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Atrophic thyroid follicles and inner ear defects reminiscent of cochlear hypothyroidism in *Slc26a4*-related deafness

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Abstract

Thyroid hormone is essential for inner ear development and is required for auditory system maturation. Human mutations in *SLC26A4* lead to a syndromic form of deafness with enlargement of the thyroid gland (Pendred syndrome) and non-syndromic deafness (DFNB4). We describe mice with an *Slc26a4* mutation, *Slc26a4*^{loop/loop}, that are profoundly deaf but show a normal sized thyroid gland, mimicking non-syndromic clinical signs. Histological analysis of the thyroid gland revealed defective morphology, with a majority of atrophic microfollicles, while measurable thyroid hormone in blood serum was within the normal range. Characterization of the inner ear showed a spectrum of morphological and molecular defects consistent with inner ear pathology, as seen in hypothyroidism or disrupted thyroid hormone action. The pathological inner ear hallmarks included thicker tectorial membrane (TM) with reduced β -tectorin protein expression, absence of BK channel expression of inner hair cells and reduced inner ear bone calcification. Our study demonstrates that deafness in *Slc26a4*^{loop/loop} mice correlates with thyroid pathology, postulating that sub-clinical thyroid morphological defects may be present in some DFNB4 individuals with a normal sized thyroid gland. We propose that insufficient availability of thyroid hormone during inner ear development plays an important role in the mechanism underlying deafness as a result of *SLC26A4* mutations.

Introduction

Hearing development and maturation depends on normal thyroid gland function. Congenital hypothyroidism, as well as endemic dietary iodine deficiency, leads to hearing impairment in children (DeLong et al. 1985; Rovet et al. 1996). The murine auditory system continues to develop after birth and reaches complete hearing maturation after weaning. Thyroid hormone (TH) is highly essential during this time window, promoting the later steps of auditory development and function (Knipper et al. 1999). Hence, depletion of TH production by the thyroid gland systemically, or local defects in TH receptors at the target organ, will

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lead to a similar pathology. Cochlear regions sensitive to TH include the organ of Corti and the greater epithelial ridge (GER) (Bradley et al. 1994). The TH receptors TR α 1 and TR β are expressed in the developing cochlea and mediate TH action. Targeted deletion of TH receptors leads to deafness in mice with impaired morphological differentiation of the cochlear sensory epithelium (Rusch et al. 2001; Winter et al. 2006). TR β -deficient mice fail to develop hearing (Forrest et al. 1996), while human TR β biallelic mutations lead to deafness in children due to TH resistance (Refetoff et al. 1967). In the setting of normal expression of cochlear TH receptors, primary hypothyroidism leads to inner ear pathology and deafness. A targeted disruption of *Pax8*, a paired homeobox gene required for thyroid organogenesis, leads to athyroid mice with congenital hypothyroidism and deafness (Christ et al. 2004; Mansouri et al. 1998).

Pendrin, encoded by the *Slc26a4* gene, is a member in the solute carrier 26 family of multifunctional transporters (Dorwart et al. 2008). Human mutations in *SLC26A4* lead to a syndromic form of deafness with enlarged thyroid gland (Pendred syndrome; PS; OMIM #274600) and non-syndromic (DFNB4; OMIM #600791), where only the ear is affected (Everett et al. 1997). Pendrin is a transmembrane protein and is expressed in different tissues, including the thyroid and inner ear. In the thyroid, pendrin is localized to the apical membrane of follicular cells. The spherical follicles are filled with secreted colloid, a proteinaceous substance rich in thyroglobulin (TG), the precursor of thyroid hormone (Arvan and Di Jeso 2005). Functional studies showed that pendrin mediate apical iodide efflux from thyroid follicular cells to the extracellular thyroglobulin rich colloid (Gillam et al. 2004; Scott et al. 1999; Yoshida et al. 2002). Pendrin was shown to be regulated by number of key players proteins involved in TH metabolism. Low levels of TG were shown to increase follicular pendrin protein levels through thyroid-specific gene expression (Royaux et al. 2000). Another level of pendrin regulation is highlighted by the pituitary thyroid stimulating hormone (TSH). In addition to its thyrotrophic role, TSH stimulation mediates the translocation of pendrin to the cell membrane (Pesce et al. 2012). Furthermore, the increase in pendrin membrane abundance is correlated with reduced intracellular iodide concentration, suggesting an active role for pendrin in thyroid iodide efflux. In the inner ear, pendrin functions as a chloride/bi-carbonate exchanger (Kim and Wangemann 2011) with early onset of expression during embryogenesis. A targeted disruption of *Slc26a4* in pendrin null mice (*Pds*^{-/-}) leads to profound deafness but these mice lack systemic thyroid dysfunction. Nonetheless, it has been suggested that local cochlear hypothyroidism leads to the failure to develop hearing in *Pds*^{-/-} mice (Wangemann et al. 2009). The discrepancy of normal thyroid hormone plasma levels in pendrin null mice questions the involvement of systemic thyroid deficiency in the abnormal development of the auditory system [reviewed in (Bizhanova and Kopp 2011)].

