Cochlear Gene Therapy

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Over 450 million people worldwide suffer from hearing loss, leading to an estimated economic burden of ~\$750 billion. The past decade has seen significant advances in the understanding of the molecular mechanisms that contribute to hearing, and the environmental and genetic factors that can go awry and lead to hearing loss. This in turn has sparked enormous interest in developing gene therapy approaches to treat this disorder. This review documents the most recent advances in cochlear gene therapy to restore hearing loss, and will cover viral vectors and construct designs, potential routes of delivery into the inner ear, and, lastly, the most promising genes of interest.

Hearing impairment is the most common sensory disorder in humans, affecting approximately a half a billion people worldwide (Kral and O'Donoghue 2010; Wilson et al. 2017). Further, approximately 1.5 in 1000 newborns suffer from congenital hearing impairment (Di Domenico et al. 2011), and genetic forms of sensorineural deafness account for almost half of all patients with hearing loss (Shearer et al. 2013). Despite this ubiquity, treatment options for sensorineural hearing loss are quite limited, consisting only of hearing amplification for mild-to-severe losses, and cochlear implantation for severe-to-profound losses. These treatments, though quite beneficial for those who receive these technologies, still have great limitations and do not restore hearing to normal levels (Kral and O'Donoghue 2010). For these reasons, cochlear gene therapy has been suggested as an alternative route toward treatment for both genetic and acquired forms of hearing loss.

Application of gene therapy toward the treatment of hearing loss holds promise for several reasons. For acquired forms of hearing loss, research over the past 20 years has led to a deeper understanding of the pathways controlling cochlear development and, in particular, auditory hair cell formation and spiral ganglion development. Mammalian hair cell regeneration, once thought to be impossible, can now be achieved both in vitro and in vivo by manipulating development pathways. For genetic forms of hearing loss, there are a large number of identified single-gene defects affecting the cochlea that lead to sensorineural deafness. Further, vectors have the ability to target those specific cells in which these genes manifest their function, including hair cells and spiral ganglion neurons. In theory, introduction of the normal (wild-type) gene into these cells can restore function or prevent the degeneration, and the subsequent loss of hearing, that would otherwise occur.

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Together, these potential gene therapy applications offer a future of hearing restoration that is unachievable with current technologies.

VECTORS FOR GENE DELIVERY

The ability to target a specific cell type is a critical feature of successful gene therapy. Although viral vectors predominate the landscape, there are a number of nonviral vectors that have been described for use in gene therapy, whose potential advantages include lower immunogenicity and toxicity and better cell specificity (Table 1) (Wu et al. 2018). However, within the cochlea there have only been limited studies using nonviral vectors. One study by Qi et al. (2014) employed TAT double-stranded RNA-binding domains that were able to deliver labeled small interfering RNA (siRNA) into cochlear inner hair cells (IHCs) and outer hair cells (OHCs), as well as hair cells in the crista ampullaris, macula utriculi, and macula sacculi, when injected through the round-window membrane. Another study by Wu et al. (2013) documented successful transfer of the gene *Atoh1* (part of the Notch pathway, see below) through the round-window membrane of animals using nanoparticles. Liposomes are another nonviral vector used for gene delivery, with the potential advantage of large DNA packaging capacity and a high safety profile. However, again, there has been

very limited successes for cochlear gene therapy using liposomes, in large part as a result of the low transfection rates that have been achieved with this vector (Liao et al. 2014). One notable exception to this is the use of liposomes to deliver Cas9-guide RNA complexes to correct the hearing deficit in an Usher syndrome mouse model (Gao et al. 2018).

Despite some of these successes with non-viral vectors, viral vectors have predominated in studies on animal models of hearing loss. To date, a number of viral vectors have been described for use in gene therapy (Table 1). Lentiviruses were used as a vector in many of the critical early gene therapy studies for human disease, in part because of the large genetic payload they could carry and robust transduction rates. There have been several limited studies examining lentivirus as a vector for cochlear gene therapy (Pan et al. 2013b). However, complications, including insertional mutagenesis, have limited their use and, at least within the ear, lentivirus has largely been supplanted by adenovirus and adeno-associated virus (AAV).

