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DEVELOPMENT OF THE STRIA VASCULARIS AND POTASSIUM REGULATION IN THE HUMAN FETAL COCHLEA: INSIGHTS INTO HEREDITARY SENSORINEURAL HEARING LOSS

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ABSTRACT

Sensorineural hearing loss (SNHL) is one of the most common congenital disorders in humans, afflicting one in every thousand newborns. The majority is of heritable origin, and can be divided in syndromic and nonsyndromic forms. Knowledge of the expression profile of affected genes in the human fetal cochlea is limited, and as many of the gene mutations causing SNHL likely affect the stria vascularis or cochlear potassium homeostasis (both essential to hearing), a better insight into the embryological development of this organ is needed to understand SNHL etiologies. We present an investigation on the development of the stria vascularis in the human fetal cochlea between 9 and 18 weeks of gestation (W9-W18), and show the cochlear expression dynamics of key potassium-regulating proteins. At W12, MITF+/SOX10+/KIT+ neural-crest-derived melanocytes migrated into the cochlea and penetrated the basement membrane of the lateral wall epithelium, developing into the intermediate cells of the stria vascularis. These melanocytes tightly integrated with Na^+/K^+-ATPase-positive marginal cells, which started to express KCNQ1 in their apical membrane at W16. At W18, KCNJ10 and gap junction proteins GJB2/CX26 and GJB6/CX30 were expressed in the cells in the outer sulcus, but not in the spiral ligament. Finally, we investigated GJA1/CX43 and GJE1/CX23 expression, and suggest that GJE1 presents a potential new SNHL associated locus. Our study helps to better understand human cochlear development, provides more insight into multiple forms of hereditary SNHL, and suggests that human hearing does not commence before the third trimester of pregnancy.

INTRODUCTION

According to recent estimates of the World Health Organization, more than 5% of the world's total population suffer from some form of disabling hearing loss [1]. A large part of this hearing loss is of sensorineural nature, making sensorineural hearing loss (SNHL) the most prevalent sensorineural disorder in humans. SNHL is also the most common congenital disorder, with a prevalence of 1 in every 1000 newborns in the UK and the Netherlands (with a bilateral hearing loss $\geqslant$ 40 dB [2, 3]). Of these, over two-thirds can be attributed to genetic factors. Hereditary SNHL can be classified in syndromic (~25%) and nonsyndromic (~75%) forms [4].

Examples of syndromic SNHL include Pendred's syndrome ([MIM 274600], combining hearing loss and goiter [5, 6]), Waardenburg syndrome (hearing loss and pigmentation abnormalities [7, 8]) and Jervell and Lange-Nielsen syndrome ([MIM 220400 and MIM 612347], hearing loss and cardiac symptoms [9]). In nonsyndromic SNHL, associations with obvious abnormalities of the external ear or symptoms other than hearing loss cannot be found, but the heterogeneity of loci and genes is high: over 130 loci have been mapped and over 60 different genes have been implicated presently (Van Camp & Smith). Nonsyndromic forms are grouped in autosomal dominant (DFNA), autosomal recessive (DFNB), X-linked (DNFX) and mitrochondrial subtypes. The most commonly affected gene causing nonsyndromic hearing loss (both in autosomal recessive DFNB1A [MIM 220290] and in autosomal dominant DFNA3A [MIM 601544]) is *gap junction beta 2* (*GJB2*, [MIM 121011]), the gene encoding the connexin 26 protein (CX26) [11, 12]. Depending on the studied population, 20-50% of all recessive nonsyndromic SNHL cases can be attributed to a mutation in *GJB2* [13, 14]. For a comprehensive overview of other affected genes we refer to recent reviews [15 – 19].

In SNHL, the disorder lies either in the cochlea itself, or in any of the retrocochlear auditory structures. Cochlear hair cells are responsible for converting sound into electrical signals that travel to the brainstem via the cochlear nerve [20]. Hair cell function depends on the endocochlear potential, a positive extracellular potential ($\approx$80-100 mV relative to perilymph) in the endolymph of the cochlear duct (or scala media), generated by an unusually high concentration of potassium ions (K^+) [21]. These ions are secreted into the endolymph by specialized cells within the stria vascularis, located in the lateral wall of the cochlear duct [22]. The stria vascularis is highly vascularized and consists of three layers of distinct cell types: the marginal

cells, the intermediate cells (melanocytes), and the basal cells [23, 24]. It is generally accepted that the depolarizing K^+ flow causing hair cell activation in the organ of Corti is recycled back to the stria vascularis via the epithelial lining of the cochlear duct and the spiral ligament fibrocytes, and/or through the perilymph, as depicted in Figure 1. To maintain the endocochlear potential, this recycling system requires a specific distribution of cochlear cell types as well as selective ion channels and gap-junctions [25, 26].