Mouse models have proven invaluable in defining the developmental, physiological, and genetic bases of human disease. While gene-targeted mutagenesis has provided information about null mutations, *N*-ethyl-*N*-nitrosourea (ENU)-generated mutants have allowed specific mutations and aberrant protein function, rather than complete depletion of the protein, to provide information about the pathological consequences of the mutation. Previously, we described ENU mice carrying a recessive missense mutation in *Slc26a4* (gene symbol *loop*) that leads to impaired transport activity of its encoded protein pendrin (Dror et al. 2010). As

a result, *Slc26a4^{loop/loop}* mice are profoundly deaf and show severe vestibular dysfunction associated with pathological nucleation of novel calcium oxalate stones in the inner ear. Here we show that *Slc26a4^{loop/loop}* mice show defective thyroid gland morphology manifested by a majority of atrophic microfollicles dispersed and detached from one another. Despite these prominent thyroid defects, the gland is normal in size and serum level of the thyroid hormones triiodothyronine (T3) and thyroxine (T4) are within the normal range. Extensive characterization of the *Slc26a4^{loop/loop}* auditory system reveals a variety of molecular and morphological defects reminiscent of inner ear pathology due to hypothyroidism, including a retarded GER, a thicker tectorial membrane (TM), reduced expression of β -tectorin proteins, impaired development of the spiral limbus, absence of BK expression of inner hair cells and reduced ear bone calcification. Despite significant thyroid pathology, *Slc26a4^{loop/loop}* are systemically euthyroid, suggesting that a local TH deprivation contributes to inner ear malformations as part of the mechanism underlying *Slc26a4*-related deafness.

Materials and methods

Mice

The founder mouse carrying the *Slc26a4^{loop}* mutation was generated in an ENU mutagenesis program (Hrabe de Angelis et al. 2000). The *loop* mice colony was maintained on the original C3HeB/FeJ genetic background. All wild-type mice in this study were aged-matched controls with *+/+* genotypes as compared to homozygous *loop/loop* mutants. All procedures involving animals met the guidelines described in the National Institutes of Health Guide for the Care and Use of Laboratory Animals and have been approved by the Animal Care and Use Committees of Tel Aviv University (M-07-061).

Inner ear dissections

Whole inner ears were dissected from newborn and adult mice as described previously (Dror et al., 2010).

Scanning electron microscopy (SEM)

Whole inner ears were dissected from two-month old mice. SEM was performed as described previously (Dror et al., 2010). Images were acquired using a JEOL JSM-6701F SEM.

Antibodies

For immunohistochemistry and immunoblotting we used previously characterized primary antibodies as follows: rabbit anti-BK (1:200; Sigma Aldrich); mouse anti-parvalbumin (1:500; Swant); mouse anti-thyroglobulin (1:200; Abcam); rabbit anti-pendrin (1:100) (Frische et al. 2003), kindly provided by Jørgen Frøkiær; rabbit anti-otoancorin (1:200) (Zwaenepoel et al. 2002), kindly provided by Christine Petit; rabbit anti- β -tectorin (1:300) (Knipper et al. 2001), kindly provided by Guy Richardson; mouse anti-HSC70 (1:30000, Santa Cruz). The following secondary conjugated antibodies and actin fluorescent dyes were used: rhodamine phalloidin (1:350, Invitrogen); Alexa 488 phalloidin (1:500, Invitrogen); Alexa 488/568 conjugated donkey anti-rabbit (1:350, Invitrogen), Alexa 488/568 conjugated

donkey anti-mouse (1:250, Invitrogen). For immunoblotting, goat anti-rabbit and goat anti-mouse conjugated to HRP (1:20000, Jackson ImmunoResearch) were used.

Immunohistochemistry

For whole-mount preparations, inner ear fixation was performed as previously described (Dror et al., 2010). Immunolabeling was visualized with secondary conjugated antibodies and actin-dyes in adjustment to the source of the primary antibodies. Staining with 4'-6-Diamidino-2-phenylindole (DAPI) was used for the visualization of the cell nucleus. Four biological and technical repeats were performed. For sections, inner ear fixation, decalcification and paraffin sections were performed and prepared as previously described, as well as antigen retrieval and immunolabelling (Dror et al., 2010).

