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Sound Strategies for Hearing Restoration

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Abstract

Hearing loss is the most common sensory deficit in humans with some estimates suggesting up to 300 million affected individuals worldwide. Both environmental and genetic factors contribute to hearing loss and can cause death of sensory cells and neurons. Since these cells do not regenerate, the damage tends to accumulate leading to profound deafness. Several biological strategies to restore auditory function are currently under investigation. Due to the success of cochlear implants, which offer partial recovery of auditory function for some profoundly deaf patients, potential biological therapies must extend hearing restoration to include greater auditory acuity and larger patient populations. Here we review the latest gene, stem-cell, and molecular strategies for restoring auditory function in animal models and the prospects for translating these approaches into viable clinical therapies.

The remarkable extension of human lifespan over the past century has come at a price: it has also expanded our vulnerability to neurodegenerative disorders, including sensorineural hearing loss. Over the course of an average human lifetime the incidence of clinically significant hearing loss increases from one in a thousand at birth to one in three by 80 years of age. This dramatic rise in the incidence of hearing loss is due to overexposure to environmental toxins — including toxic noise, genetic predisposition to age-related hearing loss, or both. The problem is further compounded as the human population continues to age: estimates suggest that the number of Americans who suffer significant hearing impairment will rise from the current level of ~29 million to as many as 65 million by the year 2030 (4). This will undoubtedly have enormous socioeconomic impact with implications for patients, health care providers and policymakers.

While the epidemiology numbers are alarming, the consequences for the individual hearing loss patient can be devastating and very personal. As humans, we rely heavily on the richness of spoken language; therefore, loss of auditory function for the individual often leads to social isolation, depression and in extreme cases, suicide. Indeed, as Helen Keller famously noted in a 1910 letter to Dr. James Love: "The problems of deafness are deeper and more complex, if not more important, than those of blindness. Deafness is a much worse misfortune. For it means the loss of the most vital stimulus — the sound of the voice that brings language, sets thoughts astir and keeps us in the intellectual company of man."

Restoration of auditory function remains a lofty, but achievable goal for deaf patients and for scientists. Because hearing loss is a multifactorial problem, arising from many possible causes, it is unlikely that any single strategy will be an effective cure-all. Rather, multiple strategies may ultimately be required, tailored to target the underlying cause of the problem

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for each individual. Here we review recent progress toward development of strategies to treat hearing loss, with particular focus on four general approaches: cochlear implants, gene therapy, stem-cell therapy, and molecular therapy. These treatment strategies are at various stages of development and implementation and each carries its own unique set of advantages, risks and future promise.

Hair cells, spiral ganglion neurons and auditory transduction

The perception of sound in the human inner ear begins in the sensory hair cells of the snail shaped cochlea. Hair cells convert sound vibration into electrical signals which are transmitted via spiral ganglion neurons through the eighth cranial nerve to the brain (Figure 1). A number of problems can affect the physical transmission of air-borne sound vibrations from the external ear, through the middle ear into the inner ear. These middle ear problems can give rise to several forms of conductive hearing loss, which are often treatable through surgical intervention, placement of tubes to drain middle ear fluids or antibiotics. Problems that affect the conversion of sound signals into electrical signals or transmission of those signals to the brain are collectively known as sensorineural hearing loss, for which there is no cure.

There are approximately 16,000 sensory hair cells in the cochlea of a newborn. Hair cells are interdigitated with supporting cells that together form an epithelial layer. At the hair cell basal pole are synaptic contacts with spiral ganglion neurons. There are 30,000 – 40,000 afferent neurons (those that relay information toward the central nervous system) in each auditory organ. Hair cells, supporting cells, and spiral ganglion neurons are critically important for normal auditory function and because these cells do not spontaneously regenerate, those present at birth must last a lifetime. Unfortunately, due to the fragile cytoarchitecture of the mechanosensory organelle, the hair bundle, hair cells are particularly susceptible to damage and death. Hair cell loss often begins at the basal, high-frequency end of the cochlea and progresses toward the apical low-frequency end. At birth, the sensitive range of the human auditory organ spans frequencies from 50 to 20,000 Hz. With age, the high frequency end of the auditory spectrum creeps lower, even under the best conditions. Genetic factors, exposure to excessive noise or ototoxic drugs can accelerate the shift of the high frequency end. If the progressive hearing loss eventually reaches frequencies within the range of human speech – 1,000 to 4,000 Hz – it can interfere with normal speech comprehension in older adults and lead to misunderstanding, frustration and isolation for both the affected listener and the speaker. Hearing aids offer sound amplification and can help, but must rely on the hair cells and neurons that remain.

Auditory afferent neurons, known as spiral ganglion neurons, can also be damaged due to genetic and environmental insult. A growing body of evidence suggests that loss of these neurons and damage to the tonically active hair cell-afferent synapse is a major source of sensorineural hearing loss (5). Ninety-five percent of the afferent sensory information is transmitted to spiral ganglion neurons from a subset of hair cells known as inner hair cells. Each inner hair cell is contacted by up to 20 afferent neurons. This diversity of synaptic contacts is hypothesized to encode different aspects of the sound stimulus such as frequency and intensity, as well as temporal qualities like the beginning and ending of a sound.

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Efferent feedback from the brain to the inner ear modulates the sensitivity of hair cells and afferent neurons. Both afferent and efferent fibers travel along the eighth cranial nerve. Loss of subsets of these neurons may render a patient insensitive to certain aspects of an auditory stimulus. For example, middle-aged individuals may have near normal auditory thresholds but have difficulty distinguishing the voice of a speaker amid background noise, such as the cacophony of a crowded cocktail party. Here again, the hearing aid is woefully inadequate as it amplifies all sound and does little to boost the signal to noise ratio.

