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Cellular development of the human cochlea and the regenerative potential of hair follicle bulge stem cells

Locher, heiko

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Author: Locher, Heiko

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INTRODUCTION

HEARING: ANATOMY AND PHYSIOLOGY

Of all the human senses, hearing is the most astounding achievement of evolution. Sound, be it spoken language or originating from another source, reaches the tympanic membrane by travelling through the external auditory meatus (ear canal) (Figure 1). The sound waves are propagated by the tympanic membrane (eardrum) that passes its vibrations on to the ossicles, the three smallest bones of the human body: the malleus (hammer), incus (anvil) and stapes (stirrup). The footplate of the stapes, which is inserted into the oval window, serves as a piston so that the mechanical energy is transferred into the cochlea, which houses the sensory receptors of hearing.

The cochlea (from the Greek *κοχλίας* (kokhlias) meaning snail) is a bony structure that coils around its own axis. In humans it has approximately 2.5 turns^{footnote1}. It contains three fluid-filled compartments of which the outer two, the scala vestibuli and scala tympani, are connected in the helicotrema at the apex of the cochlea. The middle compartment, the

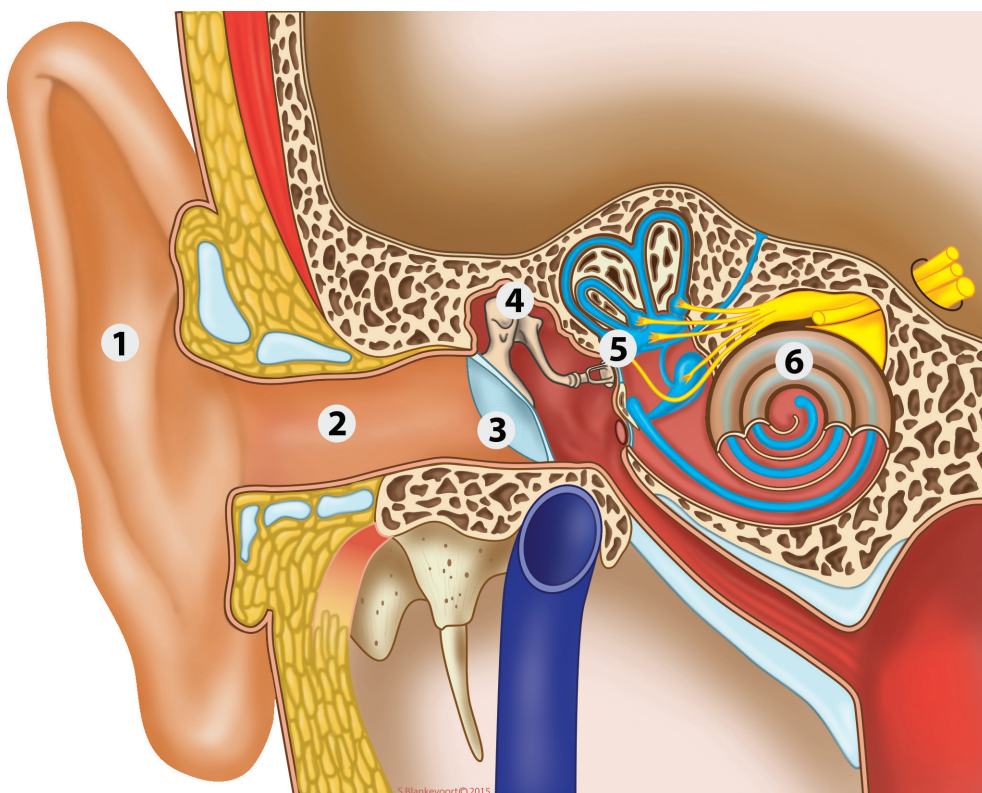


FIGURE 1. SCHEMATIC ILLUSTRATION OF THE OUTER, MIDDLE AND INNER EAR.

1: auricle (pinna), 2: external auditory meatus (ear canal), 3: tympanic membrane (eardrum), 4: the three auditory ossicles (malleus or hammer, incus or anvil, and stapes or stirrup), 5: oval window with the footplate of the stapes, 6: cochlea. Picture courtesy of S.B. Blankevoort and R.G.E. Noteboom.

cochlear duct (or scala media), contains the structure that houses the sensory receptors of hearing: the organ of Corti (Figures 2 and 3).