For the past 20 years, adenovirus has been a very popular virus for gene therapy (Lee et al. 2017). It is thus not surprising that it is also one of the two principle viral vectors used for cochlear gene therapy (Praetorius et al. 2009; Staecker et al. 2014). Some of the advantages of adenoviruses for gene delivery are their relatively

Table 1. Vectors used for gene therapy

Vector	Advantages	Disadvantages
Adenovirus	Easy to make <10 kb inserts	Immunogenic Transient expression
Adeno-associated virus (AAV)	Nonpathologic Cell division not required	<5 kb inserts Difficult to make Variable transfection
Herpesvirus	100 kb inserts Stable expression	Human disease Cytopathic
Lentivirus	Stable expression	Insertional mutagenesis Low transfection rate
Nonviral vectors (e.g., liposomes)	Easy to make No insert size limit Nonpathologic Improved cell specificity Lower immunogenicity Lower toxicity	Low and transient expression

large payload capacity, up to 10 kb, and tropism for a number of cochlear cell types. In particular, their affinity for supporting cells makes them particularly suitable for such applications as hair cell regeneration, as several approaches involve converting supporting cells into hair cells using the notch signaling pathway. Further, the duration of transgene expression is typically on the order of weeks to months, which is advantageous for applications in which a relatively short period of expression is needed. This is also one of its principle disadvantages, for example, for genetic forms of hearing loss, where long-term gene expression would be desired. Another potential disadvantage of adenovirus is that it has the potential to elicit an immune response. Although newer generations of adenovirus have been engineered to reduce this probability, if repeated delivery is required, this could potentially exacerbate immune-related side effects.

AAV has become increasingly used as the vector of choice for a number of gene therapy protocols. This is because of its excellent safety profile, broad cellular tropism (although which cells are transfected depends upon the specific AAV subtype used), and high transfection efficiency (Colella et al. 2018). These features make AAV vectors highly suitable for cochlear gene delivery. They are replication-deficient viruses and can efficiently transfer transgenes to a number of different cochlear cell types, including nondividing neurons and hair cells. Because AAV entry into the cell is mediated by specific receptors (Nam et al. 2011), different AAV subtypes can have a direct effect on the cells able to be transfected. AAV can also effectively transduce IHCs, cells that are affected by a number of genetic deficiencies. One potential disadvantage of AAVs is their reduced OHC tropism (Akil et al. 2012; Askew et al. 2015). However, this hurdle appears to have been partially overcome by the development of a synthetic “ancestral” AAV, termed Anc80, which has been shown to transfect OHCs with much greater efficiency (Landegger et al. 2017; Suzuki et al. 2017). Another advantage of AAVs is that they can incorporate into the host genome, resulting in stable, long-term expression of the transgene (Xia et al. 2012). Because long-term expression is very im-

portant for stable rescue of genetic defects, this becomes a critical advantage over a vector such as adenovirus, which traditionally has only provided transgene expression for up to several months. Further, AAV has also been shown to be safe and effective in several clinical trials (Le Meur et al. 2018). Because AAVs are not associated with any human disease or infection and demonstrate no ototoxicity, at present they thus remain the ideal candidate for use in gene therapy for inherited forms of hearing loss and other applications where long-term gene expression is desirable.

The biggest limitation of AAV as a vector for gene therapy is its relatively small packaging capacity, allowing only up to 5 kb of DNA as an insert. This represents a significant limitation as many of the genes that lead to deafness are larger than this size. However, recent advances have broken this barrier, allowing AAV to shuttle larger inserts into host cells (McClements and McLaren 2017). One study by Dyka et al. (2014) used a “dual vector” approach, splitting the desired gene into two different inserts, each containing an engineered overlapping sequence, which could individually fit into two separate AAV vectors. Once a cell was dually infected with both AAV constructs, the overlapping sequences on each DNA insert recombined, allowing the full complementary DNA (cDNA) to reform. This approach was used to successfully express the *Myo7a* gene (~6.7 kb) in vitro and in vivo with high efficiency and 100% fidelity to the predicted sequence (Dyka et al. 2014). This advance potentially opens up the door to larger genes previously not suitable for AAV-mediated gene delivery.

GENETIC REGULATORY ELEMENTS

To date, relatively little attention has been paid toward the regulatory elements controlling gene expression in cochlear gene therapy. As the field moves closer to translation into humans, however, this will take on an increasingly important role. Appropriate designed regulatory elements will allow the gene of interest to turn on or off at the appropriate time, and will help prevent “off-target” effects because of expression of the gene in incorrect cell types that are incidentally targeted by the vector. Such unintended effects

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can cause harmful side effects. These concerns have been present from the earliest gene therapy strategies that were developed (Toniatti et al. 2004). Within the fragile environment of the cochlea, this would pose a particular problem as excess production of a particular protein or expression in the wrong cell type could easily result in further hearing loss.

