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Invited Review

Gene-Based Deafness Research: Ion Transport and Hearing

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IKEDA, K. *Gene-Based Deafness Research: Ion Transport and Hearing*. Tohoku J. Exp. Med., 2004, **202** (1), 1-11 —— The cochlea is a sensory organ that converts physical (sound) stimulation into electrical signals. This process is fundamentally and substantially based upon the ion transport system. Here, I summarize the physiological and molecular biological aspects of transporters, channels and receptors expressed in the cochlea. With reference to these findings, recent advances in genetic research on hereditary deafness are discussed. ——— deafness; ion transport; hereditary deafness; DNA

The hearing organ is a sensory apparatus that converts the mechanical stimulation of sound into electrical energy in the cochlea, and then into a neural code in the central auditory pathway. Electrical phenomena in the cochlea include the endocochlear potential (EP), receptor potential of the sensory hair cells, outer hair cell motility, and neurotransmission, all of which involve the maintenance of the intra- and extracellular chemical and electrical environment (Ikeda et al. 1994a). Namely, the fundamental and substantial cellular responses of the cochlear cells are based upon an ion transport system responsible for the electrochemical properties, resulting in the efficient energy-yielding processes of acoustic transduction (Fig. 1).

In the present review, I focus on functional, molecular biological, and genetic analyses of carrier transporters, ion channels and receptors localized in the cochlea, mainly from the viewpoint of our own work, to clarify the cellular and molecular bases of pathophysiological events and responsible genes for deafness, with the hope that this will in the future contribute to the screening, diagnosis, treatment and prevention of deafness.

Na^+-K^+-2Cl^- cotransporter

Ototoxicity of loop diuretics was first reported in 1965 (Maher and Schreiner). A single case of transient acute hearing loss was reported following intravenous administration of ethacrynic acid to a group of patients with refractory edema. Establishment of the ototoxic potential of loop diuretics has now become common in medical practice. Most reports describe transient hearing loss and tinnitus following injection of high doses

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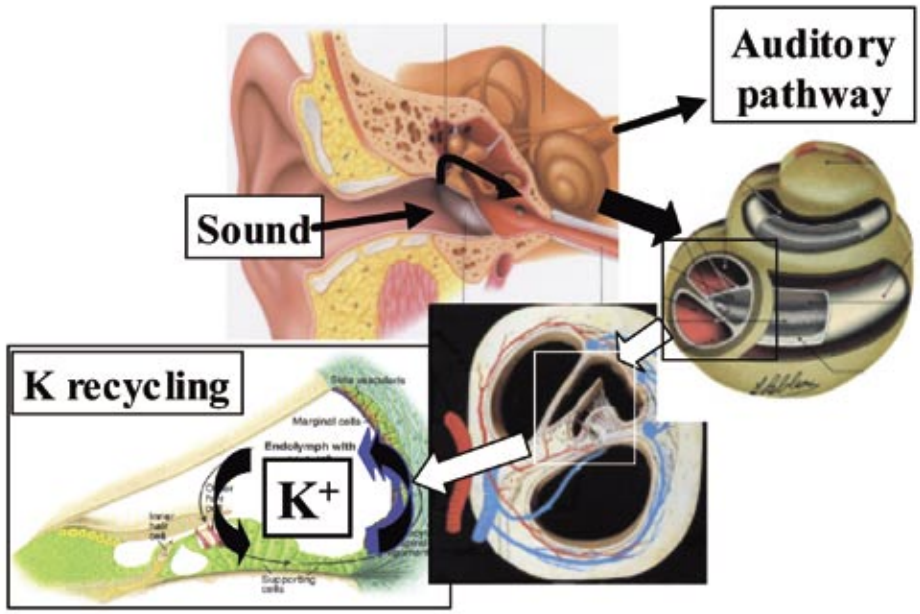

Fig. 1. Anatomical strucuture of the auditory pathway focusing on K^+ recycling in the cochlea.