Therefore, it is not surprising that many gene mutations causing SNHL either result in functional changes of the ion channels involved in K^+ homeostasis or cause an abnormal cellular morphology in the cochlea. Although progress has been made in identifying the genes responsible for SNHL in humans, knowledge on their actual expression in the human cochlea is limited and only a few studies have investigated the morphological development of the stria vascularis in the human fetal cochlea [27, 28]. In this paper, we address both aspects, focussing on the embryonic development of the lateral wall in the human fetal cochlea. We show the expression profiles of several genes involved in syndromic and nonsyndromic SNHL (Table 1) between 9 and 18 weeks of gestation (W9-W18). More specifically, we have investigated the development of the different cell types in the stria vascularis and studied the expression of K^+-regulating and gap junction proteins. Together, our data gives insight in hereditary SNHL and provides a basis for K^+-recycling models in the human cochlea.

Table 1   Selected Genes and Association with Syndromic and Nonsyndromic Sensorineural Hearing Loss

Gene	MIM	SNHL in Genetic Disorder (*MIM*)
MITF	156845	Waardenburg syndrome, type 2A (*193510*)
		Waardenburg syndrome/ocular albinism, digenic (*103470*)
		Tietz albinism-deafness syndrome (*103500*)
SOX10	602229	Waardenburg syndrome, type 4C (*613266*)
		Waardenburg syndrome, type 2E (*611584*)
		PCWH syndrome (*609136*)
		Kallman syndrome with deafness (*not defined*)
KIT	164920	Piebaldism (*172800*)
KCNQ1 (KVLQT1)	607542	Jervell and Lange-Nielsen syndrome (*220400*)
KCNJ10 ($K_{ir}4.1$)	602208	Enlarged vestibular aqueduct, digenic (*600791*)
		SESAME syndrome (*612780*)
GJB2 (CX26)	121011	DFNA3A (*601544*)
		DFNB1A (*220290*)
		Hystrix-like ichthyosis with deafness (*602540*)
		Keratitis-ichthyosis-deafness syndrome (*148210*)
		Bart-Pumphrey syndrome (*149200*)
		Keratoderma, palmoplantar, with deafness (*148350*)
		Vohwinkel syndrome (*124500*)
GJB6 (CX30)	604418	DFNA3B (*612643*)
		DFNB1B (*612645*)
		DFNB1A, digenic (*220290*)
GJA1 (CX43)	121014	DFN not defined
GJE1 (CX23)	Not defined	Unknown

METHODS

Tissue samples

Human fetal cochleas were collected from tissues obtained by elective abortion (healthy subjects, no medical indication) using vacuum aspiration. Prior to the procedure, obstetric ultrasonography was used to determine the gestational age in weeks and days. Of all collected cochleas, we discarded those which where mechanically damaged or showed marked tissue degeneration, upon inspection by light-microscopy. Cochleas of the following gestational ages were collected and used: W9, W9.1, W10.4 (2x), W12 (2x), W12.2, W14 (2x), W16 (2x), W18 (3x). The cochleas were obtained in PBS, fixed in 4% paraformaldehyde in PBS overnight at 4° C, decalcified and embedded in paraffin as previously described [29]. The use of human fetal material was approved by the Medical Ethical Committee of the Leiden University Medical Center (protocol 08.087). Informed written consent was obtained in accordance with the WMA Declaration of Helsinki guidelines.

Immunohistochemistry

The cochleas were cut in 5 or 10 µm thick sections in the sagittal plane using a RM2255 microtome (Leica). Deparaffinization and immunohistochemistry were carried out as previously described [29]. Briefly, antigen retrieval was performed (see below) and sections were consecutively incubated with primary and secondary antibodies diluted in blocking solution consisting of 1% bovine serum albumin (Sigma-Aldrich) and 0.05% Tween-20 (Promega) in PBS. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories). The primary antibodies used in this study were mouse anti-acetylated tubulin (aceTUBA, 1:500, T6793, Sigma), rabbit anti-collagen type IV (COL4, 1:200, AB748, Chemicon), rabbit anti-fibronectin (FN, 1:400, F3648, Sigma-Aldrich), rabbit anti-GJA1 (1:1000, C6219, Sigma), rabbit anti-GJA1 (1:2000, ACC-201, Alomone labs), rabbit anti-GJB2 (1:100, ACC-212, Alomone labs), rabbit anti-GJB6 (1:100, PA511640, Thermo Scientific), rabbit anti-GJE1 (1:1000, NBP2-14051, Novus biologicals), goat anti-KCNJ10 (1:100, NBP1-70371), rabbit anti-KCNQ1 (1:200, ab65092, Abcam), rabbit anti-KCNQ1 (1:100, APC-022, Alomone labs), rabbit anti-KIT (1:100, A4502, Dako), rabbit anti-laminin (LAM, 1:200, Z009701, Dako), rabbit anti-melan-A (1:500, NBP1-30151, Novus), mouse anti-microphthalmia-associated transcription factor (MITF, 1:100, M3621, Dako), mouse anti-Na+/K+-ATPase α1 (ATP1A1, 1:200, α6F, Developmental Studies