Histology

Thyroid and inner ear fixation was done in 4% paraformaldehyde (PFA) (Electron Microscopy Sciences) in Dulbecco's phosphate-buffered saline (D-PBS) for 4 hours at 4°C. Inner ear decalcification were performed and prepared as previously described (Dror et al., 2010). Paraffin sections were dewaxed in xylene and rehydrated. A standardized hematoxylin and eosin staining procedure was performed to demonstrate the histology and morphology of the inner ear. A Masson's trichrome stain using Accustain Trichrome Stains (Masson) (Procedure No. HT15) was performed to highlight cochlear TM collagen as well as colloid of thyroid in prominent blue color.

Statistical analysis of thyroid follicles area

In order to compare the follicular size of wild type and mutant mice, we measured the follicular area. The histological sections used for this comparison were taken from equivalent anatomical positions of wild type and mutant glands, taking the 3D structure of the thyroid into consideration. Representation of the marginal and central regions of the gland was equal between mutants and controls. We collected 300 measurements of the follicular area of 6 thyroid glands from each genotype. To facilitate data presentation on a plot, we normalized each measurable follicular area by dividing it with the mean area of all wild-type measurements multiplied by 100. A t-test analysis was performed to evaluate the statistical significance between the two tested groups.

Protein extraction and analysis

Western blot analysis was conducted on protein extracts from post-natal day (P)15 cochleae. Inner ears were dissected out, frozen in liquid nitrogen, homogenized and lysed using 1% NP lysis buffer. Protein quantification was performed using Bradford reagent (Sigma Aldrich) and 150ug of total protein from each sample were loaded on an 8% agarose gel. Proteins were transferred onto PVDF membrane, blocked with 5% skimmed milk and blotted using β -tectorin primary antibody and mouse anti-HSC70 (Santa Cruz) as a control. Signal analysis was conducted using ImageJ.

Thyroid hormone analysis

Blood was collected under anesthesia from the superficial vena femoralis. Blood was centrifuged for 10 min/1600g and serum was collected. Serum-free T3 and free T4 levels were measured by a solid phase analyzer 2000 chemiluminescent competitive analog immunoassay using the IMMULITE (Siemens). Quantitative measurements of circulating non-protein bound hormones were interpolated from a standard curve with calibrated free T4/free T3 concentrations, respectively. In this assay system, the normal range for free T4 was 0.8–2.0 ng/dL and 3.1–6.8 pmol/L for free T3.

RESULTS

Thyroid abnormalities present in *Slc26a4^{loop}* mice

Pendrin is expressed in different tissues, including the inner ear, kidney thyroid and endometrium (Lacroix et al. 2001; Royaux et al. 2001; Suzuki et al. 2002). Interestingly, Pendrin expression in *Slc26a4^{loop}* mice appears to be normal in all tissues tested, with exception of the thyroid. Expression analysis of P20 mice thyroid glands show that pendrin failed to reach the plasma membrane in the expressing follicular cells of *Slc26a4^{loop}* mice (Fig. 1). In contrast to the prominent staining of pendrin at the luminal side of the follicular cell seen in wild type mice, a diffused staining with indeterminate cellular localization in *Slc26a4^{loop}* follicular cells is apparent. Distribution of the aberrant pendrin protein appears in the cytoplasm and within the extracellular matrix between cells.

To further characterize the adverse effect of the *Slc26a4^{loop}* mutation on thyroid tissues, we compared the size of the thyroid gland between mutants and their littermate controls. Whereas no significant difference in thyroid gland dimensions was observed, a cross section through the tissue revealed atrophic changes at the microscopic level (Fig. 2A–H). Masson's trichrome staining on paraffin sections demonstrated the histological characteristics of the gland, including follicular cells surrounding colloids, as well as extracellular matrix between follicles. Unlike the normal appearance of the large colloid-sharing wide contact area with neighboring follicles in the control, *Slc26a4^{loop}* mice showed a majority of micro-follicles with extended extracellular space between counterparts. Moreover, atrophic colloid and follicular cells were apparent throughout the surrounding section. To compare the follicular size of wild type and mutant mice we measured the area of individual follicles. For accurate comparison, we analyzed sections from equivalent anatomical positions in the 3D structure of the thyroid gland. In each image we only measured follicles with continuous boundaries while excluding the follicles that were partially visible in the captured field. We analyzed a sum of 300 measurements from each genotype. A statistical analysis of these measurements show a significant reduction in the follicular area of *Slc26a4^{loop}* mice (Fig. 2I–J).

Serum-free T3 and free T4 levels were measured by a chemiluminescent competitive analog immunoassay, with no significant difference between wild type and mutant mice. For T3, the measurements were 4.66 ± 0.11 (mean $\pm$ SEM; wild type, N=4), 4.81 ± 0.25 (mutant, N= 5). For T4, the measurements were 2.26 ± 0.23 (wild type, N= 4), 2.58 ± 0.25 (mutant, N= 5).

