Cochlear pathology, sensory cell death and regeneration

Yehoash Raphael

KHRI, The University of Michigan Medical School, Ann Arbor, Michigan, USA

Loss of cochlear hair cells leads to permanent hearing loss. Hair cells may degenerate due to hereditary or environmental causes, or a combination of the two. Cochlear supporting cells actively participate in the process of hair cell elimination and scar formation by rapidly expanding and sealing the reticular lamina, the barrier between endolymph and perilymph. This scarring process helps preserve the remaining hair cells and hearing. Anti-apoptotic agents, anti-oxidants and several growth factors have been shown to protect hair cells and hearing against environmental insults. Characterization of the genes that regulate the development of the inner ear and its response to trauma has been helpful in designing strategies for enhancing protection of the inner ear and for inducing hair cell regeneration. This chapter discusses the potential for some of these approaches.

The hair cells in the human inner ear are born in the first trimester of embryonic development. These cells are expected to survive, without renewal, for life. Intuitively, it seems that the expectation for a cell to survive over nearly a century is rather demanding and imposing. However, several other cell types in the human body are capable of such long service, including, most notably, muscle cells and neurons. What makes the inner ear different is the fact that, unlike neurons, the total number of hair cells is very small and there is very little (if any) redundancy in this population. Thus, considering that every region in the cochlea is optimized for best function at a given frequency, loss of a single hair cell is associated with compromised hearing in a specific frequency.

Hair cell loss is the leading cause of hearing loss. Hair cell death can be caused by lack of essential growth factors, exogenous toxins (such as ototoxic drugs), overstimulation by noise or sound, viral or bacterial infections, autoimmune conditions or hereditary disease. The reader is familiar with the devastating outcome of hearing loss and with the prevalence of this sensory impairment. Sensorineural hearing loss is irreversible. This chapter describes our knowledge of the events that lead to hair cell loss and replacement, and presents possible strategies that may yield potential therapies in the future. While the proposed strategies

Correspondence to:
Dr Yehoash Raphael,
KHRI, The University of Michigan Medical School,
MSRB III Room-9303,
Ann Arbor,
MI 48109-0648, USA

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are based on current biological and technological data and concepts, some of the proposed interventions are visionary and should serve as a working plan for future research and development rather than a promise to the current population of patients.

Hair cell degeneration

Cell elimination and scar formation

As in any classical drama, the death of a hair cell is staged in a complex array of considerations involving time and space. The immediate surrounding environment is of great importance. Consider that the apical surface of a hair cell is bathed in endolymph, a fluid so rich in K^+ ions that it is toxic to neuronal endings that innervate the basal-lateral portion of the hair cell membrane. Thus, if a hair cell were to disappear and leave behind an open space, even for a short time, the hearing of the entire ear could be compromised. Nevertheless, most adult humans have lost some hair cells without having lost their entire hearing reserve. This can only be explained by a highly regulated and complex mechanism of cell death and scar formation in the organ of Corti.

The term 'scar' is used to describe replacement of the original cell type (hair cell) by a 'filling' cell, a supporting cell. The use of the term scar in the organ of Corti is justified because the scar is permanent. The immediate role of the scarring process is to prevent fluid mixing. It is especially important to prevent endolymph leakage into the fluid bathing the basal domain of inner hair cells where terminals of the auditory nerve reside. Potassium-rich endolymph would depolarize the neurons, and abolish hearing and lead to further tissue damage.

The mechanism of scar formation depends to a great extent on another important element in the immediate surroundings of the hair cell, namely, the neighbouring supporting cells. Supporting cells co-ordinate neat and organized scar formation following damage by ototoxic drugs and noise trauma[1]. Early responses of supporting cells were detected in response to hair cell trauma, as two of the four supporting cells which surround a hair cell execute a dying hair cell and seal the reticular lamina against a leak of fluids, thus preventing the mixing of perilymph and endolymph[2,3]. The involvement of supporting cells in hair cell loss, therefore, results in rapid expansion of the apical domain of two supporting cells, constricting the hair cell beneath its apical membrane, and sealing the reticular lamina prior to formation of a fluid leak (Fig. 1). Conspicuous actin cables are seen in the scarring region, suggesting that an actin–myosin system is involved in the removal of the dying hair cell (see Fig. 1).

