Fifteen hundred years ago, when a person had trouble with his or her ears, a Rabbi would prescribe the following: ‘Take the kidney of a bald goat, cut it crosswise, place it on burning coals and collect the water that begins then to flow from it. This water, when it is neither too cold nor too warm, syringe into the ears. Or one may rub in the ears with the molten fat of a big beetle….Fill the sick ear with olive-oil, then make of wheat-straw seven wicks, and with the hairs of a cattle attach to them the peel of garlic; kindle these wicks and put them into the olive-oil in the ear, taking, of course, precautions against burning the patient’ (Talmud Bavli, Tractate Avoda Zara, Chapter II). Today, we are looking towards research in genomics to provide us with approaches to cure ear ailments, particularly the loss of hearing and balance. Hearing loss (HL) is a clinically heterogeneous disorder, with differences in age of onset, severity and site of lesion. Hearing impairment is the most common disabling sensory defect in humans. Severe to profound HL affects 1 in 1000 newborns, another 1 in 2000 children before they reach adulthood, and 60% of persons older than 70 years will manifest a HL of at least 25 dB. Sensorineural HL, which affects the sensory epithelia in the organ of Corti of the inner ear (Figure 1), is most common. Approximately 60% of HL has a genetic basis and a significant proportion of genetic-based HL is non-syndromic (NSHL), the majority of which is inherited in an autosomal recessive mode [1,2]. In the past decade, many deafness-causing genes have been identified, an essential first step in understanding the molecular mechanism of auditory function and its loss. The discovery of genes and the mutations that lead to HL have been pursued for two major reasons: (1) to provide diagnostics and genetic counseling for patients, and (2) to provide biological sources for therapeutic approaches in HL. Extended families with hereditary HL have facilitated the discovery of >40 genes associated with...
NSHL. The genes known today to be involved in human hereditary NSHL encode a large variety of proteins, including molecular motors, gap junctions, ion channels, transcription factors and proteins that form the extracellular matrix of the inner ear (for updated list of genes see the Hereditary Hearing Loss Homepage, http://webhost.ua.ac.be/hhh/). However, many crucial genes in auditory transduction were found in cellular and model systems, with no apparent involvement in human disease. This could be due to the fact that some gene mutations will be lethal in humans or alternatively, a particular mutation has not been found in the human population. Therefore the use of deaf mouse models, whether spontaneous, ENU-induced or created by gene-targeted mutagenesis, have been priceless for the discovery of essential auditory genes. Much of this knowledge has been gained as a direct consequence of the unraveling of the human genome sequence, heralded as one of the most significant discoveries of modern times.

Despite the high prevalence of HL in our society, treatment today is limited to hearing aids and cochlear implants. These therapeutic tools do not completely restore our ability to hear, but for now, are the best options available. Studies are being conducted to provide alternative means of biological therapy to provide a more comprehensive treatment. There are several general approaches being considered for therapeutic intervention in HL: (1) prevention of cell death; (2) manipulation of expressed genes by gene therapy methods; (3) inhibition of negative regulators; and (4) stem cell therapy (Figure 2).

These therapeutics strategies for hearing impairment are attractive and promising for restoring HL of genetic origin. However, clinical application, particularly in the ear, is still limited. Most of the difficulties are technical ones concerning delivery of genes into the afflicted portion of the cochlea. This review will cover the current therapeutic approaches for rescuing HL by preventing hair cell death, gene manipulation and stem cell therapy.

**Prevention of cell death**

Most of the literature has dealt with prevention of hair cell death following acoustic trauma or aminoglycoside ototoxic damage. Protecting hair cells from irreversible degeneration has been a primary objective because of the finite number of hair cells in the inner ear. Hair cells stop differentiating during development and are post-mitotic, so that the number of cells we are born with (~16,000) is our lifetime supply. Little is known about the mechanisms of cell death in hereditary disease. It has been shown that in cases where hair cell damage can be predicted (although not in most genetic cases), there are several ways to moderate the damage by using neurotrophic factors [3–5], antioxidants [6,7] or anti-apoptotic agents [8,9]. It remains to be determined if any of these agents or factors can also rescue hair cells from death due to genetic disease. In this review, we will focus on anti-apoptotic agents as a promising avenue for restoring HL.