Hybridoma Bank), rabbit anti-solute family carrier 2, member 1 (SLC2A1, 1:500, ab15309, Abcam) and goat anti-SOX10 (1:50, sc-17342, Santa Cruz Biotechnology). Prior to immunostaining for COL4, LAM and FN antibodies, antigen retrieval was performed by incubating sections for 5 minutes with 20 μg/ml proteinase K (Promega) in Tris-EDTA-CaCl2 buff er (pH8.0). In all other cases, tissue sections were treated for 12 minutes at 97 ˚ C with 0.01M sodium citrate buff er (pH 6.0) and allowed to cool down. The secondary antibodies used were Alexa Fluor (AF) conjugated immunoglobulins (Life Technologies): AF 488 donkey anti-mouse (A-21202), AF 488 donkey anti-rabbit (A-21206), AF 488 donkey anti-goat (A-11055), AF 568 donkey anti-mouse (A-10037), and AF 568 donkey anti-rabbit (A-10042), all at a 1:500 dilution. As antibody specificity controls, primary antibodies were routinely omitted (negative control), isotype controls were performed (negative control) and immunostainings were performed on adult mouse cochlea (positive control). On human fetal tissue, at least three separate immunostaining experiments were performed with each primary antibody. To further verify specificity, all immunostaining results were checked to confirm the appropriate cellular location of the fluorescent signals and tissue expression patterns were compared to previous studies using experimental animals, if available.

Image acquisition and processing

Confocal images were acquired with a Leica SP8 confocal laser scanning microscope operating under the Leica Application Suite Advanced Fluorescence software (LAS AF) using Leica objectives (40x/1.3 oil HC PL Apo or 63x/1.4 oil HC PL Apo). Maximal projections were obtained from image stacks with a z-step size of 0.5 μm. Brightness and contrast adjustments consistent with image manipulation policies were performed either with LAS AF, Fiji (ImageJ version 1.48k [30]) or Adobe Photoshop CS6 (Adobe Systems) image-processing software.

RESULTS

Melanocytes invade the lateral wall epithelium at W12 and develop into the intermediate cells

The cochlear melanocytes that form the intermediate cell layer of the stria vascularis are thought to originate from the neural crest. In order to investigate the formation of the three cell layers of the stria vascularis, we first determined the cochlear location of melanocytes and monitored their invasion into the lateral wall epithelium during embryonic development of the human fetal cochlea. Immunostaining for melan-A ([MIM 605313], a marker of melanocytes) and acetylated α-tubulin (aceTUBA, which labels various types of epithelial and neural cells) allowed us to visualize melanocyte location and general cochlear architecture. At W9.1, the melan-A+ melanocytes were located both in the part of the periotic mesenchyme that will develop into the otic capsule (called hereafter: otic capsule), and in the part of the periotic mesenchyme that will form the spiral ligament and the spiral limbus (called hereafter: periotic mesenchyme), but not in the epithelium of the cochlear duct (Figure 2A-B, Figure S1). More specifically, most melanocytes were found in the apical half of the developing cochlea situated above the cochlear duct, and only a few could be observed in the periotic mesenchyme bordering the lateral wall epithelium, i.e. the future stria vascularis (Figure 2B, right arrowhead). Immunostaining for basement membrane proteins laminin (LAM) (Figure S2A), collagen type IV (COL4) (Figure S2B) and fibronectin (FN) (Figure S2C) at W9.4 showed a strictly continuous and smooth basement membrane, further confirming the mesenchymal location of the melanocytes. At W10.4, the cochlear duct had spiralled twice around the central axis of the cochlea and melan-A+ melanocytes were observed exclusively in the periotic mesenchyme of the future middle and basal turns (Figure 2C-D, arrowheads). When compared to W9.1, more melanocytes were located close to the lateral wall epithelium.

At W12, melan-A+ melanocytes were observed in all cochlear turns. In the apical and middle turns, melanocytes were still located exclusively to the periotic mesenchyme (Figure 2E). However, in the basal turn, melanocytes were observed both in the periotic mesenchyme (Figure 2F and arrowheads in Figure 2G) and in the lateral wall epithelium of the cochlear duct (the future stria vascularis), indicating that they had penetrated through the basement membrane (arrows in Figure 2G). This finding is in agreement with the observation that the basement

membrane at this location showed irregular staining patterns for LAM, COL4 and FN, in contrast to its smooth and continuous appearance near the developing Reissner's membrane and at the location of the outer sulcus (Figure 2H-I and three-dimensional reconstruction provided in Interactive PDF file 1).