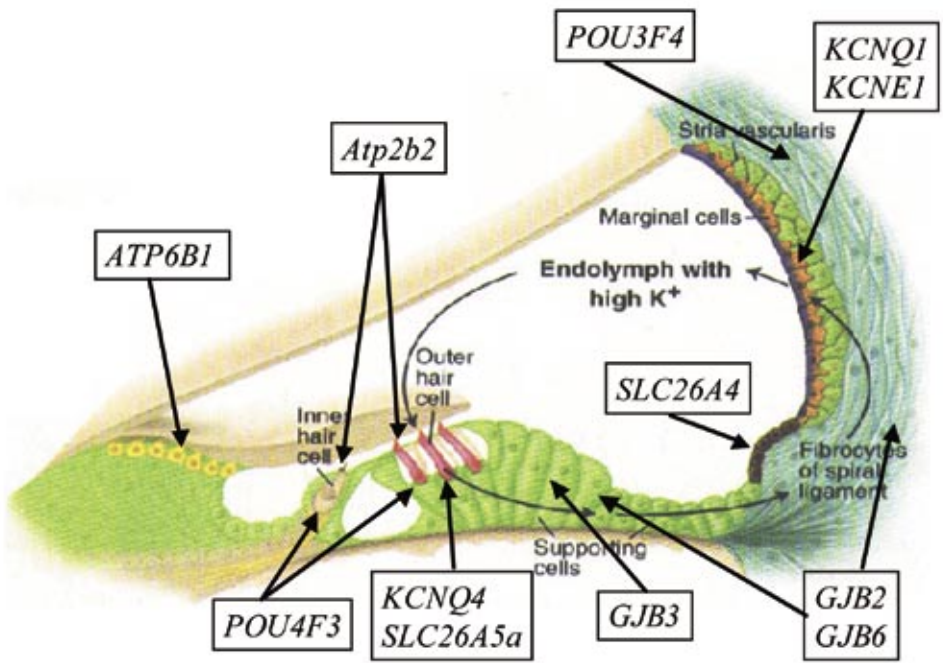

Fig. 2. Anatomical localization of the representative deafness genes in mouse or human related to the ion transport. *POU4F3* is found in both inner and outer hair cells, whereas *KCNQ4* and *SLC26A5a* (prestin gene) are specific for the outer hair cell. *Atp2b2* is mutated in the mouse model of deafness and limited to the stereocilia of the hair cell. *GJB2* and *GJB6* are located in both fibrocytes and supporting cells while *POU3F4* is located in the former cell and *GJB3* in the latter. K^+ channel units of both *KCNQ1* and *KCNE1* are found in the marginal cells of the stria vascularis. *SCL26A4* is mainly located in the outer sulcus cells below the stria vascularis. *ATP6B1* is found at the medial region of the cochlear duct epithelial cells, the interdental cells.

of loop diuretics (Schneider and Becker 1966). Permanent hearing loss following oral doses of furosemide or ethacrynic acid has, however, also been documented (Meriwether et al. 1971).

Various methods have been used to demonstrate the effects of loop diuretics on the stria vascularis in the cochlea. First, a characteristic feature is strial edema, indicating an accumulation of fluid in the intra- and/or extracellular space (Bosher 1980). Second, loop diuretics cause a substantial dose-related reduction of the EP (Cohen et al. 1971; Kusakari et al. 1978). The correlation in the time course of the changes in the strial morphology and the EP following the intravenous injection of furosemide was obtained (Pike and Bosher 1980), which further suggests that the stria vascularis is the target for the effects of loop diuretics. Third, measurements of ion concentrations in the endolymph showed that loop diuretics decreased the K^+ and Cl^- concentrations, increased the Na^+ and Ca^{2+} concentrations, but had no effect on the pH (Melichar and Syka 1978; Bosher 1979; Rybak and Whiteworth 1986; Ikeda et al. 1987). Finally, the ionic profile for monovalent ions obtained by the impalement of ion-selective microelectrodes across the in vivo stria vascularis cells suggested that the Na^+-K^+-$2Cl^-$ cotransporter exists at the basolateral membrane of the marginal cell (Ikeda and Morizono 1989).