Vibration of the stapes creates a traveling wave in the fluid (perilymph) of the scala vestibuli and scala tympani. This results in displacement of the floor (basilar membrane) of the cochlear duct, onto which the organ of Corti is located. The location of maximum displacement depends on the sound frequency: high frequencies are detected in the basal turn, whereas lower frequencies are detected in the more apically located turns. The organ of Corti contains the hair cells: in humans, it consists of one row of inner hair cells (~3,500 cells) and three rows of outer hair cells (~12,000 cells) [1]. If the displacement of the basilar membrane is large enough, mechanosensitive transduction channels in the stereocilia of the hair cells are opened. The fluid of the cochlear duct (endolymph) is rich in potassium, which is secreted by the stria vascularis, and opening of the transduction channels causes an influx of potassium ions leading to depolarization of the hair cells. Here, the mechanical energy is converted into an electrochemical signal as the hair cell activates the connecting nerve endings (synapses) of the spiral ganglion neurons by releasing neurotransmitters. Inner hair cells are the principal sensory receptors of hearing, whereas outer hair cells are thought to affect cochlear sensitivity. Multiple type-I spiral ganglion neurons (90-95% of the total population) make synaptic contacts with a single inner hair cell, while a single type-II spiral ganglion neuron contacts multiple outer hair cells. Spiral ganglion neurons are the afferent transmitters of information: upon neurotransmitter release by the hair cells, spiral ganglion

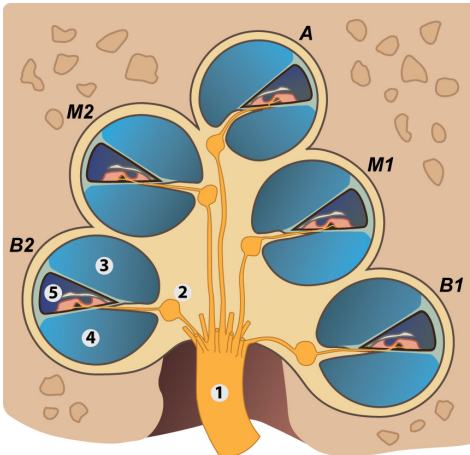


FIGURE 2. SCHEMATIC REPRESENTATION OF A MIDMODIOLAR TRANSECTION OF THE ADULT HUMAN COCHLEA.

1: cochlear nerve, 2: spiral ganglion, 3: scala vestibuli, 4: scala tympani, 5: cochlear duct / scala media. B1: lower basal turn, B2: upper basal turn, M1: lower middle turn, M2: upper middle turn, A: apex. Picture courtesy of S.B. Blankevoort.

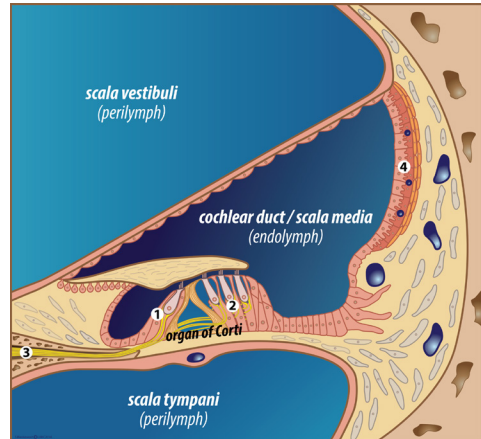


FIGURE 3. SCHEMATIC ILLUSTRATION OF THE COCHLEAR DUCT AND SURROUNDING STRUCTURES.

1: inner hair cell, 2: outer hair cells, 3: nerve fibers innervating the hair cells, 4: stria vascularis. Picture courtesy of S.B. Blankevoort.

neurons depolarize and generate action potentials that travel along the cochlear nerve (i.e., the cochlear branch of the 8th cranial nerve) to the brainstem. Efferent connections to hair cells are provided by neurons originating in the brainstem itself.