Development of hearing restoration strategies must confront the unique anatomy and physiology of the inner ear, which present both advantages and disadvantages. Among the advantages is the relatively easy access. Like the eye, the inner ear sensory organ is bathed by accessible fluid-filled spaces. The inner ear fluids are separated from the systemic vasculature by the blood-labyrinthine barrier which provides some protection from immune responses that might interfere with exogenous cells and vectors (6). The barrier may also prevent systemic dispersal of inner-ear-targeted therapeutic agents, thus limiting potential side effects in off-target tissues at remote locations. Access to the inner ear, while not as straight forward as to the eye, is possible via injection pipettes that penetrate the round window, a thin membrane that separates the middle and inner ear spaces. Injections via the round window allow entry to the inner ear fluids that bathe hair cell cell-bodies. For strategies that target the fluids that bathe hair bundles a cochleostomy approach may be required which necessitates drilling through the bony labyrinth near the cochlear apex (7). The unique fluids of the inner ear also present a significant challenge. There are two separate fluid-filled compartments: one that contains perilymph and another that contains endolymph. Perilymph is similar in ionic composition to cerebrospinal fluid and other standard extracellular fluids, while endolymph has high extracellular potassium, low sodium and low calcium. Disruption of the tight junctions that separate perilymph and endolymph can cause mixing of low and high potassium solutions and a decay of the endolymphatic potential, leading to hair cell death (8). Therefore, accessing the endolymphatic fluids without causing additional damage must be part of any strategy that targets those fluids. Furthermore, vectors and cells introduced into the endolymphatic fluids must be able to tolerate the unique high K^+ environment.

The inner ear also includes a multitude of other highly specialized cell types. Regeneration of missing cell types or designing vectors that target specific cell types will be required to address specific deficits that result from many forms of deafness. On the other hand, the limited number of inner ear sensory cells - 16,000 vs ~2 million in the eye - may prove advantageous as only a limited therapeutic load may be required.

Because defects in hair cells, spiral ganglion neurons or supporting cells can cause deafness, they are all possible targets of emerging technologies designed to treat auditory dysfunction. Indeed, all cell types must be present and functional for normal auditory perception. Although restoration of sound perception across entire human auditory spectrum is a worthy goal, lessons learned from cochlear implants suggest that even partial restoration may be sufficient to restore some aspects of sound perception.

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Cochlear Implants

The cochlear implant is, arguably, the most successful device at the machine-brain interface (9). The device was recently acknowledged with the 2013 Lasker-DeBakey Award for Clinical Medical Research, awarded to developers Graeme Clark, Ingeborg Hockmair and Blake Wilson. Cochlear implants have restored at least partial auditory function for over 300,000 patients worldwide (View http://www.youtube.com/watch?v=LsOo3jzkhYA to witness the joys of cochlear implant technology). The implants bypass the nonfunctional or absent hair cells and directly stimulate spiral ganglion neurons. The technology takes advantage of the tonotopic arrangement of the human auditory organ which allows for discrete stimulation of auditory neurons that encode different ranges of sound frequencies (Figure 2). Current implant technology provides stimulation at 8 to 22 discrete sites along the organ thereby signaling an equal number of auditory frequencies. Remarkably, this minimal auditory input (compared to the thousands of inputs in a healthy cochlea) is sufficient to enable speech comprehension, in some cases restoring the ability to communicate by cell phone.

Unfortunately, for many patients cochlear implants offer little hope (10). Patients who lack spiral ganglion neurons are not candidates for cochlear implants, as functional implants require these neurons to be present to convey the auditory information from the periphery to brainstem nuclei. For these patients, restoration of spiral ganglion neurons will be required either alone – if hair cells remain intact – or perhaps together with cochlear implants – if hair cells are also absent. Regardless, the success of the cochlear implant has been remarkable and has raised the bar for scientists focused on hearing restoration using biological interventions. Any alternative therapy will need to exceed the implant success rate, reduce the risks or provide hearing restoration at lower cost, if it is to be a competitive therapeutic alternative.

Gene Therapy for Genetic Deafness

With over 300 genetic loci implicated in hearing loss and the causative genes identified for about 70 of them, gene therapy is poised for development as a powerful hearing loss remedy (11–13). Many deafness mutations affect hair cell genes, while some affect neurons or supporting cells. Genetic deafness can be subdivided into two broad categories, syndromic and nonsyndromic. Syndromic mutations cause deafness and some other dysfunction, (such as cardiac arrhythmia as in the case of Jervell and Lange Nielsen Syndrome). Another prominent example of syndromic deafness is Usher Syndrome, which causes profound congenital deafness and progressive visual impairment. There are at least 15 genes associated with Usher Syndrome when mutated, 11 of which have been identified. Gene therapy trials for treatment of retinitis pigmentosa, a form of progressive blindness, are currently underway for Usher Syndrome 1B. Usher 1B is caused by mutations in *MYO7A* which encodes an unconventional myosin that is expressed in the sensory cells of eyes and ears. The human trial was approved based on successful lentiviral gene transfer of wild-type *MYO7A* into the eyes of mice with Usher 1B phenotype (14). This approach could be tested in mouse ears to treat auditory dysfunction in mouse models of Usher 1B. If successful in mouse ears, the approach could be adapted for human trails in the ears of Usher 1B patients.