A recent straightforward strategy to elucidate the structural basis of channel and carrier proteins is to probe using molecular biological tools. The molecular anatomies of the known types electroneutral cation-Cl^- cotransporters (ENCC) can be applied to auditory biology, resulting in new and powerful experimental approaches. We designed a technique to identify the ENCC isoforms expressed in the cochlear lateral wall (Hidaka et al. 1996). Degenerate primers designed from sequences of high homology between the rat bumetanide-sensitive Cl^- cotransporter (BSC1, ENCC2) and rat thiazide-sensitive Cl^- cotransporter (TSC, ENCC1) were used to amplify a product from the mRNA of the rat cochlear lateral wall by a reverse transcriptase-polymerase chain reaction (RT-PCR) technique. The PCR product obtained from the cochlear lateral wall was characterized by restriction digestion and Southern hybridization, indicating that neither ENCC1 nor ENCC2 was expressed. The nucleotide sequence analysis revealed that the isolated clone was highly homologous to the mouse BSC2 (ENCC3) rather than to the ENCC1 and ENCC2. These results demonstrated that ENCC3 is expressed in the rat cochlear lateral wall. Furthermore, from the functional aspects of ENCC isoforms, the cochlear lateral wall expressing ENCC3 could be characterized as secretory epithelia. The localization of ENCC3 in the basolateral side of the marginal cells was proved by immunohistochemical (Mizuta et al. 1997) and in situ hybridization methods (Goto et al. 1997).

The mechanism of loop diuretic ototoxicity can be explained by the presence of the Na^+-K^+-$2Cl^-$ cotransporter in the cochlea, which is reversibly inhibited by loop diuretics. On the other hand, the absence of the thiazide-sensitive ENCC1-type cotransporter in the cochlear lateral wall is consistent with the fact that there are no reports concerning the adverse effects of thiazide diuretics on hearing acuity. The molecular type of ENCC in the cochlea rather than the diuretic potency explains why reversible ototoxicity tends to be induced by massive and rapid administration of loop diuretics (Ikeda et al. 1997). Although recent genetic analyses of a mouse model lacking the Na^+-K^+-$2Cl^-$ cotransporter encoded by *Slc12a2* revealed that this molecule is essential for the generation of endolymph (Delpire et al. 1999; Dixon et al. 1999; Flagella et al. 1999), there have been no reports concerning the possible relation of this molecule to human hereditary deafness yet.

Na^+-H^+ exchanger

The Na^+-H^+ exchanger (NHE) is a plasma membrane transporter which mediates the electroneutral exchange of extracellular Na^+ for intracellular H^+ (pHi) in a one-to-one stoichiometry (Aronson 1985), and is found in virtually all mammalian cells. The NHE plays an essential

role in pHi homeostasis (Grinstein et al. 1989). Studies from our laboratory have established that a variety of cochlear tissues and cells of the guinea pig including the outer hair cells (Ikeda et al. 1992) and the stria vascularis cells (Ikeda et al. 1994) regulate pHi via NHE. Another laboratory demonstrated physiological evidence for the presence of the exchanger in the gerbil vestibular transitional cells (Wangemann et al. 1993) and dark cells (Wangemann et al. 1996).

Five distinct NHEs, NHE-1 to -5, have been identified in a variety of tissues and found to share significant amino acid sequence identity. NHE-1, the first member of the NHE gene family to be cloned (Sardet et al. 1989), is the housekeeping isoform which probably serves a cell maintenance function and is distributed ubiquitously in both epithelial and nonepithelial cells.