Melanocytes in the stria vascularis express MITF, SOX10 and KIT

Next, we investigated the expression of two genes, *MITF* [MIM 156845] and *SOX10* [MIM 602229] that are expressed in the melanocytic lineage. At W12, MITF was expressed in all turns in both the melan-A+ melanocytes in the periotic mesenchyme and in the melanocytes that had invaded the developing stria vascularis (Figure 3A). Previously, we have shown that SOX10+ cells are present in the spiral ganglion and throughout the cochlear epithelium [31]. In the present study, we also detected SOX10 expression in the presumptive melanocytes bordering the stria vascularis (Figure 3B, arrowheads).

Double immunostaining for MITF and KIT [MIM 164920], the latter protein being expressed in both the neural crest and the melanocytic lineage, at W12 showed that KIT was expressed by all MITF+ melanocytes (Figure 3C). In addition, KIT expression was found in the cochlear duct epithelium, in a few remaining mesenchymal cells within the developing scala tympani (Figure 3C-D), and in the spiral ganglion (data not shown).

In the developing stria vascularis, melanocytes integrate with the marginal cells in a spatiotemporal order

At W12, we detected a clear spatial gradient in the location of melanocytes, with epithelial invasion commencing in the basal turn (Figure 2E-F). To monitor this development, we immunostained cochleas at W14, W16 and W18 for melan-A and aceTUBA. At W14, melan-A+ melanocytes were still confined to the periotic mesenchyme in both the upper middle (M2) and lower middle (M1) turns (Fig 4A, B). In the upper basal turn (B2), melanocytes were observed invading the lateral wall epithelium (Figure 4C) and in the lower basal turn (B1) most melanocytes were found integrating with the developing marginal cells (Figure 4D). At W16, integrating melanocytes were found in greater numbers and observed throughout all turns (Figure 4E-H). In addition to melanocytes in or near the lateral wall epithelium, we also consistently observed the presence of melan-A+ melanocytes

along the edges of the otic capsule, here shown in the M2 turn at W16 (Figure 4I, arrowheads) and the B1 turn at W18 (Figure 4J, arrowheads). At W18, melanocytes were found tightly integrated with the developing marginal cells in the B1 turn, and some melanocyte processes were also observed around the developing strial capillaries (Figure 4K, arrowheads). Expression patterns of aceTUBA (Figure 4K) and the basement membrane proteins LAM, COL4 and FN better accentuated the development of other regions in the lateral wall epithelium (Figure 4L-N, Figure S2D-L and Figure S3, and three-dimensional reconstruction provided in Interactive PDF files 2 and 3). In particular, with these immunostainings the spiral prominence is clearly emerging, thereby demarcating the stria vascularis from the future root cells in the outer at W18.

Until W18, SLC2A1+ basal cells were not detected in the stria vascularis

In the stria vascularis, in addition to the marginal cells and the melanocytes, we also investigated the formation of the third and innermost strial layer, i.e., the basal cells. However, until W18, we were unable to detect any basal cells by immunostaining for solute family carrier 2, member 1 (SLC2A1, also known as GLUT1, [MIM 138140]), a glucose transporter known to be expressed by the rodent cochlear basal cells and vascular endothelial cells [32]. SLC2A1 expression was found in the developing human stria vascularis but this expression was confined to capillaries and erythrocytes (Figure S4). Interestingly, we did observe consistent expression of SLC2A1 on the apical (luminal) membranes of all epithelial cells between the organ of Corti and the spiral prominence (Hensen's cells in the organ of Corti as well as Claudius' cells and root cells in the outer sulcus) at all studied stages (from W12 to W18, Figure S4).

Dynamics of KCNQ1 in the developing human cochlear duct

To investigate the onset of expression and developmental distribution of ion channels and enzymes involved in cochlear K^+ transport and the generation of the endocochlear potential, we immunostained for the K^+ channel protein KCNQ1 [MIM 607542]. At W10.4 (Figure 5A) and W12.2 (Figure 5B), reactivity was observed at the basement membrane delineating the lateral wall epithelium. At W14, this pattern had changed to reactivity in the developing Reissner's membrane (arrowhead in Figure 5C) and the future outer sulcus (arrow in Figure 5C). From W16 (Figure 5D, 5F) to W18 (Figure 5H, 5I) increased KCNQ1 expression was observed at the

apical (luminal) membrane of the developing marginal cells in the stria vascularis, in addition to reactivity in Reissner's membrane (Figure 5E, 5H) and the future root cells of the outer sulcus (Figure 5G, 5J). However, a different anti-KCNQ1 antibody clone (Alomone) that also recognizes the C-terminus of KCNQ1 did not show any reactivity in Reissner's membrane or in the root cells of the outer sulcus. It did, however, show identical reactivity in the developing marginal cells at W16 and W18 (data not shown). Although we cannot explain this difference as both antibodies should recognize the same KCNQ1 transcript, expression of KCNQ1 in Reissner's membrane has been reported previously [33].

