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Review Article

Emerging Gene Therapies for Genetic Hearing Loss

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ABSTRACT

Gene therapy, or the treatment of human disease using genetic material, for inner ear dysfunction is coming of age. Recent progress in developing gene therapy treatments for genetic hearing loss has demonstrated tantalizing proof-of-principle in animal models. While successful translation of this progress into treatments for humans awaits, there is growing interest from patients, scientists, clinicians, and industry. Nonetheless, it is clear that a number of hurdles remain, and expectations for total restoration of auditory function should remain tempered until these challenges have been overcome. Here, we review progress, prospects, and challenges for gene therapy in the inner ear. We focus on technical aspects, including routes of gene delivery to the inner ear, choice of vectors, promoters, inner ear targets, therapeutic strategies, preliminary success stories, and points to consider for translating of these successes to the clinic.

Keywords: gene therapy, viral vectors, genetic deafness, hair cell, inner ear, spiral ganglion neuron, cochlea, vestibular

INTRODUCTION

Hearing loss is the most common neurological disorder on the planet. In the USA alone, the cost to society runs well into the billions of dollars. About 2 to 3 of every 1000 children in the USA are born with a clinically significant hearing loss in one or both ears. Estimates suggest that 50 % of congenital hearing loss has a genetic etiology with approximately 4000 babies affected each year. Furthermore, with improved genetic testing and expanded focus on moderate to severe forms of hearing loss, additional genetic contributions have been identified (Imtiaz et al. 2016; Naz et al. 2017). Unfortunately, there are currently no biological treatments for hearing loss. Current state-of-art treatments focus on sound amplification and implanted electrodes that stimulate the auditory nerve. These strategies offer partial recovery of function for a limited patient population but do not come close to restoring natural hearing. Clearly, there is strong need for development of biological treatments for restoration of auditory function, and gene therapy has emerged as a possible strategy for treatment of some forms of genetic deafness (Géléoc and Holt, 2014). Ultimately, which forms of genetic deafness and which gene therapy strategies, vectors, promoters, routes of delivery, genetic sequences of the therapeutic agent, time points of administration, patient populations, and costs will need to be determined before clinical trials can commence. Here, we review the current state of the field and the prospects for development of gene therapy for treatment of genetic deafness in humans.

ROUTES FOR DELIVERY

The inner ear is an attractive target for gene therapy. Like the eye, the inner ear is a relatively accessible, enclosed fluid-filled space. This provides several distinct advantages, as well as a few disadvantages. Because there are significant physical and diffusion barriers, including the blood-labyrinthine barrier, systemic distribution of therapeutic agents is limited (Harris and Ryan 1995). While the blood-labyrinthine barrier is not as tight as the blood-brain barrier, it provides an impediment that may

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slow distribution of large molecules, such as viral vectors and other gene therapy reagents. These features can be advantageous, because the blood-labyrinthine barrier allows therapeutic agents injected into the cochlea to remain within the target organ at high concentration while minimizing distribution, and perhaps toxicity, in off target organs and tissues.

Multiple routes of delivery to the inner ear have been explored. These include injection into the perilymphatic spaces via the round window membrane (RWM) and via the oval window and injection into the scala tympani or scala vestibule via cochleostomy (Rivera et al. 2012; Chien et al. 2015a, b). Distribution throughout the perilymphatic spaces has been demonstrated for all these routes of delivery. Furthermore, it has been demonstrated that advection flow through the cochlea and vestibular organs can facilitate distribution of therapeutic agents from the site of injection to more distant regions of the inner ear (Borkholder et al. 2014).

