]. (Tg[E]<sup>Δ</sup>× Tg[R]<sup>Δ</sup>)<sup>Δ</sup>N<sup>Δ</sup>-Tg[E]<sup>Δ</sup> mice were crossed to generate experimental animals.
comprised a 2-minute denaturation at 94°C, 35 step-cycles of 30 sec at 94°C, 30 seconds at 55°C, and 45 seconds at 72°C, followed by a 10-minute elongation at 72°C.

dox. Drinking water containing 0.2 g dox hyclate (Sigma-Aldrich) and 5 g sucrose (MP Biomedicals) per 100 ml of reagent-grade water was prepared twice weekly and administered ad libitum. For the dox initiation paradigm (denoted by I), dox water was substituted for dox-free water at the indicated embryonic or postnatal age estimated from the date of maternal vaginal plugging.

For the dox discontinuation paradigm (denoted by D), dox water was provided to the mother from the time of mating and substituted with dox-free water at the indicated embryonic or postnatal age. Dox initiation or discontinuation is denoted by an I or D, respectively, followed by the developmental time point (day) at which the switch occurred.

Antibodies. Anti-pendrin antisera were generated by immunizing rabbits (Covance) with a synthetic peptide (NH2-CEELDVQDEAM-RRLAS; Princeton BioMolecules) that had a noncoding aminoterminal cysteine for linkage to an affinity matrix and amino acids 766–780 of the C terminus of mouse pendrin (NCBI NP_035997). Three rabbits were immunized to generate PB824, PB825, and PB826 antisera. We purified anti-pendrin IgG with immobilized protein A (Pierce) followed by affinity column purification using an AminoLink Plus Immobilization Kit (Pierce). Polyclonal rabbit antibodies against rat Kcnj10 (K-4.1, Kir1.2) were purchased from Alomone Labs (#APC-035).

Immunohistochemistry. Whole-mounted mouse cochleae, vestibules, and endolymphatic sacs were immunostained essentially as described for pendrin (15) and Kcnj10 (16) with some differences: sections were 10-μm thick, followed by developmental time point (day) at which the switch occurred.

Figure 6
Morphology of the endolymphatic sac and duct and vestibular aqueduct. Slc26a4+/− control (A and E), Tg[E];Tg[R];Slc26a4Δ/Δ IE16.5 (B and F), Tg[E];Tg[R];Slc26a4Δ/Δ IE18.5 (C and G), or Slc26a4Δ/Δ control mice (D and H) were sacrificed at P3 for paint-fill analysis (A–D) or between P28 and P109 for cross-sectional histopathologic measurement of the vestibular aqueduct (VA, shaded pink) adjacent to the common crus (CC, shaded blue; E–H). (I) Mean ± SD values are shown for cross-sectional areas of the vestibular aqueduct. Numbers of mice are indicated. Scale bars: 500 μm (A, applies to A–D; E, applies to E–H). Progressive delays of initiation of pendrin expression to E16.5 and E18.5 result in abnormal enlargement of the endolymphatic duct and sac and vestibular aqueduct. ES, endolymphatic sac; ED, endolymphatic duct; S, saccule; CO, cochlea.