HEARING: DEVELOPMENT

The human cochlea reaches its final size and shape in the fetal stages of development. Early during gestation in the growing embryo, a specific area of thickened surface ectoderm called the otic placode arises, which is destined to develop into the inner ear (Figure 4). The otic placode invaginates to form the otic vesicle (or otocyst) during the 6th week of gestation^{footnote2} [2]. In the subsequent weeks, the otic vesicle polarizes and transforms into the membranous labyrinth, a continuum of fluid filled canals and chambers of both the vestibular organ

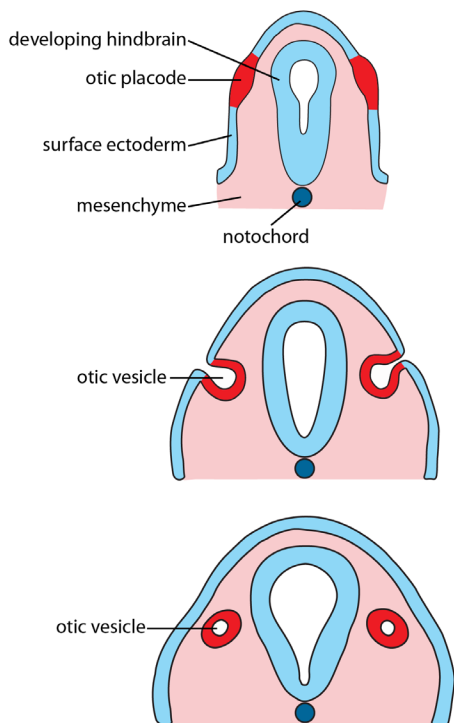


FIGURE 4. THE OTIC PLACODE.

A specialized area of the surface ectoderm called the otic placode is destined to form the otic vesicle and forms the basis of the cochlea. Early in development, it can be recognized as a thickened group of ectodermal cells (red) located near the hindbrain. Around Carnegie stage 13 (ca 6th week of gestation), the placode invaginates and pinches off from the surface to form the otic vesicle.

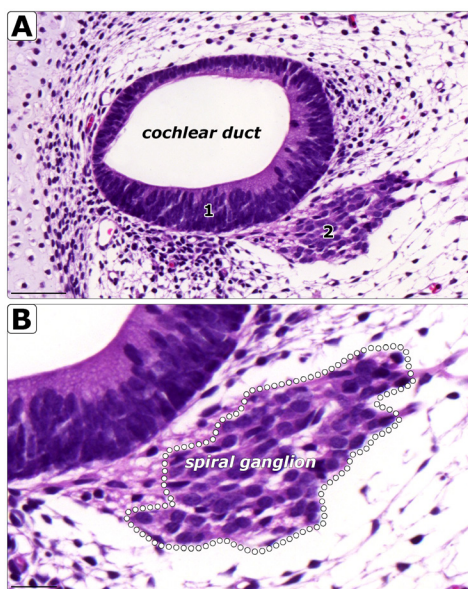


FIGURE 5. HUMAN FETAL COCHLEA AT THE 10th WEEK OF GESTATION.

(A) Hematoxylin-eosin staining of a transection through the lower basal turn. Both the epithelium of the cochlear duct (1) and the nearby spiral ganglion (2) are visible. Cells from the epithelium are microscopically undifferentiated at this stage. (B) Higher magnification of the area of the spiral ganglion (delineated with white dots). The large round nuclei belong to the developing spiral ganglion neurons. The smaller, darker and more elongated nuclei belong to the developing glial cells. Scalebars = 50 μ m (A) and 20 μ m (B).

and the cochlea. During a process called delamination, some cells from the otic vesicle/labyrinth detach from the otic epithelium and migrate into the surrounding mesenchyme, and from this group of cells the spiral ganglion neurons originate [3]. The cochlear part of the labyrinth (the cochlear duct) spirals around a central axis and reaches its final 2.5 turns at the end of the 10th or at the start of the 11th week of gestation [4, 5].

At this stage, the cells forming the epithelial lining of the cochlear duct (all derived from the otic placode/vesicle) are microscopically still undifferentiated (Figure 5A). The delaminated spiral ganglion neurons group together with the peripheral glial cells in the neighbouring spiral ganglion (Figure 5B). At the 12th week of gestation, a general developmental aspect of the cochlea can be observed: a spatiotemporal gradient in a basal-to-apical direction (Figure 6A). The development of the basal turn is ahead of that of the apical turn by one or two weeks, which is a consistent finding during maturation of the cochlea. Also, the spiral ganglion and the cochlear duct move away from one another, the scala vestibuli and tympani are formed, and the first hair cell becomes visible in the epithelial lining of the basal

turn (Figures 6B-C). At 14 weeks of gestation, one row of inner hair cells and three rows of outer hair cells can be discerned (Figure 7). Although the major structures in the cochlea are readily recognizable, differentiation and maturation continue through subsequent stages of development. Based upon the structural development of the human fetal cochlea and insights gained from animal studies, it has been estimated that the onset of cochlear function occurs around 20 weeks of gestation [6, 7].