Dynamics of Na^+/K^+ ATPase in the developing cochlear duct

Already at W10.4, immunostaining for the alpha-1 subunit of Na^+/K^+-ATPase (ATP1A1, [MIM 182310]) was observed in different degrees throughout the entire epithelium of the cochlear duct, with a moderate intensity in the future stria vascularis (Figure 6A). However, at W12 ATP1A1 expression increased in the developing stria vascularis on the basolateral membranes of the developing marginal cells (Figure 6B). At W14 and W16, ATPA1 expression became even more prominent and was located in cells adjacent to the increasing numbers of invading melan-A+ melanocytes (Figure 6C-E). Immunostaining diminished in most parts of the epithelium, and at W18 ATP1A1 expression was found on the basolateral membranes of the marginal cells, on the basolateral membranes of the developing hair cells in the organ of Corti, as well as on both the apical and basolateral membranes of the Claudius' cells and the root cells in the outer sulcus (Figure 6F-H). Curiously, at this stage, pillar cells in the organ of Corti were also consistently found to stain positive for melan-A in the basal turn, although these are not of melanocytic origin (Figure 6G). Focusing on the stria vascularis, we found melan-A+ melanocyte processes penetrating between the marginal cells towards the lumen of the cochlear duct (Figure 6I, arrow). We also observed ATP1A1 expression around the melan-A+ melanocyte cell bodies (Figure 6I, arrowhead) and around melanocyte processes encircling the strial capillaries (Fig 6J, arrows).

KCNJ10 expression is confined to the outer sulcus at W18

From W16 onwards, we observed KCNJ10 in the future root cells cells of the outer sulcus (Figure 7). However, we did not observe KCNJ10 expression by strial melanocytes at any of the investigated stages (data not shown).

Distribution of GJB2, GJB6, GJA1 and GJE1 in the human fetal cochlea

To investigated the developmental expression of gap junction proteins we immunostained for GJB2 (CX26), GJB6 (CX30, [MIM 604418]), GJA1 (CX43 [MIM 121014]), all known to be expressed in the human cochlea [34 – 36], and GJE1 (CX23 [MIM unknown]), which expression pattern has not been investigated previously in the cochlea (see Discussion).

Throughout the investigated stages (W10.4-W18), the GJB2 and GJB6 expression patterns remained identical to each other (Figure 8). At W10, immunostaining for GJB2 and for GJB6 showed a diffuse and punctated pattern in both Kölliker's organ and the cells of the future outer sulcus (Figure 8A, B). By W12, expression of GJB2 and GJB6 had greatly increased and could clearly be observed on both sides of the developing organ of Corti (Figure 8C, D). In the subsequent weeks, up to W18, this pattern remained consistent as both in the Kölliker's organ and in the cells lining the outer sulcus (Claudius' cells and future root cells) expression of GJB2 and GJB6 was observed (Figure 8E-J). Although expression of both GJB2 and GJB6 has been reported in the spiral ligament fibrocytes in the adult human cochlea [35], no expression could be observed in these cells at least up to W18 (Figure 8J). We also did not detect expression in the organ of Corti itself.

Table 2   Spatiotemporal Expression Patterns of Investigated Proteins in the Developing Human Fetal Cochlea

Protein	Expression
SLC2A1 (GLUT1)	W12–W18: apical membrane of epithelial cells between organ of Corti and root cells in the outer sulcus (i.e. Hensen's cells, Claudius cells), capillaries, erythrocytes
ATP1A1	W10: throughout cochlear duct epithelium
	W12: increased expression in lateral wall epithelium
	W14–W18: basolateral membranes of marginal cells, basolateral membranes of inner and outer hair cells, basolateral membranes of epithelial cells in outer sulcus, Kölliker's organ.
KCNQ1 (KVLQT1)	W10–W12: basement membrane lateral wall epithelium
	W14: Reissner's membrane, developing root cells in outer sulcus
	W16–W18: apical membrane marginal cells, Reissner's membrane, developing root cells in outer sulcus
KCNJ10 (K_{ir}4.1)	W10–W14: no expression observed
	W16–W18: epithelial cells in outer sulcus, including root cells
GJB2 (CX26)	W10–W12: increased expression in epithelium of cochlear duct floor, with the exemption of the prosensory domain
	W14–W18: expression in Kölliker's organ and epithelial cells in outer sulcus
GJB6 (CX30)	Identical pattern to GJB2
GJA1 (CX43)	W10–W12: no expression observed
	W14–W18: increased expression in type I fibrocytes in the spiral ligament
GJE1 (CX23)	W10–W12: basolateral membranes of epithelial cells in lateral wall
	W14: basolateral membranes of marginal cells, melanocytes
	W16–W18: exclusive to melanocytes

Immunostaining for GJA1 showed weak expression by a significant subgroup of spiral ligament fibrocytes at W14 (Figure 9A). In the subsequent weeks, this expression became more prominent and at W18 could be clearly defined as belonging to the type I fibrocytes (Figure 9B, C).