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Nonsyndromic hearing loss affects the ear alone and includes 43 autosomal recessive mutations, designated DFNB1-DFNB93 and 25 dominant mutations, designated DFNA1-DFNA64, with some overlap in the affected genes. The most common hereditary hearing loss, DFNB1, affects supporting cells in the inner ear and is responsible for deafness in 40% of patients with genetic hearing loss. Several DFNB1 mutations have been identified in the *GJB2* gene, which encodes connexin-26, a component of inner ear gap-junction channels. Connexin-26 channels are expressed in a network of supporting cells that connect the Organ of Corti through the basilar membrane to the cells of the stria vascularis. This network is critical for recycling potassium ions released from the hair cell basolateral membrane back to the potassium-rich endolymph that bathes hair bundles. Disruption of the K^+ recirculation network leads to a decline in endolymph K^+ concentration and a decay of the endolymph potential (~+100 mV), which together result in profound deafness. Several hearing loss genes, both syndromic and non-syndromic have been identified that affect K^+ recirculation including three gap-junction genes and the Jervell and Lange Nielsen Syndrome gene, *KCNQ1* which encodes a potassium channel. Gene restoration studies that target the K^+ recirculation network are currently underway, with several focused on connexin-26 in a mouse model of DFNB1. A recent *in vitro* study presented compelling proof-of-concept evidence that gene transfer of *GJB2* can restore functional gap-junction channels in the supporting cell network required for K^+ recirculation (15). In a more recent study, Yu et al. (16) used a similar adeno-associated viral (AAV) vector approach *in vivo* in *Gjb2* knockout mice and reported widespread expression of connexin-26 in the supporting cell network. Although there was no significant hearing improvement, the potential to move this line of investigation forward in animal studies and - if successful - in human studies is tantalizing. Since mutation of *GJB2* is the most common cause of genetic deafness (up to 50% in some populations; 17), effective strategies to restore gap-junction function in the cochlear supporting cell network could benefit a significant number of patients with genetic hearing loss.

A number of other mutations affect hair cell genes, the largest group of these affecting development, maintenance and function of the mechanosensory hair bundle. For genes that affect hair bundles, the design of effective therapeutic strategies faces several significant challenges. First, gene delivery systems must be capable of targeting hair cells. Second, because hair cells undergo a precise developmental sequence, it may be necessary to provide the correct gene within the correct developmental time frame. For some genes required early in development (prenatally), this may limit the therapeutic window. Lastly, current expression systems provide little control over expression level. Even within the right cells and at the right time, achieving the optimal expression level while minimizing toxic overexpression will add to the challenges of hair-cell gene therapy. Nonetheless, a number of studies have reported successful use of viral transfection of mouse cochlear hair cells *in vivo* at both neonatal (18, 19) and adult stages (Figure 3; 20).

Moreover, numerous studies have demonstrated adenoviral and adeno-associated viral vector transfection of rodent hair cells *in vitro*. For example, Kawashima et al. (21) used adenoviral vectors with reduced toxicity to replace wild-type *Tmc1* or *Tmc2*, driven by a *MYO7A* hair-cell promoter. The strategy successfully restored hair-cell mechanotransduction

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in non-functional hair cells that lacked endogenous *Tmc1* and *Tmc2*. Although the precise molecular function of TMC1 and TMC2 is controversial (22), it is clear that mutations in *TMC1* cause deafness in mice and humans (23). As such, gene augmentation in this and other mouse models of human deafness may provide powerful experimental paradigms for investigating gene therapy strategies to restore auditory function *in vivo*.

In an *in vivo* model system, Akil et al. (24) demonstrated successful replacement of vesicular glutamate transporter 3 (VGLUT3) in a *Vglut3* knockout model (see commentaries by Holt and Vandenberge (25) and by Martin and Raphael (26). In this case, adeno-associated viral vectors, serotype 1, were used to deliver the *Vglut3* sequence to the inner ear. The investigators achieved a high level of transfection efficiency, 100% of the targeted cells, and restored some auditory function as measured by auditory brainstem responses and auditory startle reflexes. Although the vectors carried a promoter with broad activity, chicken-beta actin, the *Vglut3* transgene was only expressed in the targeted auditory inner hair cells. The authors suggested that endogenous regulatory mechanisms may govern expression level and thus may help limit off-target expression. If similar mechanisms regulate expression of other hair-cell genes, they may help minimize concern over the need to develop exogenous systems with precise control over expression level. While the Akil et al. (24) study presented some of the first data showing gene-therapy-mediated recovery of auditory function in a knockout mouse model, deafness-causing mutations in human *VGLUT3* are not common and when present are dominant (DFNA25), suggesting the utility of gene augmentation strategies for *VGLUT3* mutations in humans may be limited.

Other mutations, such as those causing the recessive nonsyndromic DFNB9 (27), affect neuro-transmission at the hair cell afferent synapse. The mutated gene in DFNB9 encodes otoferlin, which functions as a calcium sensor required for fusion of synaptic vesicles and release of neurotransmitter (28). Reisinger et al. (29) investigated the possibility that synaptotagmin1 might replace otoferlin and used AAV-mediated gene transfer of the synaptotagmin1 gene into mouse hair cells deficient in otoferlin. Although the strategy failed to restore synaptic transmission, the possibility remains that gene transfer of the wild-type otoferlin sequence may restore function for DFNB9 patients. AAV-mediated gene transfer of otoferlin was not attempted because the coding sequence was too large to be packaged into AAV vectors, which further highlights the need for development of larger capacity vectors.