Promoters can roughly be divided into two types, those that are constitutively or ubiquitously active, and those that are temporally regulated. Constitutively, active enhancer–promoter regions have the advantage of continuous gene expression, regardless of the cell type or developmental stage, and typically lead to robust protein expression (Guo et al. 2008). Examples of these include the cytomegalovirus, elongation factor 1 α (EF1 α), and chick β -actin promoters. Because of the consistent levels of protein expression, they are often used when attempting to search for any effect of the expressed protein, and thus commonly used for in vivo or in vitro research studies (Staecker et al. 2014). One of the earliest studies to examine various promoters for cochlear gene therapy was by Luebke et al. (2001) who documented variable cellular expression of a reporter gene depending upon the promoter used, including cytomegalovirus (CMV) (IHCs and pillar cells), platelet-derived growth factor (PDGF) (cochlear blood vessels), neuron-specific enolase (nerve fibers), and EF1 α (spiral limbus cells).

In contrast to constitutively active promoters, other promoters are much more tissue specific, or only expressed at specific developmental stages or during certain portions of the cell cycle. In theory, these would be much more beneficial for cochlear gene therapy as they would limit off-target effects and limit protein expression only within the desired cell type (e.g., hair cells, spiral ganglion neurons). To date, a number of specific promoter regions have been identified that can target single cell types, including spiral ganglion neurons, hair cells (both inner and outer, together or separately), or the cells of the stria vascularis (Ryan et al. 2009).

One class of regulatory elements that has also been overlooked in the cochlear gene therapy literature involves the untranslated regions

(UTRs) of genes. These sequences, on either side of a coding sequence (upstream in the 5'UTR, downstream in the 3'UTR), are responsible for many regulatory aspects of gene translation, including allowing correct protein folding, and cellular targeting and trafficking. As an example, one recent study in a transgenic animal model of Usher 3a (because of loss of *Clarin1* function) documented that only with inclusion of the UTR regions could gene therapy, with *Clarin1*, prevent the degeneration of hearing that would otherwise occur (Geng et al. 2017).

GENE DELIVERY METHODS

There are essentially four ways in which vectors can be effectively introduced into the cochlea: round-window membrane diffusion, round-window membrane injection, cochleostomy, or canalostomy (Fig. 1). Round-window membrane diffusion is the least invasive of these techniques. It involves placing the vector into the middle ear and relying upon diffusion across the round-window membrane. Clinicians have been using this as a drug delivery route for such conditions as sudden hearing loss (steroids) or Meniere's disease (gentamicin). The primary advantage of this delivery route is the ease of delivery, as for clinical applications it could be performed in the outpatient setting, using instrumentation that exists in every otolaryngology office. The primary disadvantage is the barrier presented by the round-window membrane itself, which could be thicker in patients with a history of otitis media, or may represent an impediment to larger molecules. A number of animal studies have attempted round-window membrane diffusion for viral gene therapy and identified similar hurdles. However, techniques such as using hyaluronic as a carrier agent or enzymatic digestion of the round-window have improved the delivery across the membrane (Shibata et al. 2012; Wang et al. 2012).

In animal models, gene delivery via a cochleostomy or directly through the round window have been described in detail (Akil et al. 2015). This is a method that has been employed in a number of gene therapy studies in animal models of deafness (Akil et al. 2012; Askew et al.

Figure 1. There are several means of delivering a gene therapy construct into the cochlea. Although there are reports of intraperitoneal injection (Lentz et al. 2013), a majority of studies use either round-window diffusion, round-window membrane injection, cochleostomy, or canalostomy. There are advantages and disadvantages of each of these approaches, but all can allow delivery of vector with minimal cochlear damage.

2015; Emptoz et al. 2017; Landegger et al. 2017; Pan et al. 2017; Dulon et al. 2018). The procedure involves a post-auricular, trans-bulla approach to the cochlea. The round window can be punctured with a glass-pulled micropipette for gene delivery, or a 25-gauge needle can be used to perform a cochleostomy. Multiple studies over many years have documented that the approach is safe, relatively easy to perform, and poses little risk to residual/native hearing (Praetorius et al. 2007; Kawamoto et al. 2009; Akil et al. 2015). Additionally, supporting its use in both animals and preclinical studies, it has been shown that preservation of hearing via cochlear implantation in humans is possible using either a round window or cochleostomy approach (Friedland and Runge-Samuelson 2009).

