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Gene Therapy in the Inner Ear Using Adenovirus Vectors

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Abstract

Therapies for the protection and regeneration of auditory hair cells are of great interest given the significant monetary and lifestyle impact of hearing loss. The past decade has seen tremendous advances in the use of adenoviral vectors to achieve these aims. Preliminary data demonstrated the functional capacity of this technique as adenoviral-induced expression of neurotrophic and growth factors protected hair cells and spiral ganglion neurons from ototoxic insults. Subsequent efforts confirmed the feasibility of adenoviral transfection of cells in the auditory neuroepithelium via cochleostomy into the scala media. Most recently, efforts have focused on regeneration of depleted hair cells. Mammalian hearing loss is generally considered a permanent insult as the auditory epithelium lacks a basal layer capable of producing new hair cells. Recently, the transcription factor *Atoh1* has been found to play a critical role in hair cell differentiation. Adenoviral-mediated overexpression of *Atoh1* in culture and in vivo have shown the ability to regenerate auditory and vestibular hair cells by causing transdifferentiation of neighboring epithelial-supporting cells. Functional recovery of both the auditory and vestibular systems has been documented following adenoviral induced *Atoh1* overexpression.

Introduction

While gene delivery is now a relatively well-established research tool within the field of auditory neuroscience, the first reports of molecular genetic therapy for the inner ear were published only slightly more than a decade ago. These early efforts were prompted by the successful use of gene transfer in several other organ systems, including the central nervous system [1–4]. Several properties of the inner ear suggested that this would be a hospitable environment for gene therapy intervention. First, the organ is surrounded by the temporal bone and isolated within the otic capsule, reducing the risk of inoculating adjacent tissues. Second, the inner ear anatomy is composed of fluid-filled spaces that permit widespread diffusion of a locally introduced vector. Finally, the inner ear is composed of several distinctive cell types including spiral ganglion neurons, supporting cells, and hair cells. Thus, the impact of genetic manipulation on each of these cell types can be studied by quantitative, structural, and physiological analysis.

The ultimate aim of gene delivery is the expression of a gene product within the target tissue. A number of variables affect the precise approach to this goal. Gene transfer can be accomplished by both in vivo and ex vivo techniques. In vivo transfer involves the introduction of a vector directly into the target organ. The gene may be taken up directly by host cells and act locally, or the gene product may be secreted to influence the surrounding environment. This method offers the advantage of being able to genetically manipulate quiescent cell populations such as those found in the inner ear. The technique of ex vivo gene transfer involves transduction of a population of cells in culture which are then introduced to the target organ. Ex vivo manipulation allows a greater number of cells to be transduced and thus more copies of the gene of interest may be introduced. It also avoids direct exposure of inner ear cells to viral particles and thus holds promise for reducing an immune response. However, ex vivo transfection is limited to use with genes encoding secreted proteins, since the cells of the target organ will not be transduced themselves. The duration of transgene expression and the duration of survival of the transduced cells also influence the outcome of ex vivo procedures.

An important variable in gene therapy is the choice of vector. As large nucleic acid molecules do not readily penetrate the plasma membrane, they require packaging into a vector that is readily taken up by the target cells. Nonviral vectors such as liposomes and even naked plasmids have been used in prior investigations and are advantageous in that they are associated with fewer side effects than virally derived vectors. However, their transduction efficiency is quite low [5], and they are thus largely limited to in vitro use where cells can be exposed to very large quantities of vector. Viral vectors have proven to be much more efficient at gene transfer, but also have the potential to produce cytotoxicity or an immune response. A number of different viral vectors have been used to treat the inner ear including adeno-associated virus [6–8], herpes simplex virus [9], vaccinia virus [10], retrovirus [11], helper-dependent adenovirus [12] and adenovirus vectors [5, 10, 13–17]. Each of these vectors offers distinct advantages and disadvantages. Advanced generations of adenovirus have become among the most frequently used viral vectors in the inner ear. Adenovirus vectors are associated with a minimal side effect profile, can be prepared in high titers, and may enable transgene expression up to several months [18, 19]. With regard to gene therapy in the inner ear, adenovirus vectors hold an advantage over retroviruses in that they are not dependent on cell replication and can thus transfect quiescent cells of the cochlea.