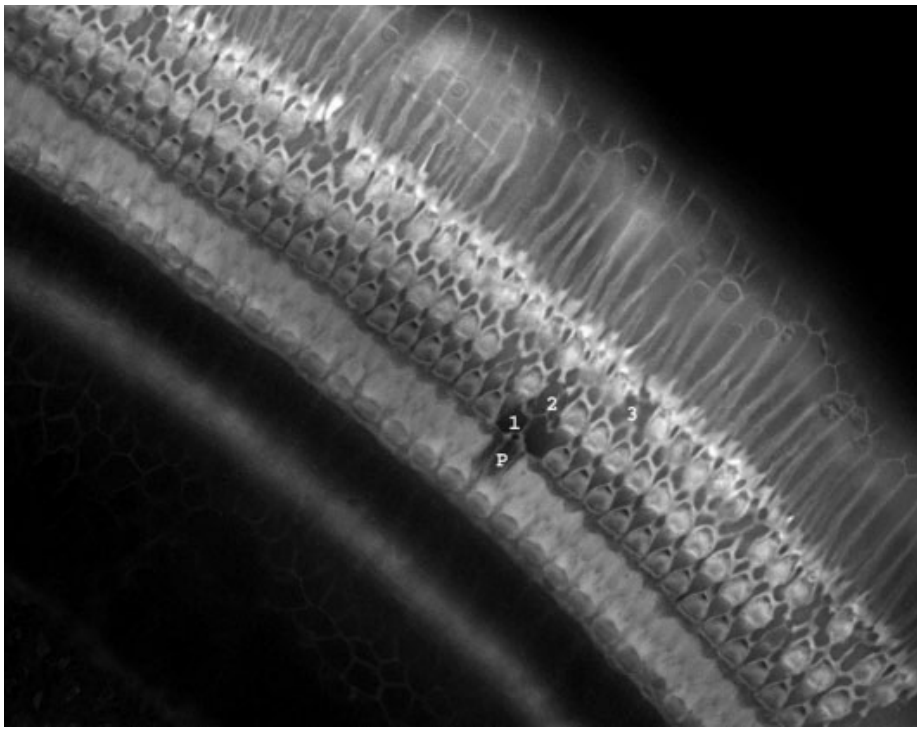

Fig. 1 A whole-mount (surface preparation) of the guinea pig organ of Corti (3rd cochlear turn) labelled with rhodamine phalloidin (a specific F-actin stain) and photographed using epifluorescence. One pillar cell is missing in the row of pillars (p). Scars made by supporting cells are seen in several sites where hair cells are missing (1, scar in the first row of outer hair cells; 2, scar in the second row of hair cells; 3, scar in the third row of hair cells) but the overall structure of the organ of Corti is preserved.

Studies on epithelial cell death in other systems have demonstrated similar mechanism to that described above[4]. At the molecular level, inhibition of Rho, one of the members of a family of small GTPases, blocks the scarring[4]. The formation of the actin–myosin rings and the process of cellular replacement (scarring) was shown to occur very early in the apoptotic cascade, prior to procaspase activation and cell changes in morphology of the dying cells. Interestingly, similar to the observation in the organ of Corti, the epithelial surface remained intact during the scarring, as determined by constant electrical resistance measured in the face of extensive cell death[4].

This scarring mechanism implies that the hair cell or, at least, most of the hair cell body remains within the epithelium. The hair cell body can be eliminated by phagocytosis, either by macrophages or by supporting cells. Evidence for macrophages is provided by their occasional presence in the auditory epithelium after lesions. However, the small number and

infrequent identification of macrophages implicates supporting cells as phagocytes for dead hair cells. Evidence from other epithelial tissues such as the retinal pigment epithelium point to similar mechanisms whereby supporting elements eliminate sensory cells that die within the epithelium[5].

An insult can lead to sub-lethal pathology in hair cells, resulting in morphological and functional changes that can be defined as hair cell injury. While some hair cells gradually degenerate, others are likely to recover and regain normal function. This implies that supporting cells do not create a scar to replace injured hair cells. It is unclear at present how supporting cells chose their action: thumbs-up to allow the survival of an injured hair cell versus thumbs-down to order and perform its execution. Regardless of the mechanism, the possibility that injured hair cells survive under the reticular lamina has important implications for hair cell regeneration. Reports of hair cells that are injured by an insult and remain under the reticular laminar surface among the supporting cells came from studies of lesions in the developing organ of Corti in culture[6].