Inner ear defects in *Slc26a4^{loop}* mutants consistent with cochlear hypothyroidism

In parallel to the thyroid characterization, *Slc26a4^{loop}* mice show a wide variety of inner ear malformations. In this study we highlighted a subset of inner ear defects that are consistent with the phenotype seen in hypothyroidism.

Among the macro malformations we identified, an abnormal bone deformity was found in the mutant inner ear. A comparison of isolated inner ears from wild type and mutant mice showed reduced calcification of the mutant inner ear (Fig. 3). While normal inner ear bones are prominently calcified at the age of P15, *Slc26a4^{loop}* bones show a significant blue staining of the skeletal preparation, an indication of impaired calcium deposition. As a consequence, the mutant ear is brittle and required no decalcification prior to histological sectioning, as opposed to the durable bone of the controls.

The cochlear fluid-filled compartments of *Slc26a4^{loop}* mice inner ears undergo a prominent distortion due to hydrops of endolymphatic compartments (Dror et al. 2011). Further investigation of these changes demonstrated that hyperplastic changes were seen within the GER, observed with a specific marker (otoancorin) for this sub-population of epithelial cells (Fig. 4). The increased cell number in the GER was followed by hypertrophic changes observed with an enlarged cell surface (inset in Fig. 4). On the other hand, the specialized sensory epitheliums of the cochlea showed greater resistance against the stretching forces of the hydrops pressure. A top view of the sensory epithelium demonstrated that the organ of Corti, which contains the cochlear hair cells, maintained its normal width despite the tension generated by the SM hydrops. Scanning electron microscopy of the sensory epithelium surface revealed massive destruction of inner and outer hair cells (Fig. 5). The structure of the stereocilia bundles of the sensory cells ranged from mildly disorganized to greatly deteriorated and complete absence of the bundle. A gradient of hair cell degeneration appeared along the snail shape of the cochlea. Whereas an inner hair cell degeneration gradient occurred from the base to the apex, the outer hair cell degeneration gradient progressed from apex to base.

The extracellular matrix of the TM has functional significance on the mechanoelectrical transduction (Richardson et al. 2008), and is affected by the *Slc26a4^{loop}* mutation. An enlarged and thicker TM was apparent in *Slc26a4^{loop}* mice in all regions of the cochlea (Fig. 6). As opposed to the condensed TM of the wild type mice, the swollen TM of *Slc26a4^{loop}* showed loose connective collagen bundles with an atypical 'basket-weave' appearance. At the molecular level, the aberrant TM expressed a lower amount of β -tectorin protein quantified by western blot analysis.

Impaired maturation of the cochlea in *Slc26a4^{loop}* mice

An additional hallmark of abnormal cochlear maturation was demonstrated with immunohistochemistry analysis of BK channel expression of inner hair cells (Fig. 7). Normal expression of BK is shown in a section from P10 mice, with typical distribution of the channel within the outer membrane of the cells above the nuclei level. BK is absent from *Slc26a4^{loop}* inner hair cells in all cochlear regions.

DISCUSSION

Mutations in *SLC26A4* are either linked to non-syndromic (DFNB4) or syndromic forms of deafness (PS). PS patients characterized with enlarged thyroid gland, which also defined as goiter in clinical examination (Everett et al. 1997). The presence of DFNB4-affected individuals that lack thyroid clinical signs suggests that the deafness entity is independent of thyroid function. On the other hand, the existence of an enlarged thyroid gland in PS patients with overt hypothyroidism raises the question of the possible involvement of thyroid dysfunction as part of the etiology of *SLC26A4*-related deafness. The important role of thyroid hormone in hearing development suggests that within the subgroup of *SLC26A4* syndromic patients, thyroid dysfunction may contribute to their deafness.