Immunostaining for GJE1 revealed a dynamic pattern of expression during human cochlear development. At W10.4 and W12, strong expression of GJE1 was observed in the basolateral membranes of the future marginal cells (Figure 10A, B). Interestingly, at W14, expression was observed both in the lateral wall epithelium and in some of the cells in the adjacent periotic mesenchyme, presumably melanocytes (Figure 10C). At W16, expression of GJE1 was downregulated by the developing marginal cells and became exclusively limited to those adjacent cells (Figure 10D). Double immunostaining at W18 for GJE1 and ATP1A1 confirmed the melanocytic origin of the signal, as no overlap in GJE1 and ATP1A1 was observed (Figure 10E, F).

An overview of the observed spatiotemporal expression patterns of selected proteins in this study is presented in Table 2.

Discussion

Are human cochlear melanocytes derived from the neural crest?

The developmental pattern of melanocyte distribution observed in the human fetal cochlea closely follows that of the mouse, where it is generally accepted that cochlear melanocytes are derived from the neural crest [37 – 39]. Therefore, we suggest that the melanocytes in the human cochlea are also derived from the neural crest. However, in all species analysed so far, the migratory route taken by melanocytes to arrive in the cochlea remains unknown. As cochlear development in humans progresses slower than in mice (as a rule of thumb, 1 day of rodent cochlear development corresponds to 1 week in humans), this permits a more explicit visualization of developmental events. Recently, it has been reported that cranial melanocytes can arise from Schwann cell precursors migrating together with outgrowing nerves [40, 41]. As Schwann cell precursors arrive in the cochlea via the cochlear nerve [42], it is tempting to propose that melanocytes (precursors) may travel along the same path. However, our data on melanocyte migration towards their target location in the cochlea, which to our knowledge has not been shown before in such clarity, suggests that cochlear melanocytes in humans migrate through the periotic mesenchyme from the opposite side. In agreement, several studies in mice embryos show the presence of neural crest derivatives or melanocytes near this part of the otic vesicle around embryonic day 10.5 [37 – 39, 41, 42]. Therefore, although the peripheral glial cells in the cochlea originate from the migratory wave of neural crest cells from rhombomere 4, we now hypothesize that cochlear melanocytes originate from a different wave of neural crest cells, namely those delaminating from the region at rhombomere 6, at the location of the developing glossopharyngeal nerve (cranial nerve IX) and the third pharyngeal arch.

Cochlear melanocytes, a major player in syndromic SNHL

Intermediate cells in the stria vascularis were identified as melanocytes in 1977, although their exact function was unknown at that time [24]. The involvement of cochlear melanocytes in generating the endocochlear potential was recognized later in a studies with 'viable dominant spotting' mouse mutants, with a mutation in the *W* locus [43, 44]. Shortly hereafter, *Kit* was found to be the gene product of the *W* locus [45, 46], and it was shown to primarily affect the survival of migratory melanoblasts [47]. In humans, *KIT* mutations can result in the neurocristopathy (a

pathology affecting normal neural crest development) piebaldism [MIM 172800], a disorder characterized by areas of skin and hair devoid of melanocytes. Although most of these patients have a heterozygous mutation, deafness has been observed in rare cases of both heterozygous and homozygous mutations [48, 49]. We show that KIT is expressed by strial melanocytes in the human fetal cochlea, suggesting that SNHL due to *KIT* mutations in humans is caused by mechanisms similar to those in mouse *Kit* mutants.

Another neurocristopathy affecting melanocyte development with pigment abnormalities and SNHL is the genetically heterogeneous Waardenburg syndrome (WS), the most common type of autosomal dominant SNHL in humans. Its four subtypes are based on clinical symptoms and multiple causative genes have been identified [8]. We show here that in the human fetal cochlea, MITF expression is confined to melanocytes (Figure 3A, 3C). Therefore, it is likely that cochlear melanocytes are responsible for SNHL in WS with *MITF* mutations (causing type IIa WS [MIM 193510]), and/or *PAX3* mutations (a gene regulating *MITF*, causative for type I WS [MIM 193500] and type III WS [MIM 148820]). In addition to *MITF*, mutations in *SOX10* can also cause type II WS in next to type IVc WS [MIM 613266] [50, 51], whereas *SOX10* has recently also been identified to play a role in Kallmann syndrome with deafness (KS, [MIM 147950, 244200, 308700, 610628, 612370, and 612702]) [52]. SOX10 expression, however, is not exclusively limited to melanocytes in the human fetal cochlea (Figure 3B). We recently observed additional expression throughout the human fetal cochlear duct epithelium [29] and in all peripheral glial cells [31]. Although it is likely that melanocytes play a major role in SNHL in WS or KS due to *SOX10* mutations, it cannot be excluded that other SOX10+ cell types in the cochlea are involved as well. Taken together, the melanocytic expression of MITF, SOX10 and KIT in the human fetal cochlea help to explain the aforementioned hereditary hearing disorders and underscore the important role of this cochlear cell type in syndromic SNHL.