More recently, several reports have described “canalostomy,” or delivery of virus through the horizontal semicircular canal (Gassner et al. 2012; Guo et al. 2017; Tao et al. 2018). The advantage of the canalostomy is potentially broader delivery throughout the labyrinth without additional risk to vestibular or auditory function. This would be particularly advantageous when both auditory and vestibular func-

tion are targeted (e.g., some forms of Usher syndrome). In a recently launched human gene therapy trial that uses the human atonal-1 (*Atoh1*) gene to transdifferentiate supporting cells into hair cells, the method of delivery is via the oval window (www.clinicaltrials.gov/ct2/show/NCT02132130, 2018). This approach was chosen in part to maximize gene delivery throughout the labyrinth.

APPLICATIONS OF COCHLEAR GENE THERAPY

Although the number of potential diseases that gene therapy can potentially help seems to be growing each year, there are several areas related to hearing where the most amount of research has been targeted, including hair cell regeneration, treatment of acquired cochlear synaptopathy, preservation and regeneration of spiral ganglion neurons, and genetic deafness (Fig. 2).

Hair Cell Regeneration

Loss of auditory hair cells underlies many forms of sensorineural hearing loss. Conventional au-

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Figure 2. Strategies used for cochlear gene therapy can be divided into “regenerative” goals and for correcting genetic defects. One regenerative strategy involves manipulating the cell cycle pathways, commonly employed for hair cell regeneration, typically by overexpression of atonal-1 (Atoh1). For spiral ganglion regeneration or preservation, this involves delivery of growth factors such as brainstem-derived neurotrophic factor (BDNF) or neurotrophic factor-3 (NT3). In contrast, when attempting to correct genetic defects leading to hearing loss, strategies include expressing a wild-type gene when the mutated gene is nonfunctional, attempting to override malfunctioning protein with the normal gene product, or in the case of heterozygous-dominant mutations, inactivating the mutant allele.

ditary physiology postulated that under normal conditions, hair cells do not regrow in the mammalian organ of Corti, though this phenomenon had been observed in birds (Stone and Cotanche 2007). However, beginning in the late 1990s with the identification of the molecular signals controlling hair cell differentiation, a number of strategies have been proposed for hair cell regeneration in mammalian systems. These strategies focus on two pathways leading to hair cell formation—the Notch and Wnt signaling pathways (Fig. 3) (Munnamalai and Fekete 2013; Waqas et al. 2016).

The Wnt/ β -catenin signaling pathway induces the proliferation of hair cell progenitor cells (*Lgr5*⁺ cells), which in turn can transdifferentiate into hair cells (Munnamalai and Fekete 2013; Shi et al. 2013; Jacques et al. 2014). The advantage of manipulating this pathway is that it can potentially increase the number of progenitor cells. However, Wnt activation alone likely cannot repopulate a pool of hair cells, and thus prior studies have either focused on combining Wnt activation with Notch inhibition, or focusing only on Notch inhibition (Ni et al. 2016).

In contrast to the Wnt/ β -catenin signaling pathway, the Notch pathway has been exploited

using gene therapy in a number of studies to induce new hair cell formation in mammalian systems. During development, activation of the Notch signaling pathway, through several mediators, blocks the activity of Atoh1, which signals a progenitor cell to become a supporting cell (Zak et al. 2015). In contrast, if Atoh1 levels are elevated, then the cell will terminally differentiate into a hair cell. Thus, overexpression of Atoh1 will induce supporting cells to differentiate into hair cells. The first study to demonstrate this phenomenon using gene therapy was by Kawamoto et al. (2003), which documented new hair cell formation using adenovirus-mediated transfection of mouse atonal-1 (Math1) into guinea pig cochleae. In a follow-up report, this same group used the same viral construct (Ad-Math1) to induce new hair cell formation in a deafened animal model, with partial recovery of hearing (Izumikawa et al. 2005). Similar studies in the aminoglycoside-treated vestibular system by Staecker et al. (2007) documented regrowth of vestibular hair cells and partial recovery of vestibular function. Since these seminal studies, numerous additional reports have documented varying degrees of Math1-mediated hair cell regeneration and hearing rescue in animal models of hearing loss (Izumikawa et al.

Figure 3. Strategies proposed for hair cell regeneration include manipulating one of the two pathways identified for hair cell regeneration. By suppressing the Wnt/ β -catenin pathway, there is the potential for mitotic generation of new hair cells, increasing hair cell number. To date, there has been limited success exploiting this pathway. In contrast, by suppression of Notch, or overexpression of atonal-1 (Atoh1), existing supporting cells can be induced to transdifferentiate into new hair cells. However, this methodology requires the existence of supporting cells, and thus there may be a limit to the number of new hair cells that can be created.