Pendrin mutations lead to variable thyroid manifestation in both human and mouse

In this work we show that profoundly deaf mice with a missense mutation in the *Slc26a4* gene have abnormal thyroid gland histology and morphology. Previously reported mouse models for PS, including the *Pds*^{-/-} (Everett et al. 2001) and *Slc26a4*^{tm1Dontuh/tm1Dontuh} mice (Lu et al. 2011), were all consistently deaf but did not show thyroid histopathological alterations. Unlike other mouse models, *Slc26a4*^{loop/loop} mice fail to maintain normal thyroid histology and show a majority of atrophic microfollicles. The inconsistency of a thyroid phenotype in the different *Slc26a4* mouse models is not surprising and correlates with human thyroid clinical variation (Gonzalez Trevino et al. 2001). Several explanations for the different thyroid manifestations can be suggested, focusing on the interaction between genetics, molecular and environmental factors. Whereas *Slc26a4*^{loop/loop} mouse was established on the C3HeB/FeJ background (Dror et al. 2010), other mouse models were maintained on C57BL/6 Black Swiss (Everett et al. 2001) and 129Sv/Ev mouse strains (Lu et al. 2011). A remarkable example demonstrates how specific the genetic background of *Prop1*^{df} mutant protects against hypothyroidism-induced deafness (Fang et al. 2012). Whereas both *Pou1f1*^{dw} and *Prop1*^{df} mice has same undetectable serum level of TH, *Pou1f1*^{dw} mutant are profoundly deaf while *Prop1*^{df} hearing is mildly affected. The large number of *SLC26A4* mutations in humans, combined with the variety of genetic background, contributes to a different *SLC26A4* thyroid phenotype. Functional assays have shown that different mutations within the *Slc26a4* sequence have different effects on pendrin transport activity and thus may explain the significant clinical heterogeneity (Pera et al., 2008). We have previously reported that the *Slc26a4*^{loop/loop} S F mutation in pendrin dramatically reduces its functional transport capacity (Dror et al. 2010). Moreover, other mutations affect subcellular localization of pendrin in a way that it fails to reach the plasma membrane (Brownstein et al., 2008). The interaction between environmental and genetics factors may also trigger and exacerbate thyroid malfunction when an appropriate genetic background of thyroid susceptibility is present (Gonzalez Trevino et al. 2001). Likewise, it is reasonable that different diet conditions can either enhance or mask a clinical picture of thyroid pathology.

The potential role of pendrin in thyroid function

The prominent expression of pendrin in thyroid follicular cells (Everett et al. 1997), together with its known capacity to mediate iodide efflux (Scott et al. 1999), raised the hypothesis

that in the thyroid, pendrin functions as apical exchanger. On the other hand, the prevalence of patients homozygous for the *SLC26A4* mutation that lacks thyroid manifestation (Sato et al. 2001) suggests that other iodide channels compensate for the loss of pendrin. The CLCN5 chloride channel, located at the apical membrane of follicular cells, can potentially generate iodide efflux towards the follicular lumen (van den Hove et al. 2006). An elevated expression of CLCN5 in Pendred patients (Senou et al. 2010), together with the typical goiter phenotype of *Clcn5* null mice, supports this assumption (van den Hove et al. 2006). The well-established function of pendrin as an HCO_3^- exchanger in the inner ear suggests that it has a similar function in the thyroid (Wangemann et al. 2009). Acidification of the thyroid follicular lumen in *Slc26A4* null mice supports the potential role of pendrin as an HCO_3^- exchanger in this system. This study provides a first example for thyroid phenotypic variation in a mouse with a mutation in the *Slc26a4* gene. Histopathology analysis of thyroids from *Slc26a4^{loop/loop}* mice shows atrophic microfollicles with reduced colloid content. A possible reduction in thyroid follicular pH is a potential trigger for atrophic processes of the thyroid in *Slc26a4^{loop/loop}* mice. At the cellular level, pendrin failed to reach the plasma membrane of follicular cells but retained normal expression and localization in other organs such as kidney and trachea. It is known that *SLC26A4* mutations affect the cellular localization of pendrin protein and altered level of N-glycosylation during processing (Yoon et al. 2008). In the presence of a disease-causing mutation, a cell specific alternatively spliced isoforms (Laurila and Vihinen 2009) or altered protein environment of different cell types (Ferrer-Costa et al. 2002) can influence localization of the protein in one system but not another. Here we show how the *Slc26a4^{loop/loop}* mutation leads to pendrin mislocalization in thyroid cells exclusively in a tissue-specific mechanism.

While the actual transport properties of pendrin in the thyroid remain to be elucidated, it is clear that key players of the thyroid endocrine pathway regulates pendrin expression. Pituitary thyroid stimulating hormone (TSH) regulates pendrin translocation to the cell membrane, followed by an increase of iodide efflux (Pesce et al. 2012). Eliminating the PKA phosphorylation site of pendrin abolishes the response to PKA stimulatory pathway and results in decreased pendrin membrane abundance and iodide efflux (Bizhanova et al. 2011). Iodide within the follicular colloid undergoes an organification process (summarized in Fig. 8) that yields the production of TH T3 and T4, which are released to the blood circulation for distribution towards target organs (Dohan et al. 2003; Kopp 2005). In *Slc26a4^{loop/loop}* mice, the levels of plasma thyroxine were consistently within the normal range. This evidence suggests that despite the prominent morphological defects of the *Slc26a4^{loop/loop}* thyroid, its synthetic capacity is not affected by the pendrin mutation.