Gene Therapy for Acquired Deafness

Gene therapy strategies may also prove useful for acquired hearing loss that results from death of hair cells or spiral ganglion neurons. Hair cells are fragile and can easily be damaged by over exposure to loud sounds, bacterial and viral infections and an array of ototoxic drugs including aminoglycoside antibiotics and the anti cancer drug, cisplatin. Although lower vertebrates retain the ability to generate new hair cells, this capacity has been lost in mammals, including humans. Therefore, generation of new mammalian hair cells is an active area of research. In addition to stem cell therapies for hair cell replacement (see below), it may also be possible to convert the phenotype of endogenous supporting cells into hair cells using key regulatory elements such as the hair cell transcription factor

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ATOH1 (reviewed by Rubel et al. (30). ATOH1 is a basic helix-loop-helix transcription factor that is necessary for proper hair cell differentiation (31). Several groups have used transfection of *Atoh1* into inner ear supporting cells to drive generation of new hair cells, either in deafened guinea pig inner ears (32) or in embryonic mouse inner ears (33). In the latter case, the cells were found to be functional with hair bundles that supported mechanosensory function. In both studies exogenous *Atoh1* expression was targeted to the supporting cells of the cochlea. However, *Atoh1* may also offer therapeutic benefits in surviving hair cells that have lost their mechanosensory hair bundles. Yang et al. (34) suggested that forced expression of *Atoh1* in noise-damaged hair cells can induce repair and recovery of sensory hair bundles. If so, such an approach may offer hope for patients who suffer acute noise damage such as battlefield troops or victims of the Boston Marathon bombing, many of whom suffered significant hearing loss and are involved an ongoing clinical study. The therapeutic window may be short, however, as hair cells must be targeted shortly after the injury, before they die. Although additional data will be required to map out the temporal window of opportunity for this potential therapeutic approach, human clinical trials have recently been approved for use of adenoviral delivery of *Atoh1* into the supporting cells of the inner ear to treat auditory and balance dysfunction in an industry collaboration between Novartis and GenVec. The team filed an Investigational New Drug (IND) application with the Food and Drug Administration (FDA) for the clinical development of the approach and received approval in early, 2014.

Gene Therapy for Spiral Ganglion Neurons

Loss of spiral ganglion neurons (SGNs) may occur either as a secondary consequence following loss of trophic support or can occur directly through damage to the afferent synapse or spiral ganglion cell bodies (35). Inner ear cells, including hair cells, release at least two neurotrophins, brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT3; 37). Although the source of BDNF and NT3 is dynamically regulated during development (38, 39) it has been suggested that hair cell death leads to loss of trophic support and progressive loss of SGNs. This model has been challenged by Zilberstein et al. (40) who suggested that inner hair cells are not necessary for SGN survival and that the source of the trophic support may be cochlear supporting cells. Regardless of the cause, loss of SGNs reduces the number of information channels to the brain, impairing speech perception and limiting the therapeutic utility of cochlear implants. Since cochlear implants stimulate SGNs directly with electrical impulses, survival of SGNs is a prerequisite for implant success. Thus, a number of studies have focused on methods for providing exogenous trophic support capable of promoting SGN survival. Several viral vectors have been used successfully to mediate gene transfer of the coding sequence for BDNF into the supporting cells of the inner ear in animal models (12, 41–43). The strategy has generally proven successful for promoting SGN survival, at least over the short term. However, several important questions remain. Can the strategy be extended to promote long term SGN survival, and if so, can it be adapted for use in conjunction with cochlear implant technology in humans? Whether viral mediated expression of neurotrophins or direct perfusion of exogenous growth factors would be more suitable will also be worthy of further investigation.

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Stem Cell Therapies for Generation of Hair Cells

Stem cell therapy for treating deafness has received considerable attention over the past decade with major advances in generating hair-cell-like cells and auditory neurons *in vitro* from fetal auditory stem cells, embryonic stem cells, or induced pluripotent stem cells (44). Identification of a renewable source of sensory progenitor cells is an important first step in the quest to develop stem cell therapy for deafness.

Li et al. (45) demonstrated the feasibility of using stepwise differentiation for the generation of inner ear progenitor cells and hair cells from embryonic stem cells. Nestin-positive progenitor cells that expressed markers of the otic placode and otic vesicle were successfully obtained from embryoid-body-derived cell populations with an EGF/IGF1 treatment followed by bFGF. Differentiation was induced by removal of growth factor for 10 to 14 days after which the presence of inner ear cell types was indicated by expression of various hair cell markers including ATOH1 and MYO7A.

Stepwise differentiation protocols have been further refined to successfully lead to the generation of hair-cell-like cells. Oshima et al. (3) identified conditions that enabled differentiation of otic progenitors into hair-cell-like cells (Figure 4). These cells expressed numerous hair-cell genes, developed actin-based hair-bundle-like protrusions on their apical surfaces, and displayed interciliary links that resembled tip links. Mechanical deflection of the apical bundles evoked mechanosensitive transduction currents, however, the amplitudes were small and they appeared to lack directional sensitivity. The currents were inhibited by a mechanotransduction channel blocker, dihydrostreptomycin, suggesting that the currents were carried by bona fide mechanotransduction channels. Oshima et al. (3) also analyzed voltage-dependent currents which varied in amplitude and kinetics but resembled those of immature vestibular hair cells (46). While an important advance, the generation of hair-cell-like cells required co-culture with a feeder layer of stromal cells derived from embryonic chicken utricles. The feeder layer provided required trophic support for a short time but the specific factors needed remain unknown. The approach was also limited by its low yield, with only very few cells adopting hair-cell-like phenotypes.

More recently, Koehler et al. (47) used a precise temporal sequence of signaling molecules to recapitulate *in vivo* development in a three dimensional culture (Figure 4). Preplacodal induction was successful with an interplay of BMP activation and TGF-β inhibition. Induction resulted in self-guided formation of sensory epithelia with hair cells that possessed functional and structural properties of native hair cells. The number of cells generated by this method was astonishing, with over 1500 cells per aggregate. This tour de force is a giant step forward in the inner ear stem cell field. Demonstration that these cells display normal hair-cell functions, including mechanotransduction, will help validate this approach.