Delivery into the endolymphatic spaces has also been explored via cochleostomy into the scala media, via canalostomy (Suzuki et al. 2017) and by injection into the endolymphatic sac (Yamasoba et al. 1999; Kitahara et al. 2003; Rivera et al. 2012). These approaches have also yielded broad distribution but face the added challenge of breaching the barrier between high potassium endolymph and perilymph. Disruption of the barrier poses two potential problems. First, leakage of high potassium into the perilymphatic spaces that bathe the basolateral surface of hair cells and neurons can chronically depolarize these cells and lead to cell death. Second, breach of the tight junctions between endolymph and perilymph can lead to rundown of the endocochlear potential which typically ranges between +80 and +120 mV. Rundown of the endocochlear potential reduces the driving force for sensory transduction in hair cells and therefore leads to reduced cochlear sensitivity and elevated auditory thresholds. Avoiding these complications is particularly challenging in the adult cochlea. However, by targeting endolymphatic spaces in the vestibular system, which does not have an endolymphatic potential, but are continuous with cochlear endolymph spaces, these confounding issues may be minimized while still providing sufficient distribution within the cochlea.

The choice of delivery route may largely be dependent on the cell type to be targeted. For example, if the stria vascularis is to be targeted, as might be the case for mutations in KCNQ1 and KCNE1 genes which cause Jervell and Lange-Nielsen syndrome (combination of sensorineural hearing loss and cardiac abnormalities), injection in to the endolymphatic spaces may be required (Chang et al. 2015). On the other hand, if spiral ganglion neurons are the target, a perilymph injection would be needed. For some cell types, particularly those that reside at the junction between endolymph and perilymph, both delivery routes may need to be explored to identify the optimal approach (Fig. 1).

VECTORS

A number of viral and nonviral vectors have been developed for delivery of genetic material in various tissues and organs (Sacheli et al. 2013; Fukui and Raphael 2013; Kohrman and Raphael 2013). In most cases, these vectors are replication incompetent and pose little threat of viral-induced disease. Rather, the viral genome has been partly or fully deleted, expanding the capacity to allow inclusion of therapeutic DNA cargo within the viral capsid. Some vectors include single-stranded DNA, while others include double-stranded DNA. All viral vectors have a limited delivery capacity and some have limited expression duration. Choice of delivery vector can affect the outcome of a gene therapy approach. However, provided that the target cell type is transduced, the nature of the viral capsid may be of little consequence. By analogy, as long as she makes it to the operating room, does it really matter if your neurosurgeon drives a Chevy or a Ford? In other words, the quality of the intervention is likely to have a greater influence on the outcome than the delivery
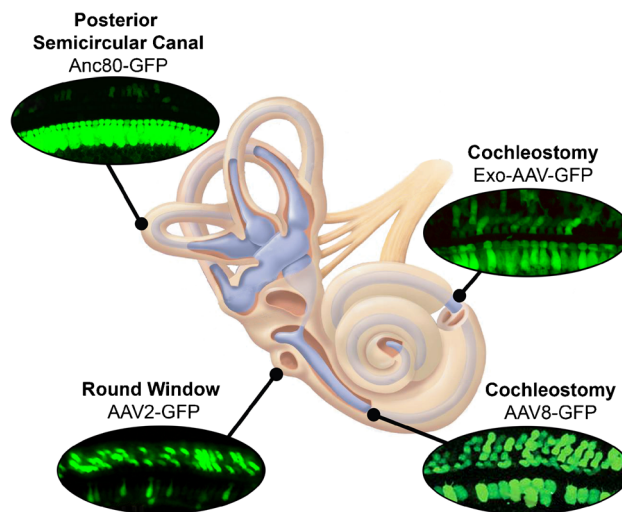
Fig. 1. Schematic diagram illustrating several routes of delivery for injecting gene therapy vectors into the inner ear. Fluorescent images showing expression of green fluorescent protein were selected from the literature to illustrate use of several AAV vector serotypes and delivery routes from the *top right, moving clockwise*, they are as follows: cochleostomy, exo-AAV-GFP (György et al. 2017); cochleostomy, AAV8-GFP (Kilpatrick et al., 2011); round window membrane, AAV2-GFP (Akil et al. 2015); posterior semicircular canal, Anc80-GFP (Suzuki et al. 2017). Each fluorescence image is oriented with a single row of inner hair cells at the *bottom* and three rows of outer hair cells at the *top*.

vehicle. Several of the more common delivery vectors have been tested in the inner ear and are reviewed below.