Another variable of particular importance when considering gene transfer to the inner ear is the means and route of delivery. Vector introduction is complicated by the fact that the cochlea and vestibular organs are isolated by the bony otic capsule, the fluid spaces of the ear are divided into individually isolated endolymphatic and perilymphatic compartments, and the structures of the inner ear, particularly hair cells, are quite sensitive to trauma. One of the least invasive means of delivery is via topical application of a vector to the round window, allowing diffusion across this membrane into the scala tympani. This method has met with some success in prior studies, although it has not proven to be highly effective for viral vectors [20]. Thus, most studies employ techniques that allow the direct inoculation of vector-containing fluid into one of the fluid spaces of the inner ear, typically performed with a micropipette. Potential methods for inoculation include directly piercing the round

window, performing a cochleostomy to access the scala tympani, vestibuli, or media, or injecting into the endolymphatic sac. Each of these has been attempted previously with differing results that will be presented later.

Significant progress has been made in the realm of inner ear gene therapy over the past decade. This chapter will focus specifically on the use of adenovirus as a vector for gene transfer to the inner ear. We will first review the general characteristics of adenoviral gene transduction. We will then describe the use of adenovirus for the protection of hair cells and spiral ganglion neurons. Finally, we will discuss the use of adenoviral therapy for hair cell regeneration.

Characteristics of Adenoviral Gene Transduction in the Inner Ear

The Adenoviridae family, named for its discovery in human adenoid tissue, is composed of nonenveloped icosahedral viruses containing double-stranded DNA. The 51 known serotypes are divided among 6 species and cause infections ranging from respiratory infection to gastroenteritis. This is considered a relatively simple family of viruses whose replication is heavily dependent on the host cell. This simple genomic backbone offers several advantages as a vector. Adenovirus can be 'guttled' to a minimal genome including the genes necessary for host cell recognition and endocytosis. Such a stripped-down genome facilitates packaging with large transgene fragments and may also reduce the immunogenicity of the virus. The workhorse adenovirus for inner ear gene therapy is a modified form of serotype 5 [1, 21]. Portions of the genome, including sequence E1A and E1B as well as part of E3, have been deleted to render the virus replication deficient. In addition, the *Escherichia coli* lacZ gene has been inserted under control of either the Rous sarcoma virus (Ad-RSVlacZ) or cytomegalovirus (Ad-CMVlacZ) promoter to allow identification of transfected cells after incubation with X-Gal. This basic genome has been utilized for preliminary studies including transfection efficiency and localization. More recently, advanced generation viral vectors have been developed. One goal of the advanced design is to increase the insert size available for the transgene [18, 22]. Another important improvement is the decrease in side effects such as cytotoxicity and immune response, accomplished by deletion of additional viral genes [19].

The feasibility of adenovirus-mediated inner ear transfection was demonstrated using the Ad-RSVlacZ vector, introduced into the perilymphatic space of healthy adult guinea pigs via the round window membrane [13]. Transfected cells were identified throughout all turns of the experimental cochlea with a stable level of infection persisting for 3 weeks. Among epithelial cells, the most readily transduced cell population included the fibrocytes lining the perilymphatic compartment and connective tissue cells within the spiral ligament. The membranous labyrinth epithelium was not transduced. Contralateral control ears demonstrated no evidence of infection. While no major morphologic changes were noted in the experimental cochlea, introduction of the Ad-RSVlacZ vector was associated with a moderate inflammatory infiltrate of T cells around the fibrocytes within the walls of the perilymphatic space.

These results were further developed in a follow up study using both in vivo and ex vivo transfection techniques in both normal guinea pigs and those deafened by kanamycin and ethacrynic acid [23]. An identical pattern of transfection was identified in both normal and deafened animals, again dominated by infection of the fibroblasts lining the perilymphatic compartment. Transduction efficiency in the deafened group was slightly greater [24]. The ex vivo experiments involved introduction of transduced fibroblasts, also via micropipette injection, through the round window [23]. After 1 week, a confluent layer of transduced fibroblasts was found lining the osseous spiral lamina of the basal turn with a few additional cells in the second and third turns of the cochlea. Immunohistochemical analysis of T-cell infiltration showed no significant difference between the in vivo and ex vivo techniques. While one might expect that avoiding direct exposure to viral particles via an ex vivo transfection technique may reduce the inflammatory response, these data suggest no significant advantage over in vivo inoculation. It is hypothesized that the limited immune response seen in these experiments may be attributed to the relative immunologic isolation of the cochlea and an intact blood-perilymph barrier. By demonstrating the ability to transfect multiple cell types within the cochlea with no evidence of significant cytotoxicity, these initial studies opened the door for further characterization of adenoviral-mediated gene therapy for the inner ear.