Although NHE-2, -3 and -4 isoform mRNAs could be detected in the cochlear tissue, the NHE-1 message was predominant. Cloned guinea pig NHE-1 to -4 partial cDNA fragments were highly homologous to the corresponding rat NHE isoforms. NHE-1 isoform mRNA was distributed in the hair cells, marginal cells, spiral ligament fibrocytes, spiral prominence cells and spiral ganglion cells (Goto et al. 1999). NHE-1 localized in a variety of cochlear cells would contribute to their differential functions.

Na^+-Ca^{2+} exchange

Intracellular Ca^{2+} ions of the sensory cells are closely linked to a variety of neuronal processes such as changes in membrane conductance, synaptic activation, and transmitter secretion (Ross 1989). The cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) in various cells is maintained 10^3–10^4 times lower than the external Ca^{2+} concentration. Regulation of $[Ca^{2+}]_i$ is accomplished by a number of mechanisms such as Ca^{2+} influx, efflux, and uptake and release by the intracellular organelle as well as Ca^{2+} buffering within the cell.

The hair cell of the mammalian cochlea is surrounded by two extracellular fluids of different ionic composition, endolymph and perilymph. The perilymph contains 1-2 mM Ca^{2+} as is the case in other extracellular spaces, whereas the endolymph contains only nominal amounts of Ca^{2+} (10-30 μM) (Ikeda et al. 1987). Nevertheless, the electrochemical gradient for Ca^{2+} favours the Ca^{2+} entry through both the apical and basolateral membrane of the hair cell. The presence of the Na^+-Ca^{2+} exchange mechanism in the isolated hair cell was revealed by an optical technique together with Na^+ and Ca^{2+} sensitive fluorescence probes (Ikeda et al. 1992). First, removal of external Na^+ induced a marked rise in $[Ca^{2+}]_i$, and readmission of external Na^+ restored the $[Ca^{2+}]_i$ to the initial value. Second, an elevation of external Ca^{2+} caused a decrease in the cytosolic Na^+ concentration ($[Na^+]_i$), and the return to normal external Ca^{2+} concentration resulted in a return to the initial $[Na^+]_i$. Third, external Na^+-dependent Ca^{2+} fluxes were inhibited by La^{3+} and an amiloride derivative.

Molecular biological studies have revealed several spliced isoforms of the Na^+-Ca^{2+} exchanger (NCX) of the cochlea. The cochlear lateral wall and the organ of Corti expressed only a single isoform of NCX1. On the other hand, five isoforms of NCX1 and four isoforms of NCX3 were detected in the cochlear modiolus. The alternative splicing may account for the diverse functionsof the NCX in the cochlea (Oshima et al. 1997)

ATP receptor

Cell surface receptors for extracellular adenine nucleotides are distributed in a variety of mammalian tissues and cell types. P2 receptors, activated by either or both adenine and uridine nucleotide derivatives, and the previously termed purinoceptors are distinguished using two separate nomenclature systems as P2X and P2Y receptors.

It appears very likely that extracellular ATP acts via a number of signalling pathways within the cochlea to modulate hearing function. The effects of perfusion of ATP and P2 receptor agonists and antagonists within the perilymph sup-

port a P2 receptor mediated suppressive effect on auditory nerve discharge and distortion product otoacoustic emission via actions on the organ of Corti and the auditory nerve (Kujawa et al. 1994). An elevation of endolymphatic ATP produces a fall in the endocochlear potential and auditory compound action potential (Munoz et al. 1995). Physiological and pharmacological studies have suggested that a variety of cells in the organ of Corti have P2 receptors (Ashmore and Omori 1990; Nakagawa et al. 1990; Ikeda et al. 1991; Dulon et al. 1993). Using the agonist profile for various nucleotides, ATP was shown to induce the intracellular Ca^{2+} mobilization via P2Y1 receptors in the guinea-pig cochlear lateral wall (Ikeda et al. 1995; Suzuki et al. 1995). The P2 receptors in the gerbil marginal cells which modulated the K^+ secretion had pharmacological similarity to both P2Y1 and P2Y2 subtypes on the basis of the agonist potency (Liu et al. 1995). The inositol 1, 4, 5-trisphosphate second messenger system was coupled to P2Y1 receptors in the guinea-pig cochlear lateral wall (Ogawa and Schacht 1995). Radioligand binding assays showed that the lateral wall tissue of the guinea-pig cochlea possesses P2X and P2Y receptor sites (Mockett et al. 1995).