Lentivectors

Lentiviruses form a genus of the Retroviridae family and have a single-stranded DNA genome of approximately 9 kb packaged into enveloped virions. The lentivectors (LVs) used for gene therapy are based on human immune deficiency virus type 1 or feline immunodeficiency virus. LVs are usually pesudotyped, meaning the vector is combined with foreign viral envelope proteins to expand cell tropism, such as glycoproteins from viruses with broader tropism, i.e., vesicular stomatitis virus (Cronin et al. 2005). To date, several generations of LVs have been developed with the ability to deliver up to 8 kb of foreign DNA (Table 1). Apart from large cargo capacity, the major advantage of LVs is their ability to efficiently transduce both dividing and nondividing cells. To enhance biosafety and prevent consequences of LV integration and replication, self-inactivating and integration-deficient LVs were developed (Logan et al. 2004; Wanisch and Yáñez-Muñoz 2009; Yang et al. 2013a). Since the integration-deficient LVs persist in host cells as nonreplicative episomes, they are applicable for stable transgene expression in nondividing cells, like cells of the inner ear.

In recent years, several studies have demonstrated LV's ability to transduce inner ear cells. In vitro LV transduction in cochlear explants from neonatal rats yielded numerous transduced cell types, including spiral ganglion neurons, glial cells, and supporting cells, although transduction of the sensory cells was not observed. In vivo, via in utero injection into the developing otocyst, LVs showed low-level transgene expression in outer hair cells (OHCs) and supporting cells, but no expression in inner hair cells (IHCs) was detected (Bedrosian et al. 2006). At more mature stages, in vivo intracochlear perfusion of LV into guinea pig inner ear revealed restricted virus dissemination limited to the periphery of the perilymphatic space (Han et al. 1999). Likewise, introduction of LV into perilymph via the round window membrane led to transgene expression in cells lining the scala tympani and scala vestibuli without affecting cells of the organ Corti or the spiral ganglion (Pietola et al. 2008). LV injection into endolymph via scala media lateral wall cochleostomy resulted in expression of transgene in the marginal cells of the stria vascularis, the organ of Corti, and spiral ganglion cells, although was accompanied by hearing loss caused by the surgical approach (Wei et al. 2013). However, LV injection into scala media of neonatal mice did not induce hearing loss and provided transgene expression in Hensen's cells in the organ of Corti, marginal cells, and intermediate cells in the stria vascularis. It is of note that in all the described cases, IHCs and OHCs were minimally transduced (Wang et al. 2013). Although some concerns about LV ototoxicity have been raised, the possible ototoxicity can be eliminated by decreasing the LV dosage (Bedrosian et al. 2006). Thus, other than their lack of ability to target hair cells, LVs may be broadly useful vectors for inner ear gene delivery.

Adenovirus

Adenoviruses (AdVs) are members of the Adenoviridae family. They represent nonenveloped icosahedral nucleocapsids of 90–100 nm in diameter and contain a 36-kb double-stranded DNA genome (Table 1). Although humans can be infected by more than 50 serotypes of AdVs, those used for gene transfer are based on the most-studied serotypes, 2 and 5. AdVs can broadly infect a large number of different cell types, both dividing and nondividing, with high-level and possibly long-term transgene expression. These properties have made AdVs promising gene transfer vectors for inner ear gene therapy (Shu et al. 2016).

Several generations of replication-deficient AdVs have been developed with increased biosafety and cargo capacity due to removal of regions that include the E1/3 genes (first generation, 7 kb of capacity) or the E1/2–4 genes (second generation, 14 kb of capacity) (Räty et al. 2008). At the same time, due to high transduction efficiency and expression of residual viral genes, these vectors induce considerable innate and adaptive immune responses. The third generation of "gutless" AdVs does not contain any viral DNA except for packaging signals and inverted terminal repeats. Production of advanced generation AdVs is facilitated by providing the deleted AdV gene sequences in packaging cell lines. Deletion of AdV genome allows increased cargo capacity up to 36 kb, significantly lowers immune responses, and prolongs transgene expression (Alba et al. 2005). Nonimmunogenic transgenes delivered with gutless AdVs in mouse demonstrated a lifetime expression, although it is usually confined to several months in case of AdVs of earlier generations (Chen et al. 1997; Morral et al. 1998; Moorhead et al. 1999).