So far, diseases associated with impaired scarring mechanism in supporting cells have not been described. However, such impairment would certainly be detrimental to the organ of Corti during hair cell degeneration. I propose, hypothetically, that some forms of sudden deafness may be related to faulty scarring, during which the integrity of the reticular lamina is compromised. Accordingly, treatment of such hearing loss should be based on manipulating supporting cells and their ability to form scars rapidly and effectively.

Apoptosis and necrosis

The study of cell death is not a new one; yet, it has received much attention and effort in the last decade[7]. Of significant importance is the impressive enhancement in our understanding of active cell death, or apoptosis, including genes that encode necessary participants in the active cell death cascade, and other proteins that may inhibit active cell death. Understanding the molecular mechanism of cell death should help us design the means to manipulate active cell death.

Active cell death is a physiological process important for normal development and tissue homeostasis as well as in cell removal in response to a variety of pathological conditions. The initial signal for active cell death can be stress (of several types), absence of a trophic factor or a diffusible molecule that binds to a specific cell-surface receptor. The receptors are called death receptors. One of the better studied receptors mediating cell death is Fas. This is a transmembrane receptor found in a variety of cell types[8]. Once activated, the Fas receptor leads to direct activation of caspase-3, or to activation of

caspase-8 which, in turn, leads to the release of cytochrome c from mitochondria. Cytochrome c then activates caspase-3. Mitochondria have an alternative pathway for participating in the active cell death cascade, in which they release Smac/Diablo, which in turn activates caspase-3[9]. Molecules from the Bcl-2 family can inhibit apoptosis at this stage by preventing mitochondrial changes[10].

The main pathway of active cell death terminates by activation of specific proteases from the caspase family which serve as the cell 'executioners'[11]. The apoptotic initiators act upstream and include caspase-2, caspase-8 and caspase-9 while the downstream executioners are caspase-3, caspase-6 and caspase-7[12]. Caspase-3 is the most common molecule for inducing active cell death. Once activated, it acts on several targets, each of which leads to break-up of a different cellular compartment. To degrade chromatin, caspase-3 acts on a set of DNAses and nucleases[13]. To degrade the cell membrane and the cytoskeleton, caspase-3 acts on gelsolin and fodrin[14].

Natural inhibitors of caspases have been shown to exist in mammalian cells. These inhibitors can serve as agents for cell protection against death. For instance, a protein named X-linked inhibitor of apoptosis (XIAP) has been show to have robust anti-apoptotic activity[15]. Active cell death inhibition is accomplished by binding of the XIAP to the caspase, and inhibiting its function.

In many types of cells, identification of active cell death in terms of the criteria above is conclusive. However, in other tissues and conditions leading to cell death, the distinction between active cell death and necrosis is not perfectly clear[16]. Overlap in molecular and morphological manifestations between active cell death and necrosis, along with artefacts inherent in the technology for detection of active cell death complicate our ability to distinguish between these two types of cell death.

The degeneration of hair cells in the organ of Corti has not been easy to study. The paucity of cells precludes most biochemical analyses. TUNEL staining and other types of DNA labelling have been performed by several laboratories, identifying organ of Corti hair cells that are presumably undergoing active cell death[17,18]. Other studies clearly demonstrate false-positive apoptosis staining in hair cells and warn of the inability of present technology to distinguish reliably between active cell death and necrosis[19].

To complicate the picture further, there is a growing body of evidence that TUNEL-positive cells in several tissues are undergoing necrosis. In practical terms, the identification of proteins that are involved in the demise of the hair cells is important because this knowledge can lead to the development of preventive measures. Thus, while the distinction between active cell death and necrosis is not always clear, the similarities between them enable use of a common set of intervention means to prevent cell death (see below).

Prevention of cell death

Three major agents have been investigated for preventing hair cell loss due to ototoxic and acoustic insults. These agents are molecules that belong to the families of neurotrophic factors, anti-oxidants or anti-apoptotic agents. All three types of molecules have the potential to become clinically applicable for similar types of trauma. However, such therapy is usually effective for preventive medicine and not helpful for treating a lesion that has already occurred.