Successful adenovirus gene therapy in the inner ear is dependent on the ability to target the vector to the appropriate tissue. To that end, the transfection patterns of various inoculation techniques have been assessed. Adenoviral transfection via cochleostomy into the basal turn of the scala tympani was found to be more efficient than a round window approach [24]. In both groups, transfection was most efficient in the mesothelial cells lining the fluid spaces, particularly the scala tympani. However, the cochleostomy group demonstrated more intense and wide-spread labeling, sometimes reaching all turns of the cochlea. Possible reasons for this difference include a deeper entry into the scala tympani with cochleostomy, mechanical differences of injection between the two techniques, or altered cochlear homeostasis induced by cochleostomy. However, both techniques failed to transfect cells within the membranous labyrinth (lining the endolymphatic space), including clinically important targets such as the marginal cells of the stria vascularis and the organ of Corti.

Other approaches have produced successful transfection of target cell populations in the endolymphatic space. One technique used inoculation into the endolymphatic sac of healthy guinea pigs [25]. Transfected cells were identified in the endolymphatic sac and duct of all animals. Within the vestibular system, expression was most notable in the transitional epithelium of the utricle and saccule, and to a lesser extent in the semicircular canals. During injection, some of the animals demonstrated swelling of the endolymphatic sac. These animals were found to have transfected cells in the endolymphatic space of the cochlea. Specifically, infection was noted in marginal cells of the stria vascularis, Hensen's cells in the organ of Corti, and occasionally spiral ligament, connective tissue, and Reissner's membrane. Hair cells of the vestibular system and organ of Corti were not affected. The drawback with this technique lies in the distance between the endolymphatic sac and cochlea. Access to the cochlea was limited to those animals that received a sufficient bolus to cause visible expansion of the endolymphatic sac, and even in these cases, transfection was not sufficient for consequential biological applications.

The inability to accomplish transduction of the cochlear epithelium (membranous labyrinth) motivated the design of an alternative approach which involved a cochleostomy passing through the stria vascularis and into the scala media. This technique demonstrated reliable and high efficiency transgene expression in the membranous labyrinth [26]. Interestingly, transgene expression was not identified in hair cells. Rather, all types of supporting cells (nonsensory cells) in the organ of Corti were seen to express the reporter transgene. The extent of transfection was highest in the second and third turns, near the cochleostomy site. Although these methods are complex and are not clinically applicable at present, they nonetheless demonstrate the ability to induce transgene expression within the organ of Corti via adenoviral exposure to the apical domain of the auditory epithelial cells within the scala media.

The presence of the Coxsackie adenovirus receptor (CAR) is often associated with increased uptake of adenovirus by cells [27]. CAR is present in cells of the auditory epithelium but its distribution does not completely explain the pattern of cellular transduction in this epithelium, during development and in the mature tissue [28, 29]. Lack of transgene expression in hair cells is especially intriguing, considering the presence of CAR in these cells.