Susceptibility of inner ear cells to AdV transduction is determined by the presence of adenovirus receptors—coxsackievirus and adenovirus receptor along with $\alpha v\beta3/\alpha v\beta5$ integrin co-receptors (Zhang and Bergelson 2005; Husseman and Raphael 2009). In rat cochlea, these receptors are expressed in inner ear cells transduced by Ad5 vectors in vivo. Upon perilymphatic perfusion, only those cells lining the perilymphatic spaces expressed transgene, but no expression occurred in cells of the organ of Corti, stria vascularis, or spiral ganglion. Upon endolym-

Table 1

Summary of viral vectors used for inner ear gene therapy

Vector	Serotype	Capacity	Targets	Advantages	Disadvantages	References
Lentiviruses: HIV-1, FIV	Pseudotypes with glycoproteins from vesicular stomatitis virus, measles virus, murine leukemia virus, adenovirus, and many others	Up to 8 kb	In vitro: spiral ganglion neurons, glial cells, supporting cells. In vivo: periphery of the perilymphatic space, scala tympani and scala vestibuli, marginal and intermediate cells of the stria vascularis, Hensen's cells	Efficiently transduce both dividing and nondividing cells. Provide stable transgene expression in nondividing cells. Self-inactivating and integration-deficient vectors are available.	Minimal transduction of IHCs and OHCs. Possible ototoxic effects	Yang et al. (2013a), Bedrosian et al. (2006), Han et al. (1999), Pietola et al. (2008), Wei et al. (2013), Wang et al. (2013)
Adenoviruses (AdV)	AdV-2, AdV-5	1st generation: up to 7 kb 2nd generation: up to 14 kb 3rd generation: up to 36 kb	Cells lining the perilymphatic spaces and scala media, Hensen's, Deiters', pillar and phalangeal cells, satellite cells of the spiral ganglion neurons, hair cells	Efficiently transduce both dividing and nondividing cells. AdVs of 3rd generations may provide very long transgene expression	AdVs of 1st and 2nd generations can induce considerable immune response. Limited efficiency in hair cells	Zhang and Bergelson (2005), Husseman and Raphael (2009), Venail et al. (2006), Li Duan et al. (2002), Dazert et al. (1997), Kesser et al. (2007), Sacheli et al. (2013), Chien et al. (2015a, b), Yang et al. (2013b), Takada et al. (2015)
Adeno-associated viruses (AAV)	AAV1–8, pseudotyped AAVs, engineered AAVs-Anc80L65	Up to 4.8 kb	*AAV1, 2, 4–7, 8*: spiral limbus, spiral ligament, and spiral ganglion cells. *AAV1, 2, 3, 5, 6, 8*: IHCs. *AAV5*: Claudius cells, spiral ganglion, and inner sulcus cells. *AAV2/1*: progenitors of IHCs and OHCs. *AAV2/2*: IHCs. *And80L65*: IHCs and OHCs, type I and type II hair cells of the utricle and semicircular canals	Exceptionally long-term transgene expression. Safety and broad tropism to various cell types. Anc80L65 —exceptional efficient transduction of IHCs and OHCs	Limited packaging capacity requires additional techniques to deliver large transgenes	Jero et al. (2001), Li Duan et al. (2002), Liu et al. (2005), Kilpatrick et al. (2011), Chien et al. (2015), Bedrosian et al. (2006), Konishi et al. (2008a, b), Landegger et al. (2017)

phatic perfusion, transduction was found in the cells lining scala media, Hensen's, Deiters', pillar and phalangeal cells, and the satellite cells surrounding the spiral ganglion neurons. Transduction of sensory hair cells was not generally seen, although they express the required receptors. Some IHCs and OHCs were transduced after syringe injection into basal turn of the scala tympani (Venail et al. 2006). Likewise, direct injection of first-generation AdVs into the basal turn of the guinea pig cochlea in vivo transduced spiral ganglion cells, but not hair cells (Li Duan et al. 2002). Transduction of neonatal rat cochlear cells by AdVs was demonstrated after 72 h with the highest transgene expression level in IHCs (Dazert et al. 1997). Thus, AdVs may be broadly useful for gene transfer to the inner ear, targeting within confined fluid-filled spaces. In organotypic cultures of human vestibular epithelia, first- and second-generation AdVs type 5 efficiently targeted both hair cells and supporting cells, which suggests that successful implementation of AdVs for human inner ear gene therapy in vivo may be possible (Kesser et al. 2007).