Figure 7
Morphology of the cochlear duct and organ of Corti at P25 to P35. (A–D) Light microscopic images of plastic-embedded sections of the middle cochlear turn stained with toluidine blue. (E–H) Scanning electron micrographs of the organ of Corti from the middle cochlear turn. Scale bars: 100 μm (A, applies to A–C); 200 μm (D); and 10 μm (E, applies to E–H). Note the different scale bar in D for the enlarged cochlear duct of Slc26a4Δ/Δ mice. SLm, spiral limbus; TM, tectorial membrane; SLg, spiral ligament; OHC, 3 rows of outer hair cells; IHC, single row of inner hair cells. The Reissner membrane of Tg[E];Tg[R];Slc26a4Δ/Δ IE18.5 ears is collapsed onto the spiral limbus and tectorial membrane. The spiral ganglion, stria vascularis, organ of Corti hair cells and other structures of the cochlear ducts of Tg[E];Tg[R];Slc26a4Δ/Δ IE18.5 and DE17.5 mice look similar to those of Slc26a4Δ/Δ controls.
Figure 8
Kcnj10 and pendrin expression in the cochlea. (A–D) Anti-Kcnj10 or (E–J) anti-pendrin antibodies were used to immunostain Tg[E];Tg[R];Slc26a4/Δ IE18.5 (A, C, F, and I), Tg[E];Tg[R];Slc26a4/Δ (B, E, and H), or Slc26a4/Δ (D, G, and J) cochlea (A–G) or endolympathic sac (H–J) at P27 to P34. Insets (B–D) show magnified views of the spiral ganglion (SG). Scale bars: 100 μm (A); 50 μm (B, applies to C–G); 5 μm (inset in B, applies to insets in C and D); 50 μm (H, applies to I and J). Kcnj10 staining is evident in the intermediate layer of the stria vascularis as well as the spiral ganglion. Kcnj10 immunoreactivity in the lateral wall of Tg[E];Tg[R];Slc26a4/Δ IE18.5 cochlea appears normal or slightly reduced. Pendrin immunoreactivity is reduced in the cochlea and endolympathic sac of Tg[E];Tg[R];Slc26a4/Δ IE18.5 mice.
and the transepithelial potential were made using double-barreled microelectrodes. Procedures were developed by modifying previously described protocols (13, 37). Measurements were made in the basal turn of the cochlea by a round-window approach through the basilar membrane of the first turn. After placing electrodes in perilymph, the surgical cavity was covered with liquid Silgard 184 (Dow Corning Corp.). This maneuver was designed to prevent the measurement of artificially elevated perilymphatic pH values due to the loss of tissue CO$_2$ into ambient air. Data were recorded digitally (DIGIDATA 1322A and Axs0Scope 9, Axon Instruments) and analyzed using custom software written by P. Wangemann in LabTalk (Origin 6.0, The Origin Company). Calibration consisted of taking a reference value and obtaining the slope of the electrode in situ at 37°C. This method was devised to minimize the contribution of electrode drift and differences between reference electrodes. pH-sensitive electrodes had a slope of 56.7 ± 0.4 mV/pH unit (n = 43). Three calibration solutions with different pH values were used. Calibration solution contained pH 6: 130 mM NaCl, 20 mM MES; pH 7: 130 mM NaCl, 20 mM HEPES; and pH 8: 130 mM NaCl, 20 mM tricine. Double-barreled glass microelectrodes were manufactured from filament-containing glass tubing (1B100F-4; World Precision Instruments) using a micropipette puller (PD-5; Narishige). Prior to silanization, microelectrodes were baked at 180°C for 2 hours to ensure dryness. The longer ion-selective barrel was mounted in the lid of a beaker. The beaker was heated to 210°C and silanized by a 90-second exposure to 0.02 ml dimethion-silane (Alcan Packaging). After silanization, microelectrodes were baked at 180°C for 3 hours and tips were broken to a final OD of approximately 10 μm. The reference barrel was filled with 1 M KCl, and the ion-selective barrel was filled with the liquid ion exchanger (Hydrogen ionophore II – Cocktail A, 95297; Fluka) and back-filled with buffer solution (500 mM KCl, 20 mM HEPES, pH 7.4) (13, 37).

**Statistics.** A 2-tailed Mann-Whitney U test, with a significance criterion of P < 0.05, was used to compare the thickness of the stria vascularis.

**Study approval.** All animal experiments and procedures were performed according to protocols approved by the Animal Care and Use Committees of the National Institute of Neurological Diseases and Stroke/National Institute on Deafness and Other Communication Disorders and by the Animal Care and Use Committee at Kansas State University.

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**Address correspondence to:** Andrew J. Griffith, Otolaryngology Branch, National Institute on Deafness and Other Communication Disorders, NIH, S Research Court, Room 2B-29, Rockville, Maryland 20850-3320, USA. Phone: 301.402.2829; Fax: 301.402.7580; E-mail: griffita@nidcd.nih.gov.

Byung Yoon Choi’s present address is: Department of Otorhinolaryngology–Head and Neck Surgery, Seoul National University Bundang Hospital, Seoul National University College of Medicine, Seongnam, Korea.

Hyung-Mi Kim’s present address is: Department of Otolaryngology, CHA Bundang Medical Center, CHA University, Seongnam, Korea.

Kyu-Yup Lee’s present address is: Department of Otolaryngology–Head and Neck Surgery, College of Medicine, Kyungpook National University, Daegu, Korea.

Kelly Monahan’s present address is: University of Iowa Carver College of Medicine, Iowa City, Iowa, USA.

Yaqing Wen’s present address is: Duke University School of Medicine, Durham, North Carolina, USA.

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