Caspase 3 inhibitors (e.g. zVAD, BAF) can promote hair cell survival in vivo [10] and in vitro [11] after treatment with aminoglycosides. It remains to be determined if anti-apoptotic agents can also rescue hair cells from death due to genetic disease. In a Mongolian gerbil model for age-related HL, it has been shown that the HL is associated with suppression of the bcl-2 protein and activation of caspase 3 [12]. In several mouse models for HL, several apoptotic factors have been shown to be involved. Caspase 3-deficient mice suffer from severe HL, hyperplasia of supporting cells and degeneration of sensory hair cells [13,14]. Apparently, different steps in development and preservation of the auditory system are mediated by caspase 3. The transcription factor POU4F3 has a critical role in the development of sensory hair cells in mouse [15–17] and in humans [18]. Pou4f3 knockout mice have no cochlear or vestibular hair cells, resulting in complete deafness. The hair cells in these mice progressively degenerate via apoptosis during late embryonic development [19]. Cellular and molecular mechanisms seem to
be similar in HL resulting from aging, drug ototoxicity and genetic mutations. A final common pathway could be apoptosis. It is likely that anti-apoptotic factors will increasingly be considered as important candidates for intervention strategy in sensorineural HL. A more comprehensive understanding of the molecular mechanism of hair cell death might lead us to employ new therapeutic anti-apoptotic agents to alleviate hereditary HL.

**Gene manipulation**

In non-mammalian vertebrates, lost hair cells can be replaced by newly generated hair cells. Many efforts have been made to reveal the progenitor cell for newly produced hair cells. Genes that regulate the proliferation and the differentiation of hair cells and supporting cells are rapidly being discovered. The main approach for generation of hair cells is to introduce genes into the cochlea using gene delivery methods to induce non-sensory cells or damaged hair cells to transdifferentiate into functional hair cells.

**Genes regulating hair cell differentiation**

During embryonic development, cell fate is determined by a sequence of events governed by intercellular signaling...
and expression of specific genes. In the inner ear, there are several known genes that participate in hair cell differentiation. By altering genes in the existing sensory epithelia, stimulation of cell division and differentiation may occur. Among these genes is the mammalian atonal homolog 1 (Atoh1, previously known as Math1), a basic helix loop helix transcription factor, which is necessary for hair cell differentiation [20]. Mice deficient in Atoh1 fail to develop hair cells [20]. The delivery of the gene encoding Atoh1 into the inner ear resulted in new hair cells [21–24]. The major breakthrough of the past year was the study performed by the Raphael group [23]. They used drug-induced deaf guinea pigs and introduced the Atoh1 gene using adenoviruses into the cochlea of these guinea pigs. Surprisingly, they brought back lost cochlear hair cells. Not only did new hair cells appear, but they also had functional properties. Eight weeks after injection, hearing had improved substantially in the infected ears. This outcome is the first functional restoration of a damaged mammalian hearing organ at the cellular level. The reappearance of hair cells was extensive and occurred apparently at the correct site and in the correct orientation. The new hair cells were able to attract innervation. The authors speculate that the new hair cells were derived from supporting cells or from other nonsensory cells close to the organ of Corti, indicating a transdifferentiation pattern. The overall cell number in the organ of Corti increased after Atoh1 expression, suggesting that either secondary cell proliferation occurred or that cell migration into the organ of Corti took place. Cells with a mixed morphology were observed, demonstrating that the transdifferentiation was incomplete. Further investigation is required to better switch off the supporting cell repertoire and switch on the hair cell repertoire of genes to gain full transdifferentiation.

Several genes other than Atoh1 can cause developmental defects when absent from inner ear sensory epithelia, and some of these genes could be applicable for gene therapy use. The genes Hes1 and Hes5 are negative regulators of Atoh1, and mice lacking either gene have increased numbers of hair cells when compared with wild-type animals [25,26]. Hes1 knockout animals have more inner hair cells (IHC), whereas Hes5 knockout animals have more outer hair cells (OHC) [25]. These experiments have a significant therapeutic value that will set a platform for the future development of inner ear gene therapy approaches based on the expression of key developmental genes.