Examples of Ad-mediated transgene delivery into the inner ear are numerous and have been thoroughly reviewed elsewhere (Husseman and Raphael 2009; Sacheli et al. 2013; Chien et al. 2015a, b). More recently, AdVs have been used to mediate delivery of Math1 (Atoh1) transcription factor to transdifferentiate cells in the lateral epithelial ridge to hair cell-like cells (Yang et al. 2013b); Ad-mediated overexpression of heat shock protein 70 resulted in significant preservation of hair cells after ototoxic shock (Takada et al. 2015).

In summary, AdVs are very efficient gene delivery vectors. Although there is room for improvement of their transduction and targeting properties, the ability of AdVs to target multiple inner ear cell types and their large cargo capacity suggests that AdVs hold promise for gene therapy use in human inner ear disorders.

Adeno-Associated Virus

Adeno-associated viruses (AAV) were discovered more than 50 years ago (Atchison et al. 1965) and are now widely regarded as promising vectors for a number of viral gene delivery applications. AAVs are small replication-deficient adenovirus-dependent viruses from the Parvoviridae family (Table 1). They have an icosaedrical capsid of 20–25 nm in diameter and a genome of 4.8 kb flanked by two inverted terminal repeats (ITRs). After uncoating in a host cell, the AAV genome can persist in a stable episome state by forming high molecular weight head-to-tail circular concatamers, or can integrate into the host cell genome (Yang et al. 1999). Both scenarios provide long-term and high-level transgene expression.

AAVs are endemic in human populations, although multiple preclinical studies have not identified any associated diseases (Calcedo and Wilson 2013; Mingozzi and High 2013). At the same time, since AAV infection is common in childhood, up to 50 % of some populations have preexisting immunity to AAV. It is of note that such people are currently excluded from AAV clinical trials, which may skew the results of general AAV efficiency in humans (Zinn et al. 2015).

Twelve natural occurring serotypes of human AAV have been characterized to date. These serotypes have different inherent tropisms and transduction efficiencies in muscles, lung, liver, brain, retina, and vasculature. Multiple attempts of AAV pseudotyping and capsid engineering resulted in considerable improvement of tropism and efficiency of transduction (Rabinowitz and Samulski 2000; Zincarelli et al. 2008; Yu et al. 2009; Geguchadze et al. 2012; Aschauer et al. 2013; Sallach et al. 2014; Zinn et al. 2015; Castle et al. 2016; Mao et al. 2016; Marsic and Zolotukhin 2016; Payne et al. 2016; Sayroo et al. 2016; De Silva et al. 2016). As for cells of the inner ear, AAV1–4, 7, and 8 were shown to infect spiral limbus, spiral ligament, and spiral ganglion cells in vivo. Infection of IHCs was also shown for AAV1–3, 5, 6, and 8. AAV1 was the most effective and occasionally infected OHCs and supporting cells (Akil et al. 2012; Askew et al. 2015). Also, AAV5 was shown to be efficient for Claudius cells, spiral ganglion, and inner sulcus cells (Jero et al. 2001; Li Duan et al. 2002; Liu et al. 2005; Kilpatrick et al. 2011; Chien et al. 2015a, b). Among pseudotyped vectors, AAV2/1 was found to efficiently transduce progenitor cells giving rise to IHCs and OHCs in mouse cochlea (Bedrosian et al. 2006), and AAV2/2 was optimal for IHCs of guinea pig cochlea (Konishi et al. 2008a, b).