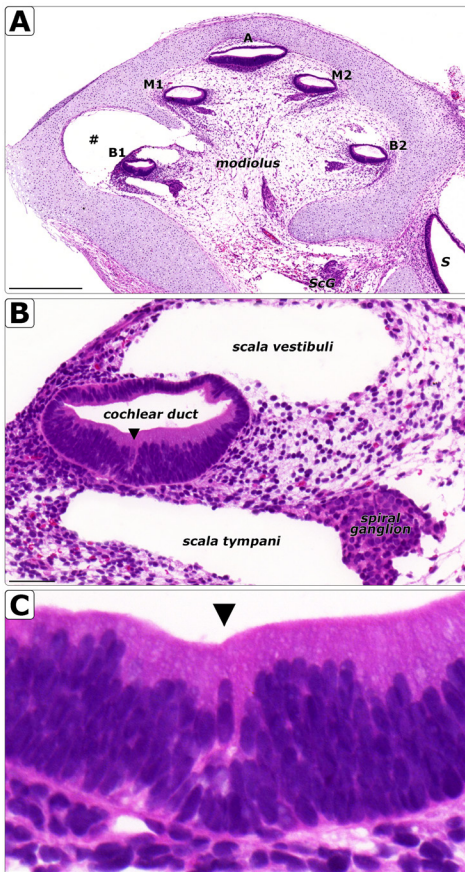


FIGURE 6. HUMAN FETAL COCHLEA AT THE 12th WEEK OF GESTATION. (A) Hematoxylin-eosin staining of a midmodiolar transection through the entire cochlea. (B) In the lower basal turn, the scala vestibuli and tympani have been formed, the distance between the spiral ganglion and the cochlear duct is increasing, and (C) the first hair cell becomes visible (arrow head). #, tissue artifact; B1, lower basal turn; B2, upper basal turn; M1, lower middle turn; M2, upper middle turn; A, apex; ScG, Scarpa's ganglion; S, sacculle. Scalebars = 500 μ m (A), 50 μ m (B) and 20 μ m (C).

HEARING: LOSS

Worldwide, 360 million individuals suffer from disabling hearing loss^{footnote3}, which is over 5% of the world's total population [8]. Although this number also includes patients suffering from conductive hearing loss (the origin of which lies in the external or middle ear), a large part is of sensorineural origin. Sensorineural hearing loss (SNHL) is therefore the most prevalent sensorineural disorder afflicting human beings. SNHL can either be acquired or congenital. Of the acquired type, hearing loss due to aging (presbycusis), ototoxicity or acoustic trauma are well-known examples. Often, outer hair cells are primarily affected [1]. Congenital SNHL is the most common congenital disorder with a prevalence of 1 in every 1000 newborns in the UK and the Netherlands [9, 10]. In many countries, national neonatal screening programs to detect SNHL have therefore been implemented. Genetic (hereditary) factors are implicated in over two-thirds of patients afflicted by congenital SNHL cases, although exact numbers are not available due to our incomplete etiological understanding. Hereditary SNHL can be divided into syndromic and nonsyndromic forms. Although the search for responsible genes for SNHL is still ongoing, our knowledge has rapidly increased during the last few decades. Over 130 loci have been mapped and over 60 different genes have been identified to date (2014) [11]^{footnote4}. Nonsyndromic forms of hereditary SNHL are grouped into autosomal dominant (DFNA), autosomal recessive (DFNB), X-linked (DNFX) and mitochondrial subtypes. Unlike acquired SNHL, only a minority of mutations affect the hair cells in hereditary SNHL. Mutations have likewise been found in various other cell types involved in the function of the cochlea or the cochlear nerve.