**Cell-cycle regulation genes**

In the cell-cycle program, the level of cyclin-dependent kinase (CDK) activity controls signals that drive cells into S-phase. The products of at least three different gene families, Ink4, Cip/Kip (for CDK interacting protein/kinase inhibitory protein) and the retinoblastoma (Rb) pocket protein family, suppress S-phase entry [27]. These gene products are named CDK inhibitors (CKI). Once the important molecules that signal mitosis in the sensory epithelium are identified, the next major step toward accomplishing hair cell regeneration will be introducing and regulating the expression of these genes in the cochlea. The Cip/Kip family includes p21(Cip1), p27Kip1, and p57Kip2. When the p27Kip1 gene is knocked out in mice, the result is ongoing cell proliferation in the mature organ of Corti, well after the developmental period when mitosis in the sensory epithelium ceases [28]. Targeted disruption of Ink4d in the mouse changes the maintenance state of sensory hair cells in post-natal mice. In Ink4d−/− animals, hair cells re-enter the cell cycle and consequently undergo apoptosis, resulting in progressive HL [29]. The gene encoding Rb is part of a tumor suppressor gene family. The major function of Rb is in the regulation of cell cycle progression. Its ability to regulate the cell cycle correlates with the state of phosphorylation of Rb. It has recently been shown that hair cells lacking the gene for Rb continue to proliferate, with extra rows of inner hair and outer hair cells appearing, while expressing differentiation markers [30,31]. In summary, it appears that Ink4d, Kip1 and Rb are involved in maintaining cellular order, suggesting a potential use of CKIs in regeneration of hair cells in the inner ear. The risk of using cell cycle genes is that malignancy might accompany any change in cell cycle regulation, so that using these genes in gene therapy will require special attention to this potential problem.

**Delivery methods**

In some ways, the cochlea is particularly well-suited for gene therapy. It is isolated from the remainder of the body by the blood–labyrinth barrier, and the perilymph and endolymph fluids permit liquids to reach the entire cochlea quickly. The advantage of the small and enclosed structure of the inner ear, however, also poses a limitation because practice is still required on how to deliver vectors to the inner ear without causing damage to existing or residual hearing. Despite significant surgical challenges, the mouse is a useful model for studying gene therapy and multiple mutants with HL are available for study [32]. Developing strategies for local delivery into the inner ear is crucial for clinical therapies based on experimental findings.

A variety of viral and nonviral gene transfer vectors have been developed for implementation of gene therapy and the delivery of therapeutic genes. Non-viral vectors include liposomes, the traditional nonviral vector used in inner ear research. The components of cationic liposomes are positively charged, whereas nucleic acids are negatively charged, so that the two materials are able to form a stable particle. The liposomes can be mixed with DNA of virtually any size. They are easily prepared in large amounts and one of their most promising features is that they are non-immunogenic (for review see [33]). An
approach used has been to transfect a liposome mixture with LacZ or GFP reporter genes into mice and guinea pig cochleae in vivo [34–36], but this technique appears to be inefficient for successful gene delivery. There are other non-viral methods, such as the gene gun [37] and electroporation [22], but their rate of transfection is low. These methods have yielded significant results so far only in vitro.

There is extensive interest in the development of viral vector delivery systems of genes to the cochlea to cure hearing impairment. Recent studies have focused on vectors based on the herpes simplex virus [38–40], lentivirus [41], adenovirus [42–44], and adeno-associated virus (AAV) [45,46]. The expression patterns of vector-encoded transgenes have been found to differ significantly between vectors.

Adenoviral vectors (Ad) are being used most widely today for cochlear gene transfer. Some of their advantages are infection of dividing and non-dividing cells, ease of production, availability of high titers and high transduction efficiency. On the other hand, they do not integrate into the genome, leading to transient expression, and they can cause a strong immune response that will be toxic to the recipient cell [47]. Hair cell transduction in vivo has been made with the replication defective (E1-, E3-, pol-) or (E1-, E3-, E4-) Ad vector [24,48]. One must note the differences in the specific expression between studies. This variation might result from different Ad vector generations, Ad concentrations, methods of injection and differences in detection assays. The major drawback is in the immune response [49,50]. Recent studies have demonstrated limited hair cell damage [51] or protection from side effects by using immunosuppression treatment [52]. For these reasons, adenoviral vectors are still considered to be an optimal delivery tool in gene therapy of the cochlea.