One of the major disadvantages of AAV is small cargo capacity. Compared to adenoviruses, which are able to package up to 36 kb, AAV with an insert size more than 4.8 kb is unstable. However, several approaches have been established to circumvent the small packaging capacity. The first approach is based on the ability of AAV genomes to undergo intermolecular ITR-mediated head-to-tail concatamerization (Trapani et al. 2013). Placing a splicing donor at the 3′-end of the 5′-half of a transgene and a splicing acceptor at the 5′-end of the 3′-half of a transgene can yield transplicing of two halves of the concatemer and formation of mature messenger RNA (mRNA) and synthesis of full-length protein. The trans-splicing-mediated dual gene delivery approach was successfully implemented in several animal models (Cao et al. 2012; Trapani et al. 2013; Colella et al. 2014; Dyka et al. 2014; Tal-Goldberg et al. 2014; Wang et al. 2014; Fine et al. 2015). The development of this method, placing ITRs from different AAV serotypes on the

ends of trans-splicing gene fragments, allows direct intermolecular recombination and enhanced efficiency of dual AAV vector trans-splicing (Yan et al. 2007). With this approach, an efficient trans-splicing of three fragments of complementary DNA (cDNA) from the DMD coding sequence (ORF size 11.1 kb) was demonstrated in muscle tissues of dystrophic *mdx* mice (Koo et al. 2014). While promising for gene sequences larger than 4.8 kb, trans-splicing has not been used for inner ear gene therapy.

Another possible method for overcoming the limited AAV cargo capacity relies on intein-mediated protein splicing (Topilina and Mills 2014). With this approach, N- and C-terminal fragments of a protein are encoded fused with N- and C-terminal fragments of trans-acting split-inteins. After dual delivery of the two transgenes into a single cell, expression of the two intein fusion proteins will form a functional intein assembly and splice itself out with accurate ligation of the two fragments of the target protein. To date, split-genes of Becker-form dystrophin (size 6.3 kb) was successfully delivered via AAV1 vectors and expressed a functional protein in muscle of a Duchenne muscular dystrophy mouse model (Li et al. 2008). In another example, intein splicing was demonstrated in a cell culture with delivery of split Cas9 in two recombinant AAV vectors (Truong et al. 2015).

Anc80L65

Anc80L65 is an effective representative of combinatorial library of AAV evolutionary ancestors (Anc80Lib) obtained by methods of maximum likelihood phylogeny from capsid sequences of 75 naturally occurring and clinically relevant AAVs (Zinn et al. 2015). In terms of production, yield of Anc80L65 is generally better than AAV2 but significantly depressed compared to the AAV8 (Zinn et al. 2015). Thus, production and purification of the vector may be a limiting factor for application in humans.

Anc80L65 demonstrated exceptional transduction efficiency in murine muscle, retina, and liver tissues, outperforming the most robust wild-type AAV8 in some of them. In addition, Anc80L65 showed outstanding efficiency of transduction of inner ear hair cells (Landegger et al. 2017). Delivery into rodent cochleas at early postnatal stages through the round window resulted in ~90 % transduction of both IHC and OHC with doses of genome-containing particles 2–3 times lower than required for other AAV serotypes. Delivery via canalostomy at adult stages leads to nearly 100 % IHC transduction, but fewer OHCs (Suzuki et al. 2017). Despite this caveat, this is the highest efficiency for AAV transduction of inner ear hair cells reported to date. Moreover, previous data showed that other conventional AAV serotypes do not target OHCs with high efficiency when delivered via the round window membrane which has limited recovery of auditory function (Askew et al. 2015). Along with IHCs and OHCs, Anc80L65 also transduced vestibular sensory organs, including type I and type II hair cells of the utricle and semicircular canals, raising the possibility for treatment of vestibular dysfunction as well. Another advantage of Anc80L65 for use in the treatment of hereditary hearing loss is its safety. In mouse, there were no detrimental effects on sensory cell function, ABRs, and DPOAEs or vestibular function. These advantages make Anc80L65 an attractive vector for inner ear gene delivery, not only for IHCs and OHCs, but also other inner ear cell types (Suzuki et al. 2017; Landegger et al. 2017).