2008; Pan et al. 2013b; Wu et al. 2013; Atkinson et al. 2014; Staecker et al. 2014).

Based on these numerous studies documenting hair cell regeneration with Atoh1, a multicenter, FDA-approved human clinical trial was begun, sponsored by Novartis, which uses an adenovirus type 5 vector, carrying the human atonal-1 gene (Hath1), under control of a glial fibrillary acidic protein (GFAP) promoter. This combined phase I/II trial seeks to determine the safety, tolerability, and efficacy of hair cell regeneration in humans. As of early 2018, a total of 15 patients have been treated but results are not yet reported. As the first cochlear gene therapy study ever performed in humans, this is being closely watched by a number of investigators seeking to advance gene therapy for other forms of hearing loss.

Spiral Ganglion Neuronal Preservation and Regeneration

The primary auditory afferents carrying the sound signal to the brain have their cell bodies within the spiral ganglion in the Rosenthal's canal within the cochlea. Loss of spiral ganglion

neurons (SGNs) with aging has been documented in both animal models of hearing loss as well as in human temporal bone archival specimens (Park et al. 2010; Makary et al. 2011). Further, the primary treatment for profound hearing loss, the cochlear implant, fundamentally relies upon a healthy population of SGNs to stimulate to propagate sound to the central nervous system. Additionally, there are now a number of studies that have evaluated the clinical outcomes of patients with cochlear implants, and many of the features associated with better auditory performance can be at least partially attributable to the SGNs and their relationship to the implant array within the cochlea (Holden et al. 2013). Thus, if SGN populations can be preserved or regenerated, this should in theory result in improved cochlear implant outcomes.

Initial studies using various neuronal growth factors, such as brainstem-derived neurotrophic factor (BDNF) and neurotrophic factor-3 (NT3) directly delivered to deafened cochlea documented preservation of SGNs as compared to nontreated animals (Landry et al. 2011; Leake et al. 2013a,b; Ramekers et al. 2015). However,

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some of these studies saw overly exuberant neuronal proliferation, with lateral neuronal growth along the cochlear duct, potentially affecting the tonotopicity of the organ of Corti (Leake et al. 2013b). Thus, investigators have also been pursuing a gene therapy approach for more directed fiber regrowth without potential loss of tonotopicity. One study by Atkinson et al. (2012) used virally mediated NT3 delivery to deafened guinea pigs and documented greater neuronal survival compared to untreated cochleae, and more directed peripheral fiber regrowth toward cells expressing NT3. Another study by Budenz et al. (2015), using AAV-mediated delivery of BDNF or NT3, documented substantial fiber regrowth and better SGN preservation with BDNF as compared to NT3 delivery. Additional studies by a number of authors have further supported these findings, raising the possibility that gene therapy could someday improve numbers of SGNs for hearing restoration or improved cochlear implant performance (Shibata et al. 2009; Wise et al. 2011).

Hidden Hearing Loss and Cochlear Synaptopathy

The pathophysiology behind both age-related hearing loss and noise-induced hearing loss has been well studied. Over the past decade, however, there is an increasing realization of how early noise exposure, even at levels that are “reversible,” can lead to a more rapid age-related hearing loss. This was first suggested in humans by Gates et al. (2000) after studying the progression of hearing loss in the Framingham Study cohort. Then in a series of animal experiments, Kujawa, Liberman, and colleagues firmly established that early “reversible” noise exposure in animals led to a loss of synapses on hair cells, with a progressive drop in spiral ganglion neurons over time (Kujawa and Liberman 2006, 2015; Lin et al. 2011; Sergeenko et al. 2013; Fernandez et al. 2015; Liberman and Kujawa 2017). These studies have also been partially expanded to humans as well (Viana et al. 2015; Liberman et al. 2016). Because auditory thresholds were often normal in these animals, despite the loss of hair cell synapses, this hearing loss has been

termed a “synaptopathy” or “hidden hearing loss.” In humans, this likely accounts for many of the challenges experienced in age-related and noise-induced hearing loss, including increasing difficulty in hearing in noisy backgrounds and reduced word understanding.

With the pathophysiology more completely understood, there have been efforts to develop therapeutic approaches for treatment. NT3 has been shown to regulate ribbon synapse density, and delivery of NT3 after acoustic trauma can regenerate cochlear synapses (Wan et al. 2014; Suzuki et al. 2016). It has thus been proposed that virally mediated delivery of NT3 can achieve similar effects in a more precise fashion as opposed to round-window delivery. However, in contrast to these findings, in a study by Lee et al. (2016), virally mediated overexpression of NT3 resulted in disruption of auditory thresholds and disorganization of peripheral nerve endings in normal hearing guinea pigs. It is thus likely that any future clinical application would likely require tighter regulation of NT3 expression than has been achieved in these animal studies.