Stem Cell Therapies for Generation of Auditory Neurons

While generation of sensory hair cells from stem cells has been an important focus of the field, it is only part of the picture. Since damage or loss of spiral ganglion neurons can be a primary cause of deafness (35, 36), development of strategies to generate new auditory neurons has become a major focus. Replacement of auditory neurons could serve two

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potential clinical needs. For auditory neuropathy patients who retain intact hair cells, generation of new SGNs capable of reinnervating hair-cell epithelia has the potential to restore function. For patients with auditory neuron and hair-cell loss, generation of new SGNs could provide a conduit for cochlear implants to communicate with auditory brainstem nuclei. Of course, both strategies will require that the new SGNs make appropriate and functional contact with the pre-existing neuronal circuitry of the auditory brainstem.

Neural progenitors have been successfully derived from cochlear stem cells (44, 48), neural stems of the lateral ventricle (49) and human embryonic stem cells (50, 51). Several groups have identified factors capable of promoting stem cell differentiation into glutamatergic neurons with otic-like, SGN phenotypes (52, 53). To investigate integration of neural progenitors into the damaged inner ear, Corrales et al. (54) deafened gerbils with ouabain which selectively killed type I spiral ganglion neurons, yet preserved the sensory hair cells. After 10 weeks, transplanted otic-like neural progenitors (ONPs) successfully engrafted into the modiolus forming ectopic ganglia with differentiated neuronal-type cells that projected to the sensory cells within the organ of Corti. Some fibers were observed at the point they exited the modiolus and projected toward the brainstem. To assess whether such progenitors could restore function, Chen et al. (51) adopted a similar approach and found that transplanted ONPs not only survived and differentiated but also grew neurite projections and improved auditory evoked responses. Otic epithelia progenitors (OEPs) and ONPs were obtained from human embryonic stem cells treated with FGF3 and FGF10, signaling factors necessary for placode induction. ONPs further differentiated into otic-like neurons using a stepwise approach that included several growth and differentiation factors. As early as 4 weeks post-transplantation, improvement of hearing thresholds was evident and by 10 weeks transplanted animals had recovered ~46% of their auditory function. While the restoration of proper anatomical connections using ONPs was observed by both Corrales et al. (54) and Chen et al. (51), the functional recovery has yet to be replicated. Validation of these remarkable results will bode well for the development of stem-cell-based restoration strategies for generation of new auditory neurons in humans (55).

Molecular Therapies for Generation of New Hair Cells

Pharmacological compounds that could induce formation of new hair cells would be particularly attractive for treating patients with hearing loss that results from hair cell death. When hair cells die, the capacity for normal hearing fades. Unfortunately, mammalian cochlear hair cells do not grow back. However, a handful of studies have now reported generation of new hair cells by manipulating endogenous signaling pathways in supporting cells. The possibility that these pathways could be targeted by pharmacological agents is enticing.

White et al. (56) collected cochlear supporting cells that expressed the cell cycle inhibitor $P27^{Kip1}$, and grew the cells on a mesenchymal feeder layer *in vitro*. The supporting cells downregulated $P27^{Kip1}$ expression and re-entered the cell cycle generating new cells, some of which became hair cells. This finding suggests that pharmacologic manipulation of cell

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cycle genes may be a viable strategy for generation of new inner supporting cells and new hair cells.

In another line of investigation, a population of cochlear cells was identified that expressed LGR5, a marker associated with stem cells (48, 57–59). The LGR5-positive cells were capable of generating new hair cells, confirming that LGR5-positive cells can act as sensory progenitors *in vivo* (60, 61). Since the cells were responsive to small molecule Wnt agonists and antagonists, it is plausible that manipulation of the Wnt pathway in the mature cochlea may hold the potential for generation of new hair cells in the human inner ear.

While these studies have helped define important inner ear signaling pathways, the work has not yet been developed for *in vivo* application. Recently, Jeon et al. (62) used a γ-secretase inhibitor that blocks notch signaling and removes lateral inhibition between hair cells and supporting cells. The team found that inner ear progenitor cells can be directed toward a hair cell phenotype by manipulating γ-secretase activity. The investigators introduced the inhibitor into cochlear explants *in vitro* and found a large number of new hair cells generated from supporting cells. This observation was confirmed and extended by another group who used γ-secretase inhibitors to generate hair-cell like cells from supporting cells in cochleas damaged with ototoxic drugs (63). When Mizutari et al. (64) introduced γ-secretase inhibitors via round-window injection into mature noise-damaged mouse cochleas *in vivo*, they found that organ of Corti supporting cells were converted into outer hair cells in the mid- to low-frequency regions. The conversion of supporting cells to hair cells was confirmed using linage tracers and antibodies to the outer-hair-cell-specific protein, prestin. Importantly, generation of new hair cells *in vivo* was sufficient to restore partial auditory function, as determined by lower sound thresholds in auditory brainstem recordings. While systemic application of the γ-secretase inhibitor caused severe side effects, local delivery to the inner ear appeared to overcome those effects. The Mizutari et al. (64) results will likely motivate the search for compounds or delivery methods that can selectively inhibit the notch signaling pathway in cochlear supporting cells.