An important clinical tool for evaluating iodide organification and TH synthesis is the perchlorate test. Whereas some patients show a partial organification defect (Morgans and Trotter 1958), others exhibit normal perchlorate testing (Albert et al. 2006; Gonzalez Trevino et al. 2001). It has been suggested that endemic nutritional iodide abundance is a contributing factor for hypothyroidism development in *SLC26A4* affected individuals. Pendred's patients with a normal iodide diet in Korea (Park et al. 2005) and Japan (Tsukamoto et al. 2003) have clinically normal thyroid function as opposed to hypothyroid Pendred patients in iodide-deficient regions of Mexico (Gonzalez Trevino et al. 2001). The development of thyroid deficiency is also highly variable within families among affected

siblings that carry the same bi-allelic *SLC26A4* mutation (Fraser 1965). Despite the absence of goiter in *Slc26a4^{loop/loop}* mice, a significant tissue deformation is observed. Our findings suggest that thyroid histopathological defects may be present in the patients with non-syndromic deafness (DFNB4) as well. Support for this hypothesis is highlighted by a study reporting that *SLC24A4*-affected individuals had partial organification defects regardless of the existence (PS) or absence (DFNB4) of thyroid enlargement and goiter (Pryor et al. 2005).

Hypothyroidism-induced deafness associated with the *Slc26a4* mutation

The data presented in this study suggest that pendrin dysfunction exerts its deleterious effect on the auditory and thyroid systems. The function of pendrin in the inner ear as a HCO_3^- exchanger regulates acid base balance of endolymphatic fluids (Wangemann et al. 2007). The reduction in endolymphatic pH in *Slc26a4*-null mice interferes with normal cochlear histology and function (Kim and Wangemann 2011; Scott et al. 1999). In addition, failure of fluid absorption in the developing cochlea leads to subsequent hydrops of inner ear compartments and distortion of its normal structure (Kim and Wangemann 2010). The presence of additional inner ear manifestations in *Slc26a4^{loop/loop}* mice, reminiscent of the phenotype seen in cochlear hypothyroidism, raised the possibility that a TH-associated mechanism contributes to *Slc26a4*-related deafness. The abnormal cochlear hallmarks related to hypothyroidism include, but are not limited to, a thicker TM, reduced β -tectorin protein levels (Knipper et al. 2001), retarded bone calcification and delayed BK-channel expression in cochlear inner hair cells (Brandt et al. 2007). Here we show that *Slc26a4^{loop/loop}* mice present all of the above auditory manifestations, with additional morphological and molecular defects consistent with hypothyroidism. The concordance of thyroid pathology with atrophic micro follicles raised the hypothesis that possible systemic hypothyroidism contribute to the *Slc26a4^{loop/loop}* deafness. Nonetheless, *Slc26a4^{loop/loop}* mice show normal plasma thyroxin similar to *Slc26a4*-null mice (Wangemann et al. 2009). The normal range TH level suggests that the cochlear phenotype of *Slc26a4^{loop/loop}* is partially attributed to local hypothyroidism that is limited to the inner ear. The prominent auditory hydrops of *Slc26a4* deficient mice likely affects capillary circulation by physical tension, altering local availability of essential hormone and nutrients. Endolymphatic acidification, secondary to pendrin dysfunction, can also lower the activity of certain pH sensitive enzymes that are required for TH activation. The type 2 deiodinase (D2), an enzyme that expressed in the inner ear, is responsible for converting the circulating thyroxin to its locally active form T3 that exert TH receptors upon binding. Spatial and temporal activity of D2 confers the inner ear with the ability to regulate the availability of T3 hormone in critical developmental time window (Ng et al. 2004). A decrease in cochlear *Dio2* expression in *Slc26a4*-null mice leads to reduction of T4 to T3 conversion, with impaired TH availability to tissue (Wangemann et al. 2009). The effect of local deprivation in the active form of TH, T3, can leads to auditory defects that are morphologically indistinguishable from systemic hypothyroidism. In both scenarios, as with other causes of hypothyroidism, a similar hallmark of inner ear pathology is apparent.

In conclusion, our study shows that similar to the thyroid phenotypic variation in humans, a mutation-specific mechanism of *Slc26a4^{loop/loop}* leads to thyroid defects in mice. Despite

the normal sized thyroid gland of *Slc26a4^{loop/loop}* mice, the abnormal histology suggests that pendrin function is essential for maintaining morphological integrity of the thyroid, at least with a particular genetic background. Nonetheless, the morphological defects of the thyroid do not affect the systemic availability of T3 and T4 hormones of the thyroid. The typical inner ear defects of *Slc26a4*-related deafness are attributed to TH dependent and independent pathways. Among a variety of auditory defects in *Slc26a4^{loop/loop}* mice, the inner ear developmental delay and dysfunction is reminiscent of rather local hypothyroidism. Expanding the spectrum of *Slc26a4* mutations of mouse models under altered genetic backgrounds will further illuminate the role of pendrin in thyroid function and its related deafness. A further understanding of the molecular basis of thyroid-related phenotypic variation in PS patients will enable the development of a mutation-specific algorithm for prediction of clinical outcome and future treatment.