Much can be learned from studies investigating gene expression patterns in experimental animals as well as from studies on developmental abnormalities in animal models of hereditary SNHL. However, this knowledge does not yet extend to the full scale of known mutations causing SNHL and only a limited number of studies have been performed on human tissues. Understanding etiologies and pathologies of disorders is essential to the advancement of therapeutic

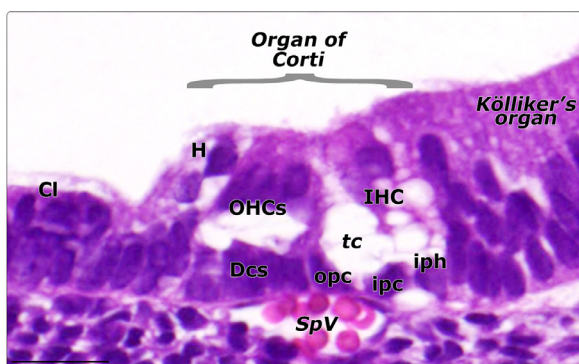


FIGURE 7. HUMAN ORGAN OF CORTI AT THE 14TH WEEK OF GESTATION. Hematoxylin-eosin staining of a transection through the organ of Corti located in the lower basal turn. IHC, inner hair cell; OHCs, outer hair cells; H, Hensen's cells; Cl, Claudius cells; Dcs, Deiters' cells; opc, outer pillar cell; ipc, inner pillar cell; iph, inner phalangeal cell; SpV, spiral vessel; tc, tunnel of Corti. Scalebar = 20 μ m.

options. For both acquired and congenital SNHL, increased knowledge of gene expression patterns and cell fate specification in the human inner ear has the potential to aid in the development of gene and cell-based therapeutic strategies.

HEARING: REGENERATION

The options for improvement of hearing in patients affected by SNHL are generally limited to hearing aids, varying from ‘simple’ types worn behind the ear to cochlear implants requiring surgery for implantation. However, their function is largely dependent on the quality of the remaining cellular structures within the cochlea and the integrity of the cochlear nerve. Hair cell loss, stria dysfunction, or neuronal degeneration can severely limit the hearing aid’s functionality. One remaining therapeutic option is the auditory brainstem implant: by bypassing the entire cochlea as well as the cochlear nerve, the brainstem is directly stimulated. However, as the quality of hearing with such an implant still remains very low [12], a different approach would be to induce tissue regeneration, i.e. to restore damaged hair cells or to regenerate the cochlear nerve. This might either improve the hearing aid’s functionality, or could possibly restore hearing itself.

Several different approaches to achieve restoration of damaged cochlear structures are currently being investigated. One interesting option involves introducing neurotrophic factors (proteins that stimulate growth and survival of neurons) into the cochlea aiming to promote spiral ganglion neuron survival or even regrowth of their peripheral processes [13]. The authors speculate that application of exogenous neurotrophic factors enhances the function of cochlear implants or may even create direct contact between the electrode and the nerve fibers [14 – 16].

Another approach is gene therapy, for example to generate new hair cells from supporting cells. Introduction of viral vectors that induce expression of *Atoh1* (the master regulator of hair cell differentiation) could convert targeted cells into new hair cells, potentially improving hearing [17 – 21]. However, it is difficult to target the right cells without affecting other cell types and disturbing cochlear architecture and function.

Regeneration of degenerated structures by stem cells is yet another, promising, approach. The rationale is that stem cells have migratory capabilities allowing them to reach appropriate locations, and that they subsequently differentiate into the desired cell type. Knowledge on the developmental origin of the damaged cell types as well as the factors that trigger differentiation is advantageous in the selection of the appropriate type of stem cell. Recently, otic progenitor cells induced from human embryonic stem cells were used in an animal model of SNHL, and led to an improvement in auditory thresholds [22]. Functional hair cells have also been cultured out of mouse embryonic stem cells [23]. These studies offer

more insight into inner ear development and pave the way for transplantation studies using in vitro grown hair cells.

HEARING: THE HAIR FOLLICLE OPTION

The development of stem cell based therapeutic strategies for restoration of auditory function starts with the search for the appropriate type of stem cell. In 2004 and 2005, two groups showed that pluripotent neural crest stem cells reside within the bulge of the hair follicle (Figure 8) in the whisker pads of adult mice. These cells were capable of differentiation into neurons, Schwann cells and melanocytes in vitro [24, 25]. Further studies showed that these cells could form neurons in vivo and promote axonal regeneration [26, 27]. Theoretically, these stem cells could be candidates to regenerate or repair the auditory nerve or its glial cells.