The mRNA expression of the three UTP receptors (P2Y2, P2Y4 and P2Y6) and that of the P2Y1 ATP receptor in both sensory and secretory structures of the rat inner ear have been reported (Teixeira et al. 2000). Both P2Y2 and P2Y4 purinergic receptors were immunolocalized at the cochlear marginal cells (Sage and Marcus 2002). Furthermore, reverse transcriptase-polymerase chain reaction (RT-PCR) and in situ hybridization data (Housley et al. 1995; Housley and Ryan 1997) demonstrated that P2X2 receptor subunits of the ATP-gated ion channels were expressed in the organ of Corti as well as in the spiral ganglion cells. However, P2X receptor messages were considerably small in the lateral wall of the cochlea.

The source of the endogenous ATP that acts in these receptors may be the marginal cells in the lateral wall stria vascularis (White et al. 1995), in which quinacrine fluorescence labelling of putative vesiculated purine stores was demonstrated. This quinacrine method was recently confirmed to be reliable for the identification of ATP-containing granules (Suzuki et al. 1997). Application of ATP to the endolymph resulted in a large decrease of the EP (Munoz et al. 1995). Taken together, the P2Y receptors on the lateral wall may modulate the cochlear potential by an autocrine or paracrine mechanism as speculated to occur in respiratory epithelial cells.

Gap junction proteins

It has been reported that the gap junction consists of at least 13 protein subunits, which may dock with each other to form heterotypic channels or homotypic channels. Gap junctions are known to serve as intercellular pathways for a variety of small molecules of substances and ions. An extensive network of gap junctions exists in two sets of cochlear cells: an epithelial cell system that surrounds the hair cells, and an adjacent connective tissue system of fibrocytes of the lateral wall. Immunostaining demonstrated that connexin26 (Cx26) is expressed by both systems (Kikuchi et al. 1995). The distribution and expression of Cx30 were found to be nearly the same as that of Cx26 (Xia et al. 2001). The dense immunoreactivity of Cx31 was observed in the fibrocytes of the spiral ligament and the spiral limbus, but not in the epithelial gap junctional system (Xia et al. 2000). The pattern of expression of Cx31 gradually decreased from the basal turn to the apical turn. On the other hand, the distribution patterns of both Cx26 and Cx30 were obviously different, as they were expressed in the basal cells of the stria vascularis and the supporting cells and uniformly in the fibrocytes throughout the turn. The expression and distribution of these three connexins well explain each characteristic frequency-dependent pattern and the severity of hearing loss.

Mitochondrial DNA mutations

Several lines of studies (Schoffner et al. 1989; Goto et al. 1990; Prezant et al. 1993) in-

dicate that sensorineural hearing loss (SNHL) is associated with mitochondrial DNA (mtDNA) mutations. MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes) is a well-known syndrome associated with the A→G mutation at position 3243 of mitochondrial leucine transfer RNA^{UUR} and represents a high incidence of SNHL (Goto et al. 1990). Aminoglycoside-induced deafness has been also associated with mtDNA point mutation (1555A→G) (Prezant et al. 1993). The most frequently observed deletion of the mtDNA in Kearns-Sayre syndrome (KSS), in which SNHL is one of the characteristic symptoms, is the 4977 nucleotide pair deletion (mtDNA4977) (Schoffner et al. 1989). We found 3 patients associated with mtDNA mutation (3243A→G) from among 100 patients with bilateral SNHL of unknown origin, implying that SNHL associated with mtDNA mutation is highly prevalent in the Japanese population (Oshima et al. 1996, 1999). Furthermore, 4 patients of 46 deaf-mute subjects possessed A1555G mtDNA (Oshima et al. 2001). Patients with SNHL had a significantly high rate of the mtDNA4977 deletion compared to controls (Ueda et al. 1998). The detection rate of the mtDNA4977 deletion was significantly increased with the deterioration of the hearing threshold.