Neurotrophic factors

Neurotrophic factors are relatively small peptides that are secreted to act in a paracrine or autocrine fashion. Upon binding with specific receptors on the membrane of the target cell, they activate an intracellular signalling cascade leading to one or more of their functions. These include signals that are necessary for development, maintenance of the differentiated state, survival in the face of insults and re-growth or axonal regeneration. The term neurotrophic factor implies an influence on neurons, but in reality most neurotrophic factors have been shown to also influence a large variety of non-neuronal tissues, including the auditory epithelium. The two neurotrophic factors found to be important for normal development of auditory neurons are brain derived neurotrophic factor (BDNF) and neurotrophin 3 (NT-3)[20]. NT-3 is also important for the developing auditory sensory epithelium[21]. Both BDNF and NT-3 were found to be protective against trauma in mature auditory hair cells, as were other neurotrophic factors such as glial cell-line derived neurotrophic factor (GDNF)[22–24].

While neurotrophin protection of hair cells and hearing against drug and noise trauma appears to be statistically significant, the extent of protection is less than ideal. However, because they act on early stages up the cascade of cell degeneration, neurotrophic factors have the potential to be excellent protective agents. It is, therefore, necessary to optimize their use by developing protective interventions via combined use of several neurotrophic factors or in combination with other protective agents. The visionary future therapy for prevention of hair cell loss may be based on application of a combination of neurotrophic factors with anti-oxidants, NMDA type of glutamate receptor antagonists, nitric oxide (NO) blockers and/or anti-apoptotic agents[25]. The use of a large array of protective agents will cover several stages in the cascade of molecular events leading to cell degeneration.

Anti-oxidants

Reactive oxygen species (ROS) have been shown to play a role in several tissues in the response to a variety of insults, resulting in cell death and

replacement. Increase in ROS has been found in the inner ear following both noise and drug trauma. It was, therefore, intuitive that free radical scavengers or other anti-oxidants could be used to protect hair cells and hearing against lesions caused by environmental inner ear insults. Indeed, iron chelators such as deferoxamine or dihydroxybenzoate were shown to reduce significantly the lesion and the functional deficit caused by several aminoglycosides in the inner ear[26]. Cisplatin ototoxicity was also significantly reduced by protective agents that enhanced the antioxidant defence of the cochlea[27]. Similarly, anti-oxidant therapy has been shown to be effective for protection against acoustic trauma[28,29]. Safe and effective antioxidant therapy needs to be developed for clinical use. One of the most promising candidates which is inexpensive, safe and widely available is aspirin[30].

Anti-apoptotic agents

The active cell death cascade involves a relatively limited number of pathways and the molecules that participate in signalling in each part of the cascade are rapidly being identified. The identification of signalling molecules in this cascade can help design ways to prevent cell death. For instance, hair cell death due to gentamicin-induced ototoxicity has been shown to involve JNK activation and hair cell apoptosis. The degeneration of hair cells can be attenuated by administration of CEP-134731, a potent inhibitor of JNK signalling.

The identification of several caspase inhibitors has paved the way to rescue experiments in many types of tissues, including the organ of Corti. One general caspase inhibitor that has been used in several rescue experiments in the inner ear is *N*-benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethylketone (Z-VAD-FMK). Z-VAD-FMK has provided protection against hair cell degeneration following cisplatin[32]. In recent experiments, chinchillas were exposed to intense noise then stained with carboxyfluorescein labelled Z-VAD-FMK. This helped identify caspase-3 as the mediator of apoptosis in the organ of Corti. Interestingly, caspase-3 dependent apoptosis is necessary for normal development of the organ of Corti[33].

In the inner ear, caspase inhibitors seem effective in rescuing hair cells that are exposed to a variety of insults[32,34]. It remains to be determined if anti-apoptotic agents can also rescue hair cells from death due to genetic disease. Based on the success in hair cell rescue by pan-caspase inhibitors, it is important to develop more specific inhibitors for use in the clinic, based on knowledge of the specific caspases that are activated following insult in the human organ of Corti. Such agents should also be cell-permeable and non-toxic.

Role of the immune system

In most tissues, there are several important roles for cells and signalling of the immune system, such as phagocytosis of pathogens and of degenerating cells or cellular debris. In addition, cytokines and other molecules provide signalling that mediates the initial response to the insult and the reparative processes. Phagocytes and immune-mediated signals, especially cytokines, play important roles in the response of the inner ear to trauma. Most significantly, cytokines have been shown to participate in the signalling for hair cell turnover and regeneration in the avian basilar papilla[35], and to influence survival of inner ear neurons[36–38]. Excessive inflammatory response can be detrimental to the structure and function of the cochlea. Thus, the beneficial as well as negative roles played by the immune system in the homeostasis of the inner ear need to be understood better so as to facilitate the design of relevant therapies for protection and repair in the cochlea[39].