Hair cells themselves may also be targets of pharmacologic manipulation for generation of new hair cells. For example, Sage et al. (65) identified retinoblastoma (Rb) protein as a signaling molecule that prevents mature post-mitotic hair cells from re-entering the cell cycle. Genetic deletion of Rb was shown to promote mitosis in mature hair cells and formation of new functional hair cells. Unfortunately, the therapeutic utility of this strategy may be limited because Rb is a tumor suppressor gene and because the newly generated hair cells tended to undergo p53-mediated apoptosis (66). Nonetheless, the findings raise the possibility that cell cycle regulators may be suitable pharmacological or gene therapy targets for control of hair cell proliferation and restoration of auditory function. Of course, such strategies will depend on survival of at least some endogenous hair cells and the ability of the new hair cells to resist apoptosis.

Molecular Therapies for Genetic Hearing Loss

To target a specific gene defect, Lentz et al. (67) provided a startling example of treatment of a genetic deafness using systemic application of antisense oligonucleotides (ASO). The

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authors used a mouse model of human Usher Syndrome 1C which affects harmonin (68, 69), a component of the hair cell transduction complex (70). The mouse model was generated to mimic a recessive mutation in French-Acadian USH1C patients that introduces a cryptic splice site and results in a severely truncated protein (67, 71). Lentz et al. (67) screened 47 different ASO sequences for those that could block cryptic splicing and promote correct splicing of the harmonin mRNA transcript. An ASO was identified that provided >20% correct splicing and increased expression of wild-type harmonin protein. Remarkably, homozygous mutant mice treated with a single ASO intraperitoneal injection recovered normal vestibular function and some auditory function, particularly at lower frequencies (8–16 kHz in the mouse). The authors went on to show that the ASO treatment restored harmonin protein levels *in vivo* and restored normal cochlear hair cell morphology, but were cautious to point out that the strategy was only effective at very early developmental stages, which may limit the utility of the approach in humans. Although a number of mechanistic questions remain, including the route of ASO entry from intraperitoneal injection into cochlear hair cells, the study presents a stimulating new strategy for the potential treatment of genetic hearing loss using systemic injection of antisense oligonucleotides.

Future Directions

Despite the remarkable progress outlined above, a number of important questions and challenges remain. Generation of new hair cells or neurons will require proper integration of the new cells into an existing, mature organ. The new hair cells will also need to be properly oriented within the sensory epithelium if they are to be functional. To transduce functionally relevant stimuli, new hair bundles must be sensitive to the appropriate sound-evoked stimulus which means their bundles must be oriented with the same polarity as native cells. Furthermore, since auditory hair cells come in different varieties (inner and outer) and their biophysical properties vary along the tonotopic axis of the cochlea, newly generated hair cells must mimic the location-dependent properties of native cells, if they are to replicate the fully functional auditory system. Likewise, new neurons must be capable of innervating existing hair cell epithelia within the tonotopic arrangement, if they are to convey frequency-specific information. Importantly, the new neurons must also be capable of projecting to the brainstem and making appropriate, functional contacts that preserve the tonotopic map of the cochlear nucleus. Alternatively, if the auditory system retains the capacity for plastic rearrangement of tonotopic maps, precise connectivity may not be an absolute requirement. As successful cochlear implants and the aging auditory periphery have demonstrated, the central auditory pathways are capable of learning how to deal with imperfect or incomplete sensory information.

For strategies that aim to repair hair cells or other inner ear cells with gene therapy there is still great need to develop better control over exogenous gene targeting and expression. The technical capacity for gene transfer of wild-type sequences into the supporting cells (18) and hair cells of the inner ear currently exists. Yet, there is still significant need for better understanding of the temporal window of therapeutic opportunity which may vary depending on the specifics of the genetic defect. Also needed are better control over expression level and studies of possible detrimental effects in off-target cell types.

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Thus far, most inner ear gene therapy strategies have focused on gene replacement or augmentation approaches. These approaches may be well-suited for recessive deafness but offer little hope for overcoming the consequences of dominant forms of genetic deafness. Several novel alternatives for treating dominant deafness are on the horizon. One such approach would involve viral delivery of siRNA sequences designed to suppress expression of the mutant sequence but not the correct, wild-type sequence (72). Since many forms of dominant deafness result in single point mutations, the design of effective and specific siRNAs, with only one nucleotide mismatch, will be challenging.

Another exciting approach would be the use of genome editing strategies. These cutting-edge approaches have not yet been investigated in the inner ear, but could offer hope of a single treatment for restoration of the wild-type sequence in native DNA. Zinc-finger nucleases (ZFNs) or transcriptional- activator-like effector nuclease (TALENs; reviewed by Gaj et al. (73, 74) could be designed and packaged into viral vectors that target inner ear supporting cells or hair cells. RNA-guided DNA endonucleases based on clustered regulatory interspaced short palindromic repeats (CRISPR/Cas) have also recently emerged and hold enormous potential for genome editing in humans (73, 75, 76). It seems plausible that any of these genome editing strategies could be adopted for use in hair cells and supporting cells of the inner ear.

In some cases, combinations of the approaches discussed above maybe warranted. For example, in cases of genetic deafness, hair cell death may follow as a secondary consequence. If so, these patients may require stem cell or gene therapy approaches to restore hair cells. If autologous stem cells are used or if *Atoh1* is used to generate new hair cells from endogenous cells, there may also be need to repair or replace mutant genes in the new hair cells.

Another example of a dual approach might be the use of gene or stem cell therapies in combination with next-generation cochlear implants (77). One potential strategy is the use of optogenetics and the expression of channelrhodopsin in spiral ganglion neurons using gene therapy vectors (78). Cochlear implants could then be designed using fiber optics and focused stimulation of small numbers of SGNs at perhaps hundreds of locations along the cochlea. This approach could circumvent a problem imposed by electrical stimulation: the spread of electrical current to neighboring off-target neurons which severely limits the number of positions that can be stimulated along the tonotopic axis of the cochlea.