AAV vectors for gene therapy are associated with several attractive features [53]. AAV vectors induce a much less potent immune response than Ad vectors [54]. AAV is able to infect and integrate into non-dividing cells with a high rate of occurrence and lead to stable integration. A major drawback of AAV is the packaging limit of 4.5 kb of foreign DNA in AAV particles, even when almost all the genome is replaced by the gene of interest (for review see [47]). Despite this limitation, the reduced immune response makes AAV attractive for further exploration. Recently, several studies found that different AAV serotypes can act differently on cochlear explants or in vivo and yield different transduction efficiencies (Figure 3a) [55–57]. The differences between in vivo and in vitro transduction must be taken into account when planning to use a particular serotype.

In the future, it is likely that we will see the fusion of vectors that will combine the infectivity and stability of the viral vectors and the safety of non-viral vectors. These ‘super’ vectors might lead to promising application of gene therapy for hearing disorders.

**Targeting expression**

The success of gene therapy depends on the ability of gene delivery systems to selectively deliver therapeutic genes to a sufficient number of target cells, yielding expression levels that influence the diseased state. There are several methods to target cells, specifically through the use of specific promoters or receptors.

The use of tissue-specific promoters makes it feasible to selectively express the therapeutic gene at the target cell [58]. Modification of promoters yields different expression patterns as well as differences in degrees of expression. The Atoh1 enhancer had been isolated and used to analyze the expression pattern of this gene [59]. The myosin VIIa hair cell-specific promoter was identified using transgenesis (Figure 3B) [60]. Myosin VIIa driven GFP expression was restricted to the hair cells of the inner ears derived from these transgenic mice. These promoters and/or enhancers can be used to deliver therapeutic gene products to the sensory hair cells.

In the case of surface receptors, which are unique to the target cells, a ligand that serves as a gene delivery vehicle would efficiently bind to the target cell surface receptor. To date, no specific hair cell receptor has been described as a ligand for gene targeting. Finding targeted ligands and more hair cells specific promoters could improve our delivery systems for the sensory epithelia.

**Stem cell therapy**

There is a great deal of hope and promise in stem cell research. The ability to differentiate stem cells into multiple cell types has been successfully applied in the generation of dopaminergic neurons for Parkinson disease [61,62] and insulin-secreting cells for diabetes [63,64]. In 2003, there was a major breakthrough in the use of stem cells for the replacement of hair cells with the discovery of embryonic stem (ES) cells, adult inner ear stem cells and neural stem cells that can generate hair cells in vivo [65–67]. Recent studies have shown that...
auditory [68] and vestibular [66] sensory epithelia can be a source for stem or progenitor cells.

A well-established technique to generate stem cells is by using adult organs (for review see [69]). It has been shown that either neural stem cells [65,70] or inner ear stem cells [66] have the ability to differentiate to different inner ear cell types in vitro [66] and in vivo [65,70]. The generated cell types and the specific markers expressed were different between these two adult stem cells. The potential for using these stem cells in therapeutic treatment lies in the ability to induce the cells to proliferate and differentiate into sensory hair cells to rescue or restore HL.

ES cells are pluripotent cells derived from the inner cell mass of the blastocyst. The Heller group succeeded in deriving inner ear progenitors from ES cells in vitro [67]. The progenitor cells expressed hair cell-specific markers such as the transcription factors Atoh1 and Pou4f3 and hair cell structural proteins such as myosin VIIa, epsin and parvalbumin 3. The ES-inner ear progenitors were implanted into the inner ear of chick embryos. Only cells in the inner ear sensory epithelium expressed hair cell-specific markers, demonstrating that the surroundings cells influenced the ES-derived cells. This is the first time that ES cells have successfully generated hair cells in vivo. The next step is to see whether the same observations will repeat themselves in a mouse inner ear, which is not identical to the avian ear.