Further investigation will be required to validate Anc80L65 as a safe and effective vector for larger animal models that provides selective expression in targeted tissues of the inner ear without unwanted biodistribution. Nonetheless, it is clear that Anc80L65 and other synthetic AAV serotypes may be useful alternatives for inner ear gene therapy.

Exo-AAV (Exosome-Associated AAV)

In a recent paper, György et al. (2017) described a new way to increase transduction efficiency of conventional AAVs by associating the virus with exosomes (exosome-associated AAV or exo-AAV). Exosomes are cell-secreted membrane vesicles of nanometer size that are believed to be an important component of intercellular communication (Colombo et al. 2014).

Delivery of exo-AAV-GFP in cochlear explant cultures or into mouse inner ears by RWM or cochleostomy injection showed significant transduction efficiency in cochlear hair cells: up to 65 % for IHCs and 50 % for OHCs in vitro and more than 95 % for IHCs and around 50 % for OHCs in vivo. GFP-positive hair cells were also evident in the utricle and the semicircular canals of the vestibular system. Exo-AAV1 gene therapy partially rescued hearing and improved balance-related abnormal movement in a mouse model of hereditary deafness (György et al. 2017).

While the exosomes are potent carriers of AAVs for delivery to inner ear cells, concerns associated with possible side effects of their usage should be taken into account. Exosomes are known to carry multiple proteins and nucleic acids, including RNAs. The repertoire of biologically active contents of exosomes has not been fully characterized but may include molecules important for intercellular communication that can influence target cell function, transcriptome alteration, and abnormal activation of signaling pathways and possibly subsequent activation of pathological states, including cancer (Bakhshandeh et al. 2017;

Fevrier 2004; Guo et al. 2015; Melo 2014). For this reason, producing exo-AAV in immortalized or cancer cell lines may be suboptimal. Alternatively, packaging of AAVs into artificial exosomes could be an option for production of safe exo-AAVs (De La Peña et al. 2009; Kooijmans, 2012).

Nonviral Delivery

Several nonviral delivery vectors that have been implemented for gene therapy of hearing disorders include cationic liposomes, dendrimers, and nonliposomic polymers. These synthetic compounds can be produced in nonbiological environments and are biologically safe and nonimmunogenic. Although nonviral delivery methods are not widely used, for some specific applications, they provide a suitable alternative and may be superior to viral vectors.

Cationic bilayer liposomes (CLs) are routinely used for effective delivery of negatively charged DNA and RNA into cells. CLs provide reliable protection of cargo from degradation and neutralization by extracellular enzymes and antibodies. In addition, due to their positive charge which opposes negatively charged lipids of cell plasma membrane, CLs effectively initiate endocytosis and facilitate escape of their contents from degradative endosomes. Many cationic lipid formulations have been developed to enhance carrying capacity and transfection efficacy of single- and double-stranded nucleic acids of different sizes with low accompanying cytotoxicity. However, a common lipophilic agent Lipofectamine 2000 affects inner hair cell viability in in vitro conditions (Ren et al. 2010). Due to the cytotoxicity, use of lipofection to deliver nucleic acids to hair cells in vivo is rare (Li et al. 2006). However, in one example of cell-gene therapy, lipofection was used with NIH3T3 cells to express recombinant brain-derived neurotrophic factor (BDNF), and the cells were transplanted into the mouse inner ear. This resulted in survival of the grafted cells in the cochlea for up to 4 weeks with sustainable production of BDNF, which protected hair cells and spiral ganglion neuron in stressed conditions (Okano et al. 2006).

Apart from DNA delivery, two CL formulations, RNAiMAX and Lipofectamine 2000, were recently used to deliver genome-editing proteins Cre recombinase and Cas9:sgRNA complexes into OHCs in vivo (Zuris et al. 2014). Both Cre recombinase and Cas9 do not have determined negative charge, so they were tagged with negatively charged protein domains to achieve stable association with CL. Lipofectamine 2000 was the most efficient and resulted in 90 % Cre-mediated recombination and 20 % Cas9-mediated genome modification in the OHCs, although some cell loss was observed. It is of note that since Cas9 can repeatedly edit the same genomic region and may have off target effects, it may be less desirable to have constitutive long-term expression. This caveat suggests that delivery of gene editing proteins may be a niche for which protein delivery by nonviral vectors is more attractive than constitutive expression of DNA sequences encoding these proteins.