Repair of injured cells

It is likely that some hair cells injured by acoustic trauma or aminoglycoside ototoxicity remain in the organ of Corti for a certain time and may be induced to heal or recover structurally and functionally. Some repair of injured cells may take place spontaneously, but the extent of such repair is difficult to measure[40]. Although it is difficult to observe tissue changing *in vivo*, functional assays may be indicative of the extent of recovery, especially within the outer hair cell population. There is an urgent need for studies that will determine what kind of therapy may enhance repair of injured hair cells in the organ of Corti.

Hearing loss and ageing

Not all hair cells survive for the full life-span of the animal and their loss gradually contributes to the general deterioration in hearing shown in ageing individuals. Presbycusis, in its more severe form, may be linked to a hereditary basis, as shown in mice with mutations in the *Ahl* gene[41]. However, humans have compromised hearing at an advanced age regardless of mutations in the *Ahl* gene. A combination of environmental factors with the extracellular environment may influence the demise of those hair cells that degenerate throughout our life[42]. Protective mechanisms discussed above versus acoustic trauma and aminoglycoside toxicity may also have relevance, at least in part, for enhancing the preservation of hearing through ageing. Considering that a large portion of the patients who seek help with hearing suffer from presbycusis, it is extremely

important to determine what type of diet, chronic low-level medications or other preventive means may prevent of reduce ageing-related hearing loss[25]. It is likely that at least some of the therapies mentioned above for protection against drug and noise inner ear trauma may also be active against presbycusis. One example is ROS antagonists which have been shown to reduce age-related hearing loss[43] either directly on hair cells or indirectly by improving circulation.

Hair cell regeneration

Once cochlear hair cells are lost, they are not replaced. The term 'regeneration' is commonly used for describing generation of new hair cells. In the scientific and medical disciplines dealing with neural repair and re-growth, nerve regeneration is used to describe repair of a pre-existing cell, not necessarily implying generation of a new cell. A clear distinction between these two mechanisms, re-growth and generation of new cells, would prevent confusion.

Conceptually, there are several possible ways to attempt generation (or introduction of) new hair cells to restore function of the organ of Corti. These include generation of new cells by mitosis of supporting cells (with some of the progeny differentiating into new hair cells), conversion of the phenotype of supporting cells to hair cells (without mitosis), or implantation of stem cells or other types of cell that will be enticed to differentiate to new hair cells. At present, none of these approaches are possible in the adult mammalian organ of Corti *in vivo*. However, several lines of research are rapidly enhancing our arsenal for possible interventions to accomplish these goals (see Holley, this volume). Most importantly, genes that regulate the proliferation of hair cell progenitors and the differentiation of hair cells and supporting cells are being discovered rapidly. The genes belong to several families, which participate in cell-cycle regulation, surface receptors and several transcription factors.

The potential for cell-cycle genes to facilitate regeneration

Cells in complex organisms constantly receive multiple signals from their environment, either from soluble extracellular factors such as growth factors and hormones, which bind to their respective receptors and activate various intracellular signalling pathways, or by physical signals provided by interaction with other cells or the extracellular matrix. Among the most important signals are those that influence a cell in its decision to commit to another round of cell division, or to remain in a quiescent state. To enter the cell cycle, cells require the appropriately regulated activities of various cyclins and cyclin-dependent kinases (CDKs)[44]. Upon activation, the cyclin–CDK

complex phosphorylates and inactivates the pRb tumour suppressor protein, thus promoting entry into the cell cycle. *In vivo*, CDK molecules exist in complex with various inhibitor molecules, which inhibit CDK activity, and thereby prevent cells from entering the cell cycle. The Cip/Kip family (for CDK interacting protein/ kinase inhibitory protein) includes $p21^{Cip1}$, $p27^{Kip1}$ and $p57^{Kip2}$. $p27^{Kip1}$ has been shown to inhibit several CDK molecules and regulate the decision to divide[44].