However, it has not yet been proven that stem cells can become functional hair cells and integrate in the auditory epithelium. For hearing to be restored, it is necessary to form the appropriate neural pathways. Stem cells as cell therapy must, first of all, answer several questions: is the location of the newly generated hair cells important? Do we need to reconstruct the exact architecture of the inner ear to gain functional hearing? Will randomly oriented hair cells transfer correct information to the brain? Based upon the success of using cochlear implants, we can hope that any new hair cell that is connected to the nervous system will improve hearing abilities. Among the complex issues we will face is the need to prevent the stem cells from growing into tumors. Further experiments must be performed in vivo with an appropriate animal model for HL, which will need to face the challenges of surgical complications. Although there are ideal mouse models for hereditary [71,72] and drug-induced [73] HL, the mouse inner ear dimensions limit potential applications. Clearly, the next step in this field is to use the knowledge gathered from mouse (and other animals) and adapt it to human stem cells as a potentially therapeutic tool.

The future of therapeutics for hearing loss
To date, much of the work done thus far in the field has focused on gene therapy, that is, adding a ‘missing’ component by genetic methods. Unfortunately, gene therapy has not fulfilled expectations and many efforts to cure disease by this method have failed. Rather, modulating key components by inhibition could be a more favorable approach. For example, improving techniques for antisense or RNA interference (RNAi) are essential for the inner ear research field, as both these techniques have been heralded as having great potential for disease therapy. A new study performed by the Smith group succeeded in silencing the expression of the R75W allele variant of the GJB2 gene (connexin 26), which causes autosomal dominant NSHL. By silencing the mutant gene using the RNAi technique in vitro and in vivo, they restored hearing loss in a genetic mouse model [74]. The use of RNAi in clinical therapy still faces many obstacles. These include finding a suitable delivery method, establishing continuous and stable silencing, and coping with the interferon response (for review see [75]). Nevertheless, there is hope that RNAi can be successfully used as a therapeutic tool for HL. Furthermore, as delivery methods improve, modifying negative regulatory genes and cell cycle genes, discussed above, could be the method of choice to induce the growth of new hair cells.

Concluding remarks
The expanding knowledge of the molecular basis cell death, cell cycle and differentiation, along with advances in gene transfer technology, should aid in the development of methods for hair cell regeneration. A combination of genomic techniques will continue to reveal the regulatory networks of auditory transduction. For example, the microarray approach for elucidating regulatory pathways has proven to be useful [76] in identifying potential survival molecules, whereas the yeast two-hybrid approach has proven invaluable in elucidating the structural components of the stereocilia [77].

It is most reasonable to assume that gene therapy for HL will not be limited to one approach, but rather will integrate stem cells and gene modifications, along with drug treatment. For example, such methods might involve stem cells that will secrete a drug or express a gene that will restore functional hair cells.

The limited availability of hair cells has impeded their study and has made it logistically difficult to carry out many molecular biological and biochemical assays. For further research, improvements of some technical tools, such as the generation of more reliable hair cell lines, will be necessary to achieve significant progress in hair cell gene therapy. For clinical application, safe, effective and direct methods of gene delivery to the inner ear need to be developed to rescue HL.

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References

17. Wang, S.W. et al. (2002) Bmn-3b/Bmn-3c double knockout mice reveal an unsuspected role for Bmn-3c in retinal ganglion cell axon outgrowth. Development 129, 467–477
46. Liu, Y. et al. Specific and efficient transduction of cochlear inner hair cells with recombinant adeno-associated virus type 3 vector. Mol. Ther. (in press)
47. Sholl, M. et al. (2005) Adeno-associated virus-mediated gene transfer to hair cells and support cells of the murine cochlea. Mol. Ther. 11, 843–848
65 Tateya, I. et al. (2003) Fate of neural stem cells grafted into injured inner ears of mice. *Neuroreport* 14, 1677–1681
77 Boeda, B. et al. (2002) Myosin VIIa, harmonin and cadherin 23, three Usher I gene products that cooperate to shape the sensory hair cell bundle. *EMBO J.* 21, 6689–6699