Lastly, as a platform to translate potential therapeutic approaches from animal models into human tissue, Kesser et al. (79) developed a novel experimental paradigm using human inner tissue maintained in organotypic culture. Live vestibular tissue was excised from the inner ears of human patients undergoing resection of tumors growing on the 8th cranial nerve. The sensory epithelia were kept alive in culture dishes and exposed to adenoviral vectors that carried the wild-type coding sequence for green fluorescent protein and the potassium channel protein, KCNQ4. Both supporting cells and hair cells were transfected and KCNQ4 was properly localized along the hair cell basolateral membrane. Since mutations of KCNQ4 cause the dominant-progressive hearing loss, DFNA2, the strategy may require significant over expression of the wild-type protein. Importantly, the study

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demonstrated that vectors and strategies developed in mice can be adapted for use in human tissue. Furthermore, the *in vitro* model system may provide a platform to help validate other treatment strategies prior to initiation of human clinical trials.

Complete restoration of auditory function is a vexing problem. Perception of the rich auditory soundscape that humans encounter daily — which includes speech, music and a plethora of natural and man-made sounds — demands a system capable of exquisite sensitivity, broad dynamic range, precise frequency discrimination and high temporal fidelity. This is a tall order for any biological or mechanical system and restoration of such a system in adult humans only adds to the challenge. Yet with the commitment of government funding agencies, private foundations and teams of dedicated scientist, it is an achievable goal with a sound future.

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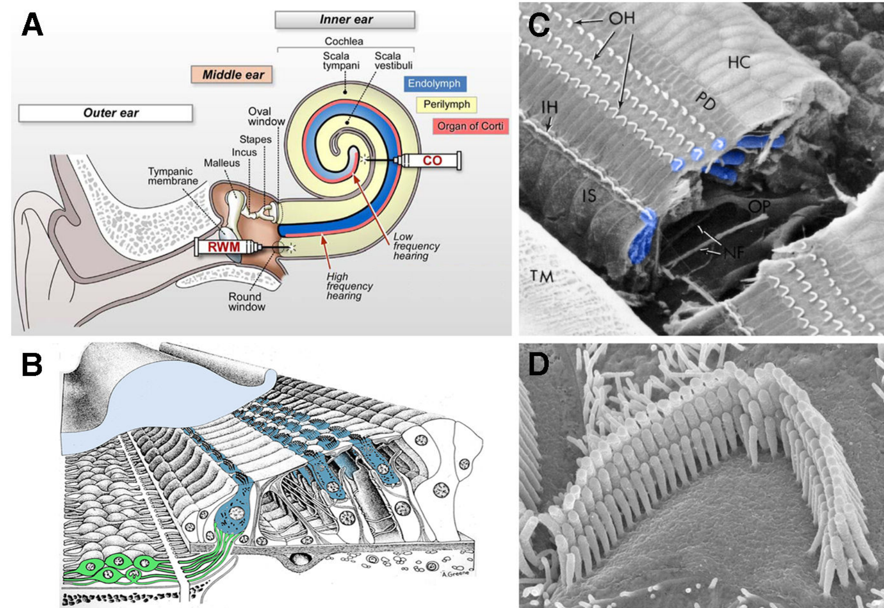

Figure 1.

Anatomy of the inner ear. A) Schematic diagram of the human inner ear (24). The spiral shaped cochlea is shown with endolymph and perilymph fluids indicated in blue and yellow, respectively. The auditory organ known as the Organ of Corti is shown in red. Potential routes of entry for therapeutics are indicated: round window membrane (RWM) and cochleostomy (CO). B) Schematic diagram of a cross-section of the Organ of Corti. The three rows of outer hair cells and the one row of inner hair cells are colored in blue and the auditory neurons or spiral ganglion neurons are shown in green. C) Scanning electron micrograph of the Organ of Corti. The hair-cell cell-bodies are pseudo-colored blue. D) Scanning electron micrograph of the hair bundle of a single outer hair cell.

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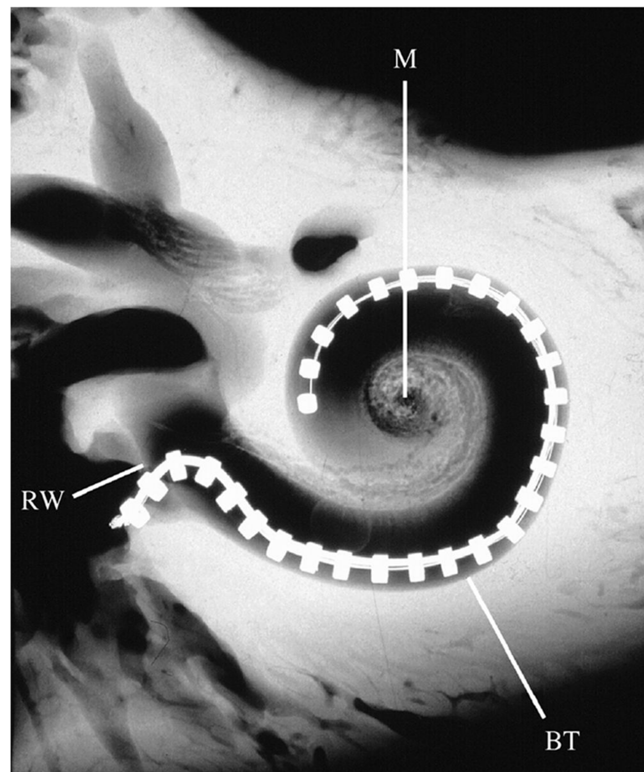

Figure 2.
X-ray image of a cochlear implant in a human cochlea. This implant includes 29 stimulating electrodes. M, modiolus; RW, round window or entry point to the basal turn of the cochlea; BT, basal turn of the cochlea.