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Fig. 1.

Pendrin is selectively mislocalized in *Slc26a4^{loop}* mice thyroid but not in other tissues. (A) Pendrin is expressed in the spiral ligament of the auditory system in epithelial cells facing the endolyphatic fluids. In the cochlea, pendrin is normally expressed in the spiral prominence (sp) and the outer sulcus (os). No difference in pendrin expression was found in *Slc26a4^{loop}* mutant inner ears. Insets, high-resolution images. (B) In the kidney, pendrin (green) is expressed on the apical side of renal non-type A intercalated cells. The abundance of pendrin is reduced in *Slc26a4^{loop}* mice. (C) The localization of pendrin in the kidney cells that retain its expression appears to be normal in *Slc26a4^{loop}* mice. (D) A transverse section of the trachea shows normal expression of pendrin in the apical side of the stratified ciliated epithelium facing the airways in both *Slc26a4^{loop}* and control mice. (E–F) In contrast to other tissues, pendrin is mislocalized in *Slc26a4^{loop}* thyroid follicular cells. While normally pendrin is expressed in the plasma membrane at the luminal side of the follicular cells, diffused expression of pendrin is observed in *Slc26a4^{loop}* tissue. Scale bars: 25 μ m. For each genotype: N=4 (P20 mice).

Fig. 2.

A majority of atrophic micro-follicles appear in *Slc26a4*^{loop/loop} mice thyroid gland. Wild-type and mutant thyroid gland morphology and histology is shown. Masson's trichrome histological colors index: blue, collagen bone and mucus; light red, cytoplasm; dark brown, cell nuclei. (A–B) Comparison of the size of the whole thyroid gland did not show a significant difference between wild-type and *Slc26a4*^{loop/loop} mice. (E–F) Macroscopic characteristic of *Slc26a4*^{loop/loop} mice show no distinct gross morphological malformations, either in shape or size of the thyroid gland. (C–D) A closer look at histological sections shows that a wild-type mouse has a majority of macro-follicles (blue) with apparent condensed distribution. Many adjacent follicles contact each other with wide adhering regions. Each follicular colloid (blue) is surrounded by a monolayer of follicular cells (red). (G–H) Higher magnification images taken from equivalent central region of the thyroid lobe show that *Slc26a4*^{loop/loop} mice display a defective morphology characterized by a majority of micro-follicles (blue) with dispersed distribution. (H) The atrophic follicles of the mutant lack the normal adhesion surfaces with their neighboring counterparts, with prominent extracellular space between one another. Several *Slc26a4*^{loop/loop} follicles are surrounded by more than one layer of follicular cells (red). (I) To compare the follicular size of wild type and mutant mice, the area of individual follicles was measured. For accurate comparison, we

analyzed sections from equivalent anatomical positions in the 3D structure of the thyroid gland. (J) The distribution of the follicular size data is presented on the plotted graph. The follicular area was normalized by dividing the measurable event by the mean area of WT follicles multiplied by 100. The differences in follicular area between wild type and mutant are statistically significant with a p-value < 0.001 . Scale bars: 500 μm in A, 100 μm in B, 50 μm in C, 25 μm in D. For each genotype: N=8 (P60 mice).

Fig. 3.

Impaired bone calcification of *Slc26a4^{loop}* mice inner ears. (A–F) A convex and concave view of wild-type (A–C) and mutant (D–F) P15 inner ear stained with Alizarin red S and Alcian blue, a differential staining of bone (red) and cartilage (blue). (A–C) During the process of inner ear maturation, the surrounding cartilaginous tissue undergoes rapid calcification and hence shows predominantly red staining. (C) A higher magnification of (B) focusing on the anterior semicircular canal shows a prominent red staining representing the calcified bone. (D–F) A distinguishable blue staining appears in all cochlear and vestibular surrounding structure of *Slc26a4^{loop/loop}* mice, an indication of high levels of cartilage, as opposed to the mineralized bone in wild-type mice. asc, anterior semicircular canal. Scale bars: 1 m in A, B–D; 0.5 m in E–F. For each genotype: N=5 (P15 mice).

Fig. 4.