In an alternative strategy, Mianne et al. (2016) used clustered regularly interspaced short palindromic repeats (CRISPR)/Cas-9-mediated homology directed DNA repair in a mouse model of age-related hearing loss (see below for a more complete discussion of CRISPR). Not only were the investigators able to correct the mutated cadherin 23 allele responsible for the phenotype, but also documented no “off-target” affects after whole genome sequencing.

GENETIC DEAFNESS

Gene therapy is ideally suited to treat genetic disorders, given the potential to override abnormal or deficient protein function. Strategies to date have focused on either restoring absent gene function, overriding abnormal gene function with normal gene expression, or inhibiting abnormal gene function in cases of heterozygosity. More recently, CRISPR/Cas9 has offered a glimpse into a new way of treating genetic disorders by rewriting the primary genetic code, although there are still many hurdles to over-

come before this can be translated into treating hearing loss in humans.

VGLUT3

A missense mutation in the human homolog of the *Slc17a* gene has been shown to be responsible for a progressive high-frequency hearing loss seen in human DFNA25, an autosomal-dominant nonsyndromic hearing loss (Ruel et al. 2008). *Slc17A* codes for vesicular glutamate transporter 3 (VGLUT3), which is localized in the IHCs and transports the neurotransmitter glutamate into synaptic vesicles before it is released into the synaptic cleft. The absence of VGLUT3 function in mice leads to deafness because of loss of glutamate release at the IHC ribbon synapse (Seal et al. 2008). Although a rare cause of hearing loss in humans, the unique features of this mouse model (lack of hair cell degeneration and limited neuronal degeneration) made it ideally suited for virally mediated gene therapy, and likely accounts for it being the first reported successful hearing restoration in a mouse model of inherited deafness. In the study, Akil et al. (2012) used AAV1-mediated delivery of VGLUT3 to newborn and P12 mice. The later delivery (P12) resulted in near normal auditory brainstem response (ABR) threshold recovery despite there being only ~40% of IHCs transfected. However, earlier delivery (P1–3) resulted in robust and longer-lasting hearing with 100% of hair cells transfected. Although the VGLUT3 mouse model was different than the human nonsyndromic hearing loss (missense mutation in the human versus null-mutation in the mouse), the successful approach nonetheless provided a strong proof-of-principle that single gene defects that lead to hearing loss could be effectively treated with a gene therapy approach and represents an important milestone.

Connexin 26

Mutations in the GJB2 gene that codes for the gap junction protein connexin 26 (Cx26) account for a large percentage of cases of genetic deafness and remain one of the most important targets of gene therapy for hearing loss (Chang

2015). Given the widespread localization of gap junctions within the cochlea, however, it is also one of the most challenging targets, as all cell types expressing gap junctions would need to be transduced to prevent hearing loss. One challenge studying Cx26 is that a germline mutation results in death in utero. Thus, the models that have been created involve some form of postnatal genetic manipulation. One early study by Maeda et al. (2007) employed an siRNA approach in a dominant-negative mutation of the GJB2 gene, which encodes Cx26. In this study, the dominant-negative mutation, when delivered to the ear, caused hearing loss. Introduction of siRNA against the mutant gene was subsequently able to knock down the exogenously delivered mutant and prevent the hearing loss. More recently, Yu et al. (2014) created a conditional knockout mouse. In this study, AAV delivery of Cx26 resulted in widespread expression of the connexin 26 protein, as well as reduced cell death and spiral ganglion neuronal degeneration. However, the hearing, as measured by acoustic brainstem response thresholds, was not substantially improved as compared to untreated mice. In another study, Iizuka et al. (2015) used AAV-mediated delivery of GJB2 to improve auditory responses and development of cochlear structures in a conditional knockout mouse model. Together, these important studies on Cx26 showed that manipulation of a mutant protein can be achieved without compromising optimal levels of the normal protein, a critical requirement for successful translation of this approach to humans.

USHER SYNDROME

Usher syndrome is the most common form of inherited deafness and blindness. The loss of vision is caused by retinitis pigmentosa, whereas the deafness results from genetic abnormalities that lead to hair cell death. Usher syndrome is divided into types I, II, and III, and there are subcategories of each. Type I is the most severe with hearing loss at birth, whereas type III is the least severe, with progressive hearing loss beginning in childhood or the early teens (Gettelfinger and Dahl 2018).