The mechanisms by which the mtDNA mutations induce SNHL are unclear. One possibility could be the large dependence of the energy metabolism of the organ of Corti and the stria vascularis on mitochondrial oxidative phosphorylation. The progressive accumulation of the mutant mtDNA with age leads to a decline in the oxidative phosphorylation capacity. Energy-dependent ATPase and the release of neurotransmitters in the cochlea are suppressed by a lowering of the ATP production by mitochondria. Another possibility is a disturbance in ion transport, leading to a reduction in the efficiency of acoustic transduction (Ikeda et al. 1994). Actually, inhibition of the respiratory chain of mitochondria in the isolated outer hair cell by applying cyanide induced the increase in $[Ca^{2+}]_i$ (Ikeda et al. 1992), presumably resulting in an alteration of the membrane capacitance. The reduced ATP production due to mitochondrial damage would activate both nonselective cation channels and I_{SK} channels in the strial marginal cells (Sunose et al. 1993) and inactivate the Ca^{2+}-ATPase in the outer hair cells (Schulte 1993).

Ion transport and deafness

Thirty-five genes responsible for non-syndromic deafness have been discovered as of May 12, 2003 and mutations have been reported to affect several components of the ion transport system such as K^+ recycling route which contribute to the homeostasis of the cochlear ionic and electrical environment. *KCNQ4* encodes a K^+ channel exclusively localized in the outer hair cells and is mutated in dominant, progressive hearing loss (Kubisch et al. 1999). Four kinds of connexins, Cx26 (encoded by *GJB2*), Cx31 (*GJB3*), Cx32 (*GJB1*), and Cx30 (*GJB6*), were found to be associated with human congenital hereditary deafness. As K^+ ions reach the stria vascularis via gap junctions in the supporting cells of the organ of Corti and the fibrocytes of the spiral ligament, these ions are pumped into the marginal cells by a Na^+-K^+ ATPase and a Na^+-K^+-$2Cl^-$ cotransporter. The cotransporter is found to induce deafness in the mouse mutant as mentioned before. Furthermore, K^+ ions flow into the endolymph through a channel of marginal cells, and both components of this channel, *KCNQ1* and *KCNE1*, cause Jervell and Lange-Nielsen syndromes (Neyroud et al. 1997; Schulze-Bahr 1997).

Five other molecules have been implicated in the homeostasis of the cochlear fluid. *ATP6B1* is believed to affect the endolymph pH and was found in patients with renal tubular acidosis and deafness (Karet et al. 1999). *PDS*, now designated as solute carrier (SCL) family, *SLC26A4*, is mutated in Pendred syndrome and in some types of non-syndromic deafness (Li et al. 1998; Everett et al. 1999). This molecule is expressed in the endolymphatic duct and sac as well as in epithelial cells of the cochlear lateral wall and acts