Transduction with viral vectors inoculated into the perilymphatic compartment has been seen in uninoculated, contralateral ears [30–32]. There is also some evidence suggesting a mild protective effects against trauma to hair cells in the contralateral control ear after unilaterally inoculating with an adenoviral construct containing the gene encoding glial cell line-derived neurotrophic factor (GDNF) [33, 34]. Further evaluation suggests that there is a volume-dependent spread of adenoviral vector likely transmitted via the cochlear aqueduct and CSF [32]. After cochleostomy into the scala tympani, contralateral infection was seen in animals that received a 25- μ l bolus but not in those given a 5- μ l injection of adenovirus. The guinea pig perilymphatic space contains a volume of 8 μ l [35], and given the rigid confines of the surrounding temporal bone, it is feasible that the excess fluid escapes the bony labyrinth, via the cochlear aqueduct. Injection of vector into the cranial or spinal CSF produced labeling of bilateral cochleae, predominantly at the base near the opening of the cochlear aqueduct. This suggests the cochlear aqueduct serves as the conduit for escape of excess fluid within the perilymphatic space and subsequent transfer of vector to the contralateral ear by means of the CSF. Intravenous injection failed to transduce the cochlea. Of note, inoculation with even the higher 25- μ l volume into the inner ear failed to produce transfection in other organs including the liver, spleen, lungs, or kidneys. Methods for inoculating viral vectors into the cochlea while minimizing transfer to the contralateral ear have been described [36].

In evaluating the clinical utility of adenovirus-mediated gene therapy, one must consider the safety of such traumatic manipulation of the inner ear. In general, the surgical procedure for inoculating into perilymph has been well tolerated by animals and minor, short-term complications such as infection or head tilt are rare [13, 23–25]. Inoculation into the perilymphatic space by cochleostomy or through the round window produced a minimal detrimental effect. Although these were associated with an influx of T cells into the tissues surrounding the perilymph space, there was no evidence of structural damage or hair cell

loss. However, inoculating into the endolymphatic space was associated with hair cell damage [26]. After endolymphatic sac inoculation, inner and outer hair cells were lost in the cochlear hook region where transfection efficiency was highest [25].

Functional results of these procedures, as ascertained by auditory brainstem response (ABR), are in agreement with histological findings. Five days after inoculation with Ad-RSVlacZ via either the round window approach or cochleostomy, the experimental ears showed a maximum threshold shift of 5–10 dB SPL [24]. There was no significant difference between these techniques. In contrast, cochleostomy into the scala media resulted in a mean threshold shift of 30 dB (measured at 4, 12, and 20 kHz) 5 days after injection of adenovirus vector [26]. The etiology of this loss cannot be attributed solely to cytotoxic activity of adenovirus as inoculation with an equivalent volume of artificial endolymph produced a slightly higher threshold shift, though not significantly different. These results demonstrate the fragile nature of cochlear hair cells and suggest they may be susceptible to local mechanical trauma induced by a cochleostomy. Fortunately, in terms of clinical application, the impact of such damage may be minimal as these techniques would likely be applied to cochleae already suffering from hair cell damage and hearing loss.

Therapy for the Protection of Hair Cells and Neurons

One potential therapeutic application of inner ear gene therapy is to prevent hair cell loss by protecting them from ototoxic insults. It is also necessary to protect spiral ganglion cells from a secondary loss which may occur following hair cell death. Hair cells are subject to injury from a number of environmental and iatrogenic sources. Preventative efforts may prove useful in cases when iatrogenic hearing loss might be expected, such as with administration of certain medications, or in ameliorating a known progressive disease process. Aminoglycoside antibiotics, a medication class frequently implicated in ototoxicity, can cause destruction of cochlear and vestibular hair cells via a free-radical mechanism with resulting permanent hearing loss or vestibular deficiency [37]. Aminoglycoside ototoxicity is an ideal model for the study of inner ear gene therapy as the extent of lesion caused by some drugs is severe and well-defined.

While a number of molecules have been investigated in attempts at inner ear protection, most of the focus has been on neurotrophins and other growth factors such as transforming growth factor- β (TGF- β) and GDNF. The latter is a member of the TGF- β family that has been characterized as a neuronal survival factor [38], and has been identified in both developing and mature mammalian organ of Corti as well as spiral ganglion [39, 40]. It has been shown to promote survival of hair cells and spiral ganglion neurons in response to noise trauma and ototoxic substances [41]. It also appears that interaction with another member of the family, TGF- β 1, enhances GDNF's neurotrophic potential [42]. Among the neurotrophin family, brain-derived neurotrophic factor (BDNF) has been shown to play an important role in the development of the vestibular system and cochlea [43]. BDNF overexpression protects vestibular hair cells from gentamicin ototoxicity [44], and cochlear infusion of BDNF promotes spiral ganglion survival in the face of hair cell loss [45].