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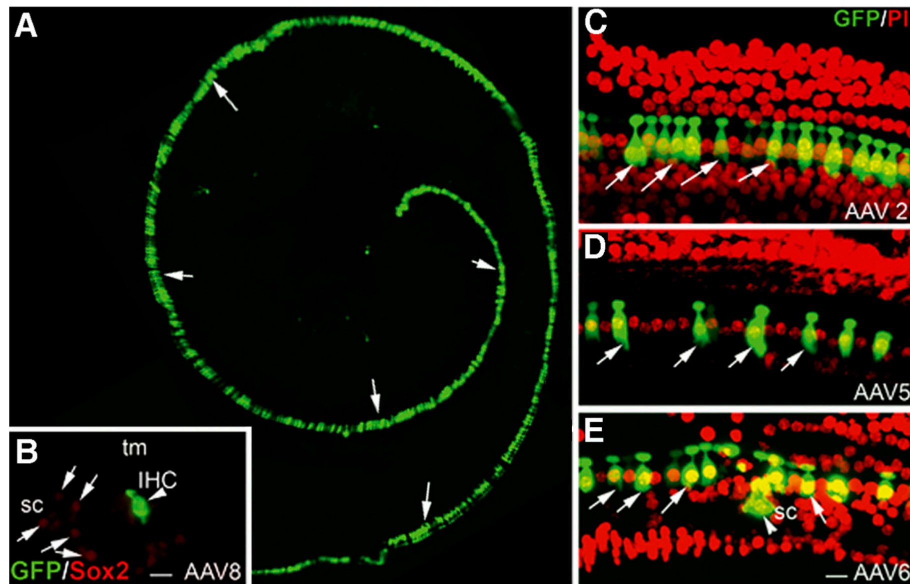

Figure 3.

Confocal images of adult mouse cochlea transfected with AAV vectors of various serotypes. A) Inner hair cells (arrows) were positive for the transgene, green fluorescent protein (GFP), 7 days after transfection with AAV2. B) Cross-section of the Organ of Corti transfected with AAV8, counter stained for Sox2 (red) which marks supporting cells. High magnification view of hair cells transfected with AAV2 (C), AAV5 (D) or AAV6 (E), counter-stained with the nuclear marker, propidium iodide (red). Scale bars = 20 μm. Reprinted from Kilpatrick et al. (19).

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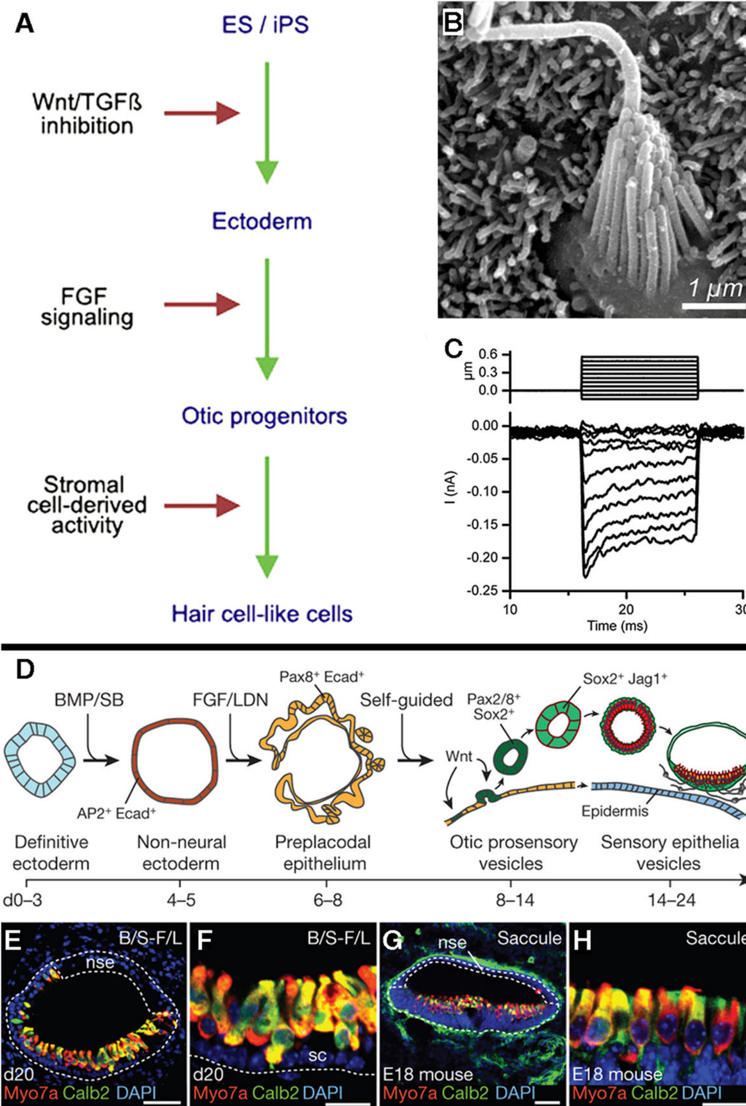

Figure 4.

Examples of stem cell-derived hair cell-like cells. A) Strategy used by Oshima et al. (45). B) Hair bundle generated from strategy shown in panel A. C) A family of mechanotransduction currents recorded from a stem-cell-derived hair cell-like cell. The hair bundle deflection protocol is shown above. D) Strategy used by Koehler et al. (47). E–F) Stem-cell derived otic vesicles and hair cells. F–G) Embryonic vestibular organ and hair cells. Immunomakers are color-coded at the bottom. Scale bar: 50 μm (E, G), 25 μm (F,H).