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Vestibular dysfunction is also a prominent feature in some forms.

The first study to document functional hearing recovery in a mouse model of Usher was by Lentz et al. (2013). Using a mouse model of Usher 1c with a mutation in the harmonin gene, the investigators used antisense oligonucleotides to correct defective pre-messenger RNA (mRNA) splicing of transcripts from the mutated gene, resulting in increased protein expression, improved stereocilia organization in the cochlea, and the rescue of cochlear hair cells, vestibular function, and hearing in mice. One interesting feature of this study was the manner in which the RNA was delivered, via direct intraperitoneal injection, as opposed to virally mediated transduction. In a related study, Pan et al. (2017) used AAV-mediated delivery of the wild-type Ush1c gene to partially correct the auditory and vestibular phenotype in the mouse model. The study was novel in that it used a newly synthesized “ancestral” AAV (Anc80) to achieve much higher OHC transfection rates than had been seen with other AAV subtypes. Because Usher-related mutations affect both IHCs and OHCs, restoration of normal function in both hair cell classes was critical to the success of these experiments. In another study, Emptoz et al. (2017), working in a model of Ush1d (causing mutations of the submembrane scaffold protein *sans*), documented a partial restoration of hearing and a complete, long-lasting restoration of vestibular function using a similar gene therapy approach with AAV.

Usher 2 is a less severe form of the syndrome than Usher 1, with hearing and vestibular loss occurring later, during the first decade of life. Usher 2D is caused by a mutation in the *WHRN* gene that encodes the protein WHIRLIN. Mutations in the *WHRN* gene loci are also responsible for the nonsyndromic hearing loss DFNB31. Using a mouse model of Ush2d, Isgrig et al. (2017) used AAV2/8 to deliver wild-type whirlin cDNA through a canalostomy (prior studies through the round window led to insufficient functional rescue) and documented improved hearing and balance for at least 4 months. In a novel experiment, Fuster-Garcia et al. (2017) used CRISPR/Cas9

on fibroblasts from an Usher 2a patient carrying a homozygous mutation, and documented successful homologous recombination repair to the wild-type state.

Usher 3 is the least severe form of the syndrome, with an even later clinical onset of hearing and visual loss beginning in the teenage years. As a result, it may potentially have the longest window for human intervention to forestall hearing loss and vestibular dysfunction. Ush3a results from a mutation in the *clarin-1* gene, and knockout mice develop hair cell defects by postnatal day 2, and are deaf by P25. Using a transgenic mouse with a delayed onset phenotype, Geng et al. (2017) showed that AAV-mediated delivery of *clarin-1* preserved hair cell bundle morphology and hearing through adult life. The study was notable on several levels. First, it documented the importance of including the UTR regions in this model to correct the hearing deficit, a phenomenon that will likely apply to other forms of gene therapy. Additionally, the study showed that for any intervention, the gene correction must occur prior to the onset of degeneration, or the gene-replacement therapy will likely not be effective.

TMC1

TMC1 is a gene integral to hair cell function and a putative component of the auditory hair cell transduction channel (Pan et al. 2013a). Mice lacking or having mutations in TMC1 have disorders of hearing and balance, and are models for both autosomal-dominant and recessive forms of hearing loss in humans, including DFNA36 and DFNB7/11, and are responsible for as many as 8% of cases of genetic deafness (Kawashima et al. 2011; Zhao et al. 2014).

Askew et al. (2015) used a transgenic mouse model that carried a targeted deletion of *Tmc1* or an ENU-generated mouse mutant, the Beethoven mouse, which carries a dominant point mutation in TMC1. Using AAV2/1 to transfect the TMC1-mutant mice with either TMC1 or TMC2, they showed robust transfection of IHCs but limited OHC transfection. Functionally, the mice had restoration of ABR thresholds and restoration of electrophysiologic transduc-

tion, but no restoration of efferent function, as would be predicted based on a lack of OHC transfection.

More recently, Gao and colleagues (2018) used CRISPR/Cas9, delivered via a cationic lipid, to target the dominant disease-causing allele. This technique substantially reduced the progressive hearing loss that occurred in the untreated mice, as well as documenting enhanced acoustic startle responses. As one of the first studies to restore hearing using the CRISPR/Cas9 system, it was a significant milestone.

CRISPR/Cas9

Traditional gene therapy attempts to correct a genetic defect by one of several mechanisms: providing missing gene function, overriding abnormal gene function, or inactivating a dominant heterozygous allele. However, none of these approaches directly address the underlying genetic defect itself. A relatively new technique can potentially solve this issue, correcting the primary DNA sequence such that future cell progeny contain the correct gene sequence.