After establishing the practicality of adenoviral gene therapy for the inner ear, therapeutic potential via gene transfer was explored. Adenovirus vector containing the GDNF gene (Ad-GDNF) was injected through the round window 4 days prior to deafening adult guinea pigs with kanamycin and ethacrynic acid [33]. Analysis of ABRs and cochlear hair cell counts demonstrated that significant protection was achieved by administration of Ad-GDNF as compared to both the control contralateral ears and to injection of artificial perilymph.

Interestingly, the data also demonstrated a trend for protection by the Ad-LacZ construct (a control group). This study also suggested a trend for protection in the contralateral ear for both Ad-GDNF and the Ad-LacZ groups in comparison to artificial perilymph, although the volume of solution injected in each group was only 5 μ l, and no reporter genes were identified in the contralateral ear upon histological evaluation. While these differences were not significant, the trend raises the possibility that a component of the protection afforded may be due to an adenovirus induced immune response resulting in a protective effect. It is also important to consider that a paracrine mechanism is likely responsible for the protective effects seen, given that inoculation into the scala tympani primarily produces transfection of the mesothelial cells lining the perilymphatic space rather than infection of hair cells themselves. Thus, it is possible that gene product may be delivered to the contralateral ear without actually transfecting the cells of that ear.

In agreement with previously reported findings in neuronal cultures, it appears the protection provided by GDNF is enhanced by co-administration of TGF- β 1 [34]. This study confirmed the protection of hair cells and hearing by administration of Ad-GDNF 4 days prior to aminoglycoside administration. In addition, when Ad-TGF- β 1 was injected simultaneously with Ad-GDNF, significantly fewer hair cells were lost in comparison to use of Ad-GDNF alone. There was also a trend to smaller ABR threshold shifts with TGF- β 1 supplementation. By inoculating via a cochleostomy into the scala vestibuli, the protective effects of Ad-GDNF can be extended to the vestibular system as well [46]. This method demonstrated that administration of Ad-GDNF was able to reduce scarring of the utricular macula in response to aminoglycoside toxicity. This study also showed that both preventative measures (Ad-GDNF given 4 days prior to deafening), as well as rescue efforts (Ad-GDNF administered concurrently with deafening agents), can successfully be used for inner ear protection.

While it is uncertain how GDNF promotes hair cell protection against aminoglycoside toxicity, one proposal is that it inhibits free radical synthesis. Another approach to preventing oxidative damage associated with aminoglycoside toxicity would be to use gene therapy to upregulate antioxidant production. This has been successfully performed using adenovirus vectors containing catalase (Ad-cat), Cu/Zn superoxide dismutase (Ad-SOD1), and Mn superoxide dismutase (Ad-SOD2) [47]. These were administered 5 days prior to deafening. Ad-cat and Ad-SOD2 produced significant protection in terms of reduced hair cell loss and improved ABR threshold shifts. These results reveal another set of molecules that may be manipulated by adenoviral gene delivery for hair cell protection.

Adenoviral gene therapy has also proven effective in promoting spiral ganglion survival in ears with severe hair cell loss. This was first achieved using the Ad-GDNF vector administered 4 or 7 days after aminoglycoside/diuretic exposure [48]. This ototoxic

combination produces severe hair cell loss which is known to result in secondary degeneration of spiral ganglion neurons [49, 50]. This is likely due to lost trophic support and disrupted neuronal interaction. Inoculation with GDNF via an adenoviral vector enhanced spiral ganglion neuronal survival based on cell counts performed 28 days after deafening. Protection was most pronounced in the basal and middle turns. Possible explanations for this phenomenon include more efficient transfection or a greater number of GDNF receptors in this region. Similar experiments were conducted to evaluate the effects of BDNF and ciliary-derived neurotrophic factor (CNTF) [51, 52]. Significant spiral ganglion protection was found with BDNF therapy alone, and a synergistic effect was seen when BDNF and CNTF were administered concomitantly. CNTF alone did not significantly enhance spiral ganglion neuron survival.