as an iodide/chloride transporter (Everett et al. 1999). A recent study of mice lacking the K-Cl cotransporter *Kcc4* revealed that removal of K^+ ions by the supporting Deiters' cells would change the ionic composition of the small extracellular space, the cortilymph surrounding the basolateral membrane of outer hair cells, eventually leading to their degeneration (Boettger et al. 2002). Prestin, a membrane protein that is highly and almost exclusively expressed in the outer hair cells of the cochlea, is a motor protein which senses the membrane potential and drives rapid length changes in the outer hair cells. Surprisingly, prestin is a gene member of SLC family 26 that encodes anion transporters and related proteins. *SLC26A5a* is the predominant form of prestin, which is responsible for recessive non-syndromic deafness (Liu et al. 2003). The visual and auditory systems have a specific requirement for H^+ disposal mediated by the sodium bicarbonate cotransporter *NBC3*. Mice lacking *NBC3* develop blindness and auditory impairment because of the degeneration of sensory receptors in the eye and inner ear as in Usher syndrome (Bok et al. 2003).

Mouse models of human hereditary non-syndromic deafness

Hereditary deafness affects about 1 in 2000 children. Without an animal model for hereditary deafness, it is quite difficult to determine the anatomical, biochemical, and cellular basis for the phenotype, or to use gene rescue to prove unequivocally that the disease-causing gene has been identified. Indeed, when a mouse model does not already exist, it is often necessary to create one as the first step in understanding how the gene mutation results in deafness.

For 30 or more genes identified as having a relationship to non-syndromic deafness, good mouse models include *Myo7a, Myo15, Pou3f4, Pou4f3, Col1a2, Tecta, Cdh23, Gjb2, Gjb6, Myo6, Slc26a4, Slc26a5, Tmc1, Tmie* and *Whrn* (see to http://www.jax.org/hmr/models.html). *Gjb2, Gjb6, Slc26a4,* and *Slc26a5* are directly related to ion transport. Additionally, mouse models of syndromic deafness are also available for *Kcnq1* and *Kcne1* encoding K^+ channel subunits, and for *Slc19a2* (Fig. 2).

Here, I introduce two mouse models of human non-syndromic deafness developed in my laboratory. X-linked non-syndromic mixed deafness is caused by mutations in human *BRN-4 (POU3F4)*, known as a POU transcription factor gene (de Kok et al. 1995). Using gene targeting technology we established a *Brn-4* deficient mouse line that exhibits profound deafness. A drastic reduction of EP, which was accompanied by severe ultrastructural alterations in the cochlear spiral ligament fibrocytes, was observed. These findings indicated the critical role of such fibrocytes in establishing the EP, probably by acting as canals for cation flow in the cochlear system (Minowa et al. 1999).

Mutations in the *GJB2* gene, which encodes gap junction protein connexin26, are the major cause of deafness in various ethnic groups (Kelsell et al. 1997; Kudo et al. 2000). However, the pathogenesis of the hearing loss caused by the *GJB2* mutations remains obscure. The generation of a mouse model to study the function of connexin26 during hearing has been hampered by the fact that *Gjb2* knockout mice are embryonic lethal (Gabriel et al. 1998). To establish viable model mice we generated transgenic mice expressing a mutant connexin26 with R75W mutation that was identified in a deaf family with autosomal dominant inheritance. We established two lines of transgenic mice that showed severe to profound hearing loss, deformity of supporting cells, failure in the formation of the tunnel of Corti, and degeneration of sensory hair cells. The EP essential for hair cell excitation was normally sustained. These results suggest that the *GJB2* mutation disturbs the homeostasis of the cortilymph, an extracellular space surrounding the sensory hair cells, due to impaired K^+ transport by supporting cells, resulting in a degradation of the organ of Corti, rather than affecting the endolymph homeostasis, in mice and probably in human (Kudo et al. 2003).

Conclusions

Tremendous progress in auditory research has been carried out by approaching the genetic basis of deafness. An increasing amount of hereditary deafness is found to be caused by defects of the carrier transporters, ion channels and receptors in the cochlea. Clarifying the physiology, and the structural and molecular framework of the ion transport systems encoded by deafness genes and resolving the underlying mechanisms of deafness could make it possible to develop fundamentally new methods of diagnosis, therapy, and prevention for millions of patients suffering from unknown origins of deafness.

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