The endocochlear potential and its relation to hereditary SNHL

Hearing depends on the highly positive endocochlear potential, generated by the interplay of various ion channels and transporters in the cell types of the stria vascularis (reviewed in [25, 53]). The location of these proteins along the membranes of the different cells of the adult stria vascularis is depicted in Figure 1B. Disrupting the function of any of these proteins (including connexin proteins) in

the stria vascularis or at other locations in the cochlea where they are thought to be involved in K+ homeostasis results in immediate SNHL [54 – 56]. In many cases of both syndromic (as mentioned above) and nonsyndromic SNHL, the endocochlear potential is likely to be affected.

The marginal cells in the stria vascularis express the voltage-gated potassium channel KCNQ1 (or KCNE1, [MIM 176261]). Mutations in *KCNQ1* cause the autosomal recessive Jervell and Lange-Nielsen syndrome [MIM 220400], characterized by SNHL and cardiac abnormalities (long QT syndrome) [9, 57]. In this syndrome, there is an impaired K^+ secretion into the endolymph by the KCNQ1/KCNE1 channel complex on the apical membranes of the marginal cells in the stria vascularis, as confirmed in a mouse model with the homozygous mutants being completely deaf [58]. Since a low number of functional channels still result in normal hearing in heterozygous patients [59], it can be surmised that, to cause deafness, mutations in *KCNQ1* have to inactive the channel complex completely so that the total K^+-secretion is reduced to a minimum. On the basolateral membranes of the marginal cells, K^+ uptake from the intrastrial space (the narrow fluid-containing space between the marginal cells and the melanocytes) is mediated by the sodium-potassium pump Na^+/K^+-ATPase [60] and the $Na^+2Cl^-K^+$ cotransporter (NKCC). Na^+/K^+-ATPase is known to be expressed in the adult human stria vascularis [61] and inhibition of Na^+/K^+-ATPase directly suppresses the endocochlear potential in guinea pigs [55, 62]. We found first expression of KCNQ1 on the luminal membranes of the marginal cells at W16. At W18, we observed that the cell processes from the developing marginal cells (expressing ATP1A1) and the strial melanocytes intermingle and form an intricate network together with the capillaries. The developmental expression of KCNQ1 and ATP1A1 indicates that both K^+ uptake and secretion could be mediated by marginal cells as early as W16-W18.

The pivotal role of strial melanocytes in the generation of the endocochlear potential is to secrete K+ ions into the intrastrial space and hence they express the inward-rectifying potassium channel KCNJ10 (also known as Kir4.1, [MIM 602208]) [63, 64]. In contrast to the potassium channels of the marginal cells, we did not observe any KCNJ10 expression in strial melanocytes up to the last stage we investigated, W18. As inhibition of this receptor directly suppresses the endocochlear potential [55], it is likely that the endocochlear potential is not yet generated at this stage. KCNJ10 is implicated in multiple syndromic disorders with SNHL. Loss-of-function mutations in *KCNJ10* cause SESAME syndrome ([MIM 612780], also called EAST syndrome), a disorder characterized by SNHL, electrolyte imbalance,

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seizures, ataxia and mental retardation. Here, the hearing loss is likely due to disrupted function of strial melanocytes [65]. KCNJ10 is also implicated in SNHL in autosomal recessive deafness-4 (DFNB4) with enlarged vestibular aqueduct (EVA) [MIM 600791], and in autosomal recessive Pendred syndrome [MIM 274600]. In Pendred syndrome, *SLC26A4* [MIM 605646] mutations account for the majority of cases [66]. The dysfunction of its protein, PENDRIN, results in loss of KCNJ10 expression in the melanocytes of the stria vascularis and subsequent loss of the endocochlear potential [67, 68]. However, mutations in *KCNJ10* itself have also been linked directly to Pendred syndrome [69]. Interestingly, KCNJ10 has also been described in the root cells of the outer sulcus, both in the adult rodent and human cochlea [64, 70]. Also, the expression pattern of KCNJ10 that we observed in the outer sulcus during cochlear development matches the known expression pattern of PENDRIN in the outer sulcus of the adult mouse cochlea [67, 71]. This provides a striking link between the two proteins and could help to explain the etiology of SNHL in Pendred syndrome.