Previous efforts have demonstrated that electrical stimulation can promote spiral ganglion neuron survival in the face of hair cell loss [53, 54]. Gene therapy experiments found the combination of Ad-GDNF and electrical stimulation provided greater spiral ganglion protection in pharmacologically deafened ears than either treatment alone [55]. Another study demonstrated the protective effect of ex vivo gene transfer via cells embedded around a cochlear implant electrode [56]. The ability to promote spiral ganglion survival in ears with severe hair cell loss is of significant clinical importance. These therapies would be ideally suited to maintain the spiral ganglion cell population in a patient awaiting cochlear implantation or hair cell regeneration, should technical advances facilitate this.

Therapy for Hair Cell Regeneration

One exciting goal for gene therapy of the inner ear is to regenerate hair cells in the organ of Corti and the vestibular epithelium. Hair cell regeneration would be of tremendous impact to the millions of individuals suffering from sensorineural hearing loss and vestibular disorders due to hair cell loss. Recent advances have brought us much closer to making hair cell regeneration a reality. Certainly, this is a technically challenging feat. While most epithelial surfaces are maintained by a basal layer of mitotically active cells that facilitate turnover of the tissue, this is not true for the organ of Corti, and therefore hair cell loss is permanent, as is the resulting hearing loss. Restoration of lost hair cells must then rely on either replacement with external cells that can appropriately differentiate, or transdifferentiation of neighboring cells already present in the organ of Corti into new hair cells. Each approach has distinct advantages and disadvantages. While embryonic stem cell approaches to hair cell regeneration are certainly under investigation, this chapter will focus on review of the efforts to achieve transdifferentiation of nonsensory cells of the organ of Corti via adenoviral gene therapy.

Study of fish and avian ears first gave hope to the prospect of hair cell restoration. Both classes have been shown capable of regenerating lost hair cells following acoustic trauma [57–59]. No stem cell population has been identified in the sensory epithelium of either fish or avian ears. Instead, supporting cells have been found to undergo mitosis in response to loss of hair cells in the avian basilar papilla [60–62] and in the fish ear [63, 64]. The process of phenotypic conversion from one identity (supporting cell) to the other (hair cell) is a form of transdifferentiation. This is a rare event in nature, though it has been demonstrated in

organ systems such as the eye [65]. Though they are only distantly related after differentiation from a common progenitor very early in the developmental process, retinal cells can transdifferentiate into lens epithelial cells. Hair cells and supporting cells have common progenitors [66, 67] suggesting a capacity for supporting cells to transdifferentiate to hair cells. This appears to happen in inner ear epithelia in all vertebrates with the exception of mammals. In mammals, transdifferentiation of supporting cells to hair cells does not occur spontaneously after cochlear hair cells are lost. Understanding the molecular signaling leading to hair cell differentiation has helped design ways to induce transdifferentiation in the auditory epithelium of mammals.

The transcription factor *Atoh1* (formerly *Math1*) is critical for the differentiation of hair cells [68]. This is a mouse homolog of the *Drosophila* gene *atonal* (the human homolog is *Hath1*). As development proceeds and hair cells are generated, the expression of the *Atoh1* gene is downregulated [69]. This transcription factor thus serves as an excellent candidate to induce transdifferentiation of hair cells from supporting cells. Overexpression of *Atoh1* in cultured rat organ of Corti has been shown to produce hair cells in immature explants [70] as well as explants of mature tissues [71].

This knowledge was utilized in an attempt to induce the development of new hair cells in an in vivo model. The *Atoh1* gene was inserted into a replication deficient adenovirus vector (Ad-*Atoh1*) and this was inoculated into 4- to 5-week-old guinea pigs through a scala media cochleostomy [72]. As had been previously seen, controls using Ad-LacZ showed reporter gene expression in supporting cells of the organ of Corti and adjacent nonsensory epithelium. Immunohistochemistry 4 days after inoculation confirmed the presence of *Atoh1* in nonsensory epithelium, primarily in the third turn near the cochleostomy. In animals sacrificed 30 or 60 days after the injection, ectopic hair cells were detected, adjacent to the organ of Corti where hair cells are not typically found. These new cells expressed a hair cell-specific marker, myosin VIIa. Additionally, neurofilament labeling identified nerve fibers growing toward the new ectopic hair cells. None of these phenomena were noted in control ears. These striking results documented the ability to induce hair cell generation from the supporting cell population by means of adenoviral-induced *Atoh1* expression in the adult cochlea. Neurofilament labeling suggested these new hair cells possessed the functional capability to attract neurite ingrowth.