CRISPR are normal DNA sequences found in bacteria and archaea, used as part of a DNA virus defense mechanism. They contain repeat clusters that are accompanied by homologous genes, and termed CRISPR-associated systems (Cas-r genes). One of these, Cas9, isolated from *Streptococcus pyogenes*, contains an endonuclease that has been engineered to allow target DNA to be cut by a guide RNA. Thus, by specifying a specific sequence of the guide RNA, Cas9 can specifically target any DNA sequence and turn it off. This feature was then exploited by Zhang and Church to allow primary DNA editing (Cong et al. 2013; Mali et al. 2013; Hsu et al. 2014). A modification of this system, termed CPfl, simplifies the Cas9 system by requiring only a single RNA, allows more precise DNA integration into the host genome, causes reduced mutations, and offers greater flexibility in choosing target sites (Li et al. 2018).

Since its description, there has been widespread enthusiasm of the ability of CRISPR to correct genetic defects and overcome many of the limitations of traditional gene therapy. To

date, it has been used in vitro and in animal models toward human immunodeficiency virus (HIV) infection, cancer, eye disease, hematologic disease, genetic immunologic disorders, neurological disorders, and respiratory disorders, among others (Mollanoori and Teimourian 2018). However, as much attention that this advance has received, there remain significant hurdles before it can become a useful clinical tool. These include the need to assess the biological distribution of the vectors used, examine off-target effects, including the potential for causing translocations, ensure only transient expression of the endonuclease occurs, and improve the efficiency in nondividing cells (Nami et al. 2018). It is these latter attributes that make CRISPR/Cas9 particularly challenging in the cochlea, with the principle targets being hair cells and spiral ganglion cells, which are both postmitotic.

Despite these challenges, there have been several noteworthy studies that have used CRISPR/Cas9 to correct hearing loss phenotypes in animals. The first of these involved the C57BL/6N mouse, a mouse model of age-related hearing loss that is caused by a mutated form of cadherin 23, part of the hair cell tip link for auditory transduction. Mianne et al. (2016) used CRISPR/Cas9-mediated homology directed toward the cadherin 23 gene in zygotes. By correcting the C57BL/6N allele to a wild-type sequence, they documented correction of the progressive hearing loss phenotype characteristic of this mouse model. Further, they documented no off-target effects by performing whole genome analysis.

As already noted above, a more recent work by Gao and colleagues (2018) applied CRISPR/Cas9 toward the mutant allele in a mouse model of nonsyndromic deafness, the Beethoven mouse, which is caused by a mutation in the putative hair cell transduction channel Tmc1. In this model, there is an autosomal-dominant heterozygous mutation that leads to the defect. These investigators used early postnatal delivery (liposome mediated) of CRISPR/Cas9 directed against the dominant mutant allele to inactivate it. This resulted in a substantially reduced progression of the hearing loss that is typically seen in the Beethoven mouse,

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along with enhanced startle responses. Thus, although not a complete correction of the auditory deficit, it nonetheless represents a significant milestone for treating autosomal-dominant forms of hearing loss.

CONCLUDING REMARKS

Cochlear gene therapy has undergone dramatic strides in the past 5 years, fueled by successes in related disciplines, coupled with advances in the understanding of the molecular biology, pathophysiology, and genetics of hearing loss. As a result, the field has witnessed dramatic hearing restorations in a number of mouse models of hearing loss, both acquired and genetic. We are also in the midst of the first gene therapy study in humans, in an attempt to regrow auditory hair cells in patients with acquired forms of hearing loss. As a discipline, we are no longer asking “if,” but “when” gene therapy will be available for the myriad forms of hearing loss that are seen. Whether the current human *ATOH1*-related hair cell regeneration study (2018) will achieve dramatic hearing results, or will simply be the first step on the long road toward multimodal therapy, remains to be seen. For genetic forms of hearing loss, the first successes will likely come from single gene disorders localized to hair cells, with a late onset and enough of a clinical window where intervention will be possible, such as Usher 3 or otoferlin-related deafness, and where treatment can begin before cellular degeneration occurs. However, in the future, it is likely that in utero or early postnatal gene replacement will be required to treat a number of forms of congenital deafness. In the end, the same rules governing outcomes with cochlear implants, such as developmental critical periods, will still apply to regenerative therapies, and thus early intervention will be crucial to achieving good hearing outcomes, regardless of the technologies employed.

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Cochlear Gene Therapy

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