Non-sensory cells hyperplasia of *Slc26a4*^{loop} cochlear duct. The mechanical stress exerted by the endolymphatic hydrops in newborn *Slc26a4*^{loop} inner ear is followed by an increase in number and size of non-sensory cells of the cochlear duct. (A–B) Whole mount cochlear preparations labeled with otoancorin (red), which stain epithelial cells of the GER, and actin (green), which label the sensory cells in the organ of Corti (OC). (B) The otoancorin expressing cells of the GER undergo massive expansion in cell number (hyperplasia) in *Slc26a4*^{loop} mutant mice. An increase in cell size (hypertrophy) is also apparent (inset in A and B). (C–D) Paraffin cross section through the cochlear duct is compared between newborn *Slc26a4*^{loop} mutant mice and their littermate controls. (D) The otoancorin (red) staining demonstrates the expanded GER facing a bulged scala media of *Slc26a4*^{loop} mutant mice. (E) The width of the *Slc26a4*^{loop} GER (red) is significantly wider as compared to wild-type mice, with a three-fold increase. The width of the sensory epithelium containing the cochlear hair cells, however, retains its normal dimension. Statistics conducted using a Student's t-test, p-value=0.001. Scale bars: 50 μm. For each genotype: N=5 (P14 mice).

Fig. 5.

A base to apex gradient of cochlear hair cell degeneration. Wild type and *Slc26a4^{loop}* SEMs of cochlear sensory hair cells are compared. (A) A typical arrangement of wild-type cochlear sensory cells includes one row of inner hair cells (ihc) and three rows of outer hair cells (ohc). Higher magnifications of single inner and outer hair cells are shown, demonstrating the normal structure of the hair bundle stereocilia in a staircase manner. (B–D) Base, mid and apical representative captions of the *Slc26a4^{loop}* mice cochlear sensory epithelium are shown. The inner hair cells of the base of the cochlea remain morphologically normal while gradual progressive degeneration is observed towards the apex, where the most disrupted hair bundles are apparent. As compared to the inner hair cells, the outer hair cells show an opposite gradient of degeneration, from apex to base. Whereas almost no outer hair cells could be detected in the base of the cochlea, a significantly greater number of outer hair cells were apparent in the apex. Nonetheless, the outer hair cells of the apical region show abnormal morphology, while their hair bundles are partially degenerated. Scale bars: 20 μ m. For each genotype: N=8 (P15 mice).

Fig. 6.

Thicker TM of *Slc26a4*^{loop} mice acquired reduced β -tectorin protein expression. Wild type (A) and *Slc26a4*^{loop/loop} (B) P15 organs of Corti from the mid region of the cochlea are compared. (A) Wild-type mice show a normal morphology of the TM (blue) attached to the spiral limbus (SL), as demonstrated by Masson's trichrome histological staining. (B) In *Slc26a4*^{loop/loop} mice, the TM is prominently thicker as compared to the control. Furthermore, the SL that serves as the TM attachment zone has lost its native spiral wave structure and is significantly smaller. Fluorescent immunostaining of α -tectorin highlights the TM morphological differences between mutant and control mice. (C) Detection of β -tectorin in the TM of P15 mice shows a prominent decrease in protein expression in *Slc26a4*^{loop} mutants. Quantification analysis of the corresponding western blot demonstrates the significant reduction in β -tectorin protein expression in the mutant TM. Statistics conducted using a Student's t-test, p-value=0.002. Scale bars: 50 μ m. For each genotype: N=8 (P15 mice).

Fig. 7. BK potassium channel expression is absent in *Slc26a4*^{loop} mice cochlear inner hair cells. (A) Schematic illustration of inner hair cells demonstrating the normal localization of BK channels at the cytoplasmic membrane of inner hair cells above longitudinal level of the nucleus (blue). (B) Staining against BK potassium channel of P10 wild type section show its normal patchy localization at the cell membrane. *Slc26a4*^{loop} mice lack BK channel expression in cochlear inner hair cells. Scale bar: 5 μ m. For each genotype: N=4 (P10 mice).

Fig. 8.

The potential role of pendrin in deafness is proposed through thyroid dependent and independent mechanisms. (A) In the inner ear, pendrin dysfunction abolishes HCO_3^- secretion into the endolymph with its subsequent acidification. Impaired inner ear homeostasis with prominent enlargement of extracellular compartments is attributed to a direct effect of pendrin insufficiency in the auditory system. Other morphological and molecular defects, such as thicker TM, reduced β -tectorin proteins expression, abnormal bone calcification and absence of BK expression in inner hair cells are all consistent with thyroid hormone deficiency. (B) The potential roles of pendrin in thyroid follicular cells are illustrated. The first role is related to pendrin participation in generating active iodide efflux into the colloid lumen. The second role is based on the well known function of pendrin in the inner ear as a HCO_3^- exchanger, suggesting that pendrin mediates HCO_3^- secretion into the thyroid follicular lumen, preventing acidification of the colloid. The hallmark of auditory defects, consistent with TH deprivation during development could be either due to local (inner ear) or systemic (thyroid) hypothyroidism. SV, scala vestibuli; SM, scala media; ST, scala tympani, TM, tectorial membrane; tg, thyroglobulin; nis, sodium-iodide symporter; tpo, thyroid peroxidase.