A subsequent study was conducted in a similar fashion, though young adult guinea pigs were deafened with kanamycin and ethacrynic acid prior to inoculation with Ad-*Atoh1* [73]. Control animals sacrificed 3 days later showed near-total absence of hair cells, though supporting cells survived the ototoxic insult. Animals killed 4 days after inoculation showed expression of *Atoh1* within the organ of Corti. Scanning electron microscopy at 8 weeks revealed numerous inner and hair outer cells in the experimental ears with relatively normal morphology and correct orientation. However, the supporting cells between hair cells were unusually narrow and ill-defined and the third row of outer hair cells was poorly organized. Functional outcome was assessed by ABR. At 4 weeks, all animals remained profoundly deaf. However, ABRs at 8 weeks showed better thresholds in experimental ears compared to contralateral control ears. Similar results were seen at 10 weeks. This study documents not

only morphological recovery of hair cells induced by adenoviral-mediated *Atoh1* expression, but also functional recovery of hearing thresholds.

If hair cells have been lost for some period of time, the cochlear sensory epithelium may de-differentiate into a 'flat' epithelium without the features of normal cochlear supporting cells. Adenoviral transduction of cells in a flat epithelium with *Atoh1* is possible. However, it does not result in the induction of hair cells [74]. Thus a certain state of differentiation of supporting cells is required to support conversion into hair cells.

One potential way to enhance hair cell regeneration is by inducing proliferation in the auditory epithelium. By manipulating cell-cycle regulatory genes, it is possible to induce proliferation in the cochlear epithelium [75]. However, it appears that by itself, the addition of new cells is insufficient for generating new hair cells. Rather, it is probably also necessary to induce transdifferentiation of the new cells into the hair cell phenotype using forced expression of transgenes such as *Atoh1*.

In a similar set of experiments, an Ad-*Atoh1* vector was used to successfully regenerate vestibular hair cells after chemical ablation [76]. The vector was administered through a scala tympani cochleostomy in adult mice 2 days after intracochlear aminoglycoside treatment. Vestibular recovery was evaluated both functionally and histologically 8 weeks later. Hair cell counts in the saccule, utricle, and lateral canal ampula showed significant regeneration in Ad-*Atoh1*-treated animals compared to aminoglycoside-only-treated animals. At 8 weeks, these mice also demonstrated functional vestibular recovery based on swim testing, with no significant difference from untreated animals. Aminoglycoside-only-treated animals showed significantly increased swim times. Of note, there was no evidence of cochlear hair cell regeneration or hearing threshold recovery in this study. These findings are consistent with prior work suggesting scala media inoculation is necessary to achieve an effect on the auditory epithelium. The data on hair cell regeneration in the cochlea and the vestibular epithelia underline the potential for adenovirus-mediated inner ear therapy.

Concluding Remarks

Certainly, inner ear gene therapy has undergone tremendous development since the initial results suggesting its feasibility just over a decade ago. We are now aware of the various transfection patterns with different cochleostomy approaches. Specifically, it has been shown that efficient transduction of clinically important structures in the endolymphatic space are dependent on cochleostomy into the scala media. Protection from hearing loss and hair cell or spiral ganglion cell death may be achieved by adenoviral-mediated neurotrophin or growth factor expression in the inner ear. Such techniques may be clinically useful when iatrogenic injury the inner ear is foreseen. Most notably, we have seen that regeneration of functional hair cells can be achieved in an in vivo model by means of transdifferentiation of quiescent supporting cells.

While these results are promising and pave the road for further research, much work remains to be done. The adenovirus vector should be optimized for greater transfection efficiency and localized targeting of specific cell subpopulations. Surgical technique can be improved to minimize the traumatic impact of cochleostomy into the perilymphatic, and especially

endolymphatic spaces. Finally, treatment for hereditary inner ear disease should also be advanced. To effectively treat genetically based hearing loss, we will need to focus our efforts at integration into the host genome for prolonged expression. While much work remains to be done, the prospect of applying these techniques in a clinical scenario is becoming more feasible.

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