The use of neural crest stem cells residing in the hair follicle has several advantages over the use of other stem cells, such as embryonic or induced pluripotent stem cells. They are easily harvested, they are not oncogenic, and there are no ethical issues involved. More importantly, hair follicle derived neural crest stem cells reside in the adult body (at least in the mouse). Another advantage of these stem cells is that they have a neural crest-like phenotype, implying that they are already committed to follow a limited number of cellular lineages, thus reducing the risk of differentiation into unwanted cell types. Finally, when used in a therapeutic setting, these cells can be used for autologous transplantation without triggering unwanted immune responses in the host.

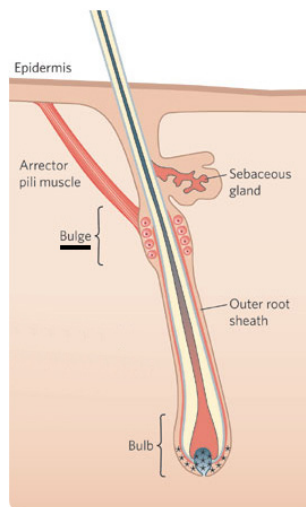


FIGURE 8. SCHEMATIC ILLUSTRATION OF A TRANSECTION THROUGH THE HAIR FOLLICLE. Neural crest stem cells are thought to reside in the hair follicle bulge. Reproduced with permission from Lin and Fisher, *Nature*, 2007.

AIMS AND SCOPE OF THIS THESIS

The aims and scope of this thesis are two-fold: (1) To gain more insight into the development of the human cochlea and (2) to investigate a possible stem cell strategy for the restoration of hearing. Although these two goals share little common ground at first sight, they are intimately associated with one another. When one pursues tissue regeneration, it is of huge advantage to have the blue print of the tissue's normal development, in order to avoid 'shooting in the dark'. Or, in other words, when something is broken, one needs to know how it normally functions in order to repair it. Knowing the origin of the degenerated cell type aids in selecting the proper replacement, and knowledge of the normal developmental steps helps in guiding a stem cell towards a specific cellular lineage.

OUTLINE OF THIS THESIS

The first and largest part of this thesis investigates the normal development of the human cochlea. *Chapter 2* focuses on the neurosensory epithelium, from which the hair cells develop, and its developmental relation with the innervating spiral ganglion neurons. In *Chapter 3*, this investigation is extended towards the third cell type involved in the hair cell and spiral ganglion neuron triad: the glial cell. Here, the focus is on the developmental distribution of the peripheral glial cells and on the onset of myelination, one of the prerequisites of normal hearing. Focusing on one of the most undervalued cochlear structures that are involved in hearing, *Chapter 4* investigates the development of the stria vascularis and cochlear potassium regulation, including its relation to syndromic and nonsyndromic SNHL.

Chapters 5-7 are related to the neural crest stem cells residing in the hair follicle. *Chapter 5* serves as an introduction to this part of the thesis and as a short summary of the findings and implications of *Chapter 6*, which is solely about skin melanocytes. These insights are further elaborated in *Chapter 7*, where we investigate and identify cells growing in hair follicle bulge explant cultures. *Chapter 8*, the general discussion, combines and discusses Chapters 2-7 and speculates on one of the most intriguing aspects of human cochlear development: the onset of human hearing.

Footnotes:

1: The size and number of cochlear turns differ between mammals. The whale cochlea has 1.5 turns, a typical mouse cochlea has 2 turns, whereas the cochlear of elephants (similar to humans) has 2.5 turns. The guinea pig rules them all: it has 3.5-4.5 turns (for images and other animals, see: <http://csi.who.edu/inner-ears-gallery>).

2: Gestational age is equivalent to weeks post conception (fetal age) plus two weeks, as gestational age is measured from the first day of the last menstrual period.

3: According to the WHO's standards, disabling hearing loss refers to a hearing loss greater than 40 dB in the better hearing ear in adults and a hearing loss greater than 30 dB in the better hearing ear in children.

4: An extensive table on involved genes, their function, gene expression and hearing loss type as well as an image showing the location of these genes within the cochlea can be found in Morton and Nance, NEJM, 2006. Also, on the Hereditary Hearing Loss Homepage (hereditaryhearingloss.org) an interactive view of many causative genes and their expression is provided.

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