Several connexins (Cx, gap junction proteins) are expressed at various locations in the cochlea and are thought to be implicated in K^+ recycling [25, 72]. Mutations in connexins are a major cause of hereditary SNHL [18]. The most frequent causative gene for nonsyndromatic SNHL is *GJB2* (CX26) [18]. Although less frequent, mutations in *GJB6* (CX30) [73, 74] and *GJA1* (CX43) [75, 76] have also been linked to SNHL. These gap junction proteins are thought to play a role in maintaining cochlear ion homeostasis and the endocochlear potential, as shown in *Gjb6*-deficient mice [56], by passively recycling K^+ ions back to the stria vascularis. The observed expression in the developing human cochlea is in support of this model. However, an interesting note can be made with regard to the observed glucose transporter (SLC2A1) expression on the luminal membranes of the outer sulcus cells. As this part of the cochlea does not have its own vascularization, one could speculate that receptor expression at this luminal location functions to take up glucose circulating in the endolymph, suggesting a rather more active role for these cells with regard to cochlear K^+ homeostasis (with a potential role for the Na^+/K^+-ATPase?).

Finally, GJE1 has not been previously investigated in the human cochlea. *GJE1* encodes the connexin 23 protein, and should not be confused with CX29/CX30.2, which used to be called *GJE1* but recently has been renamed to *GJC3* [MIM 611925]). In the developing human fetal cochlea, we observed dynamic GJE1 expression in the lateral wall, shifting from the developing marginal cells to the melanocytes between W14-W16. We speculate that GJE1 could be a suitable candidate gene for

non-syndromic SNHL, especially as a many causes of hereditary deafness remain presently unknown [77].

Maturation of the lateral wall and the onset of human hearing

In many rodent species, development of hearing extends beyond birth and the onset of hearing occurs after birth (in an altricial manner). In contrast, hearing development in humans progresses at a much slower rate, but the cochlea and hearing function are complete before birth (precocial hearing development). Although cochlear size and degree of spiralization diff ers between species, the morphological appearance of the various cochlear tissues and structures as well as the subsequent protein expression profiles are very similar. Therefore, based on similarities in morphology and protein expression patterns in adult mammalian cochleas, we conclude that the human fetal cochlea at W18 demonstrates a nearly adult phenotype, but that some essential elements for functionality are still missing. Most notably, strial melanocytes do not yet express KCNJ10, basal cells are immunohistochemically undefined, root cells have not developed their root-like basolateral processes, and GJB2 and GJB6 are not yet observed in the spiral ligament. This implies that the endocochlear potential cannot be generated at this stage and, therefore, that the human fetus at W18 is still unable to hear. Although W20 has been proposed by us [29, 31] and others [78 – 80] as the onset of human hearing based on the maturation of the organ of Corti and the cochlear nerve, it is more likely that hearing commences a few weeks later, which is in line with otoacoustic emission measurements [81] and auditory brainstem responses in preterm infants [82, 83].

In mice and gerbils, the appearance of the endocochlear potential has been observed just prior to their onset of hearing [43, 84], and coincides with morphological maturation of gap and tight junctions. Even although their auditory system does not become functional before birth, the sequence of morphological maturation is similar to what we observe in human fetuses. In view of these findings, extrapolation of our observations to later fetal stages suggests that the endocochlear potential, and therefore hearing, in the human fetus does not emerge before the third trimester of pregnancy.

CONCLUSION

We have investigated the complex embryonic development of the lateral wall in the human fetal cochlea with respect to the expression of several genes that are known to be involved both in syndromic and nonsyndromic SNHL, providing an etiological basis for these hearing disorders in humans. Although hereditary SNHL can be caused by specific mutations in various genes involved in cochlear K^+ transport, we would like to underline the major functional role of melanocytes in the cochlea and the generation of the endocochlear potential. We suggest that many cases of hereditary SNHL can be attributed to a disturbed development or migration of neural-crest-derived melanocytes.

Acknowledgments

Web Resources

Online Mendelian Inheritance in Man (OMIM), http://www.omim.org/

Hereditary Hearing Loss Homepage, http://hereditaryhearingloss.org/

GeneReviews: Deafness and Hereditary Hearing Loss Overview, http://www.ncbi.nlm.nih.gov/books/NBK1434/

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SUPPLEMENTAL MATERIAL AND METHODS

Immunohistochemistry

An additional primary antibody used in the supplementary data is mouse anti-TUBB3 (1:500, 2G10, Ab78078, Abcam).

Image acquisition, processing and reconstruction

Image restoration (deconvolution) was applied to scans shown in Figure S3. These maximal projections were obtained from images stacks acquired at sampling density according to the Nyquist rate. Deconvolution was performed using Huygens Professional version 4.3.1 software (Scientific Volume Imaging). Three-dimensional (3D) reconstructions were made from deconvoluted image stacks using AMIRA 5.3.3 (Visage Imaging), and interactive PDF files were created using Adobe Acrobat Pro Extended version 9.5.5. (Adobe Systems). For clarity, erythrocytes present in the scala vestibuli and scala tympani were manually masked with black pixels in Figure S4F. To generate the image in Figure S1A, ten consecutive tissue sections were scanned and their subsequent maximum projections were overlain manually.