Recently, expression of $p27^{Kip1}$ protein was demonstrated in the supporting cells of the organ of Corti[45], consistent with the notion that $p27^{Kip1}$ may play a critical role in cell cycle arrest and in maintaining the differentiated phenotype. In developing $p27^{Kip1-/-}$ mice, the cells of the organ of Corti continue to proliferate for more than 2 weeks after proliferation would normally have ceased[46]. Moreover, supporting cells in the $p27^{Kip1-/-}$ mice can generate new hair cells after trauma-induced hair cell loss. These findings demonstrate that overcoming or releasing the inhibition of the cell cycle in these knockout mice allows the generation of excessive number of hair cells. It is necessary to design somatic cell interventions *in vivo* to remove $p27^{Kip1}$ inhibition of cell cycle in a way that will be specific and restricted to supporting cells in the organ of Corti. It is also necessary to identify other families of molecules that may influence proliferation of supporting cells. The vestibular epithelium is a good source of information for such searches[47]. Once the important molecules that signal mitosis in the epithelium are identified, the next major step toward accomplishing hair cell regeneration will be introducing and regulating expression of these genes in supporting cells of the cochlea.

Phenotypic conversion

One potential way to generate new hair cells is by conversion of the phenotype of supporting cells without cell division (see Richardson, this volume). Such conversion likely occurs as a secondary mechanism of hair cell regeneration in the avian basilar papilla[48,49]. The genes that mediate conversion in the inner ear are unknown. Identification of this set of genes and the ability to over-express them in supporting cells of the organ of Corti may lead to means for restoring the hair cell population, without the risk of malignancy that may accompany any change in cell cycle regulation.

Genes that specify the hair cell phenotype

During embryonic development, the fate of otocyst cells is determined in a sequence of events governed by intercellular signalling and expression

sequence of specific genes. Among these genes are *Math1*[50], *Hes1*[51], *Brn-3.1*[52] *GATA3*[53] and genes from the *Notch* family[54] (see Richardson, this volume). Manipulation of the level of expression of genes that encode hair cell differentiation may influence the fate of remaining supporting cells in the organ of Corti and possibly lead to hair cell regeneration following inner ear trauma.

Cell lines and stem cell therapy

The use of cultured cells for replacing lost hair cells is a relatively new concept with exciting potential (see Holley, this volume). Candidate culture cells are hair cell lines and stem cells. Hair cell lines are cell lines that may be generated by specific selection of sub-populations from dissociated otocysts. Currently, available hair cell lines are derived from otocyst of immortomouse[55–57]. The other source of cells is multipotent self-renewing stem cells. These clonal cells keep proliferating until they receive signals that induce their differentiation. In the absence of basal cells or stem cells in the organ of Corti, it is necessary to develop the technology to introduce stem cells into the area of the organ of Corti and provide the necessary signals for their differentiation and innervation[58]. One major challenge in any introduction of foreign cells into the cochlea will be the incorporation of transplanted cells into the correct site.

Hereditary hearing losses

Approximately half of congenital deafness is thought to be due to hereditary causes. Genetics also influences the disposition of individuals to progressive hearing loss later in life. Identification of the genes involved in deafness in humans has important implications for diagnostics and prognosis. One of the major challenges we face is the development of a cure for genetic inner ear disease. Such a cure will depend on our knowledge of the mutated genes and the technology for gene transfer. The intervention (gene transfer) should be based on substituting the mutated gene (or the missing or faulty gene product) with the normal gene. A proof of the principle that such replacement may reverse the phenotype and rescue hearing and hair cells was recently provided. Specifically, germ-line insertion of a wild-type *Myo15* transgene (in a bacterial artificial chromosome) into a zygote destined to develop into an affected shaker 2 mutant mouse (deaf and circling), resulted in a phenotypic rescue. The mouse that developed was hearing and did not circle[59]. At present, as physicians refer patients for genetic counselling and diagnostic testing, the number of known families with

hereditary inner ear disease is increasing, leading to an intensive effort by the research community to identify the mutated genes. Along with development of the technology for gene transfer, the likelihood that therapy for hereditary disease will become a real option is increasing.

Conclusions and key points for clinical practice

- Better understanding of cell death mechanism may help prevent hair cell loss in the inner ear.
- Several types of molecules have been shown to have protective effects against cochlear trauma.
- Once lost, hair cells cannot be replaced. However, the expanding knowledge of the molecular basis cell cycle and differentiation, along with advances in gene transfer technology, may help develop methods for gene therapy aimed at hair cell regeneration.
- Use of stem cells or cell lines as cell replacement therapy may also contribute to restoration of inner ear function.
- Identification of the genes that are mutated in hereditary inner ear disease, along with the development of vectors that will allow gene introduction into the specific cells of the auditory system, will help develop cure for genetic disease.

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