Several additional nonviral gene delivery vectors of different compositions, such as dendrimers and polyamine nonliposomal transfection agents, have been tested for their ability to carry DNA into inner ear cells. Dendrimers are nanocomposite materials with branched structure formed by monomeric subunits diverging from a central nucleus. Combinations with different nuclei, attached monomers and surface chemical groups may allow dendrimers to have a range of properties suitable for binding nucleic acids of different structures and sizes (Shcharbin et al. 2009). Round window membrane injection of DNA in complexes with dendrimers Superfect or hyperbranched polylysine nanoparticles resulted in transfection of organ of Corti cells.

Polyamine nonliposomal transfection agents are based on complex formation between nucleic acids, nontoxic cell proteins (e.g., histones), and small amounts of polyamine polymers (Budker et al. 1997). One such reagent, Mirus TransIT, delivered transgenes into Rosenthal's canal and in the area of IHCs. At the same time, combination of nonliposomal agents and DNA condensing component Effectene was not effective. However, all the agents provided transgene expression in the spiral ganglia (Praetorius et al. 2008). The potential of linear polyethylenimine nanoparticles (Zhou et al. 2015) as well as poly(lactic-co-glycolic) acid nanoparticles (Tamura et al. 2005) was also tested but demonstrated cytotoxicity for several types of inner ear cells.

In addition to carrier-mediated gene delivery, electrical field-mediated DNA transfer (electroporation) is gaining popularity. Upon electroporation, pulses of high-voltage electric field generated by an electrode inserted into the cochlea can temporarily break cell membranes and allow the formation of pores that permit extracellular DNA to enter cells. Electroporation is advantageous for its high efficacy and absence of immune response. The main disadvantages are possible tissue damage caused by electrode placement within the cochlea or inappropriate parameters for electrical stimulation. In recent years, in vivo electroporation was used in several studies to induce transgene expression in inner ear cells. In particular, electroporation was effective for ectopic expression of transcriptional factors Neurog1, NeuroD1, and Sox2 to induce a neuronal fate in nonsensory regions of the cochlea during the early postnatal period (Puligilla et al. 2010). Cochlear implant electrode arrays have also been used for close-field

electroporation to deliver naked DNA encoding BDNF into mesenchymal cells lining the cochlear perilymphatic canals of the guinea pig to stimulate regeneration of atrophied spiral ganglion neurites (Pinyon et al. 2014).

TARGETS

Genetic hearing loss arises from mutations that affect the function of many different inner ear cell types. A thorough understanding of the biology of each cell type is required for the development of appropriate therapeutic interventions that target the various cell types. In addition to understanding cellular function and a given cell type's contributions to auditory function, a detailed understanding of gene expression is required. For example, an understanding of the precise spatial and temporal pattern of expression of a wild-type gene is needed to define therapeutic windows of opportunity for intervention. In addition to vectors that transduce the targeted cell type, promoters that drive expression at the appropriate level are also needed. In the following sections, we discuss several inner ear cell types and their ability to be targeted by emerging gene therapy approaches.

Hair Cells

Sensory hair cells, more often than the rest of the inner ear, have been a focus of gene therapy studies. This is not surprising, given that more than 50 % of gene mutations that cause hereditary deafness are expressed in hair cells (Walsh et al. 2002; Wang et al. 1998; Kurima et al. 2002a, b; Yasunaga et al. 1999; Naz et al. 2004). The most fragile and most commonly affected part of the hair cell is the hair bundle—a structure that plays a pivotal role in the auditory mechanoelectrical transduction process. The hair bundle is located at the apex of hair cells and consists of tufts of modified microvilli, known as stereocilia, which are organized in a staircase pattern and maintained by different types of links (such as the tip links and top connectors). Near the tips of the stereocilia, mechanotransduction channels are localized (Beurg et al. 2009), which convert mechanical signals into electrochemical activity. Numerous mutations in genes encoding components of the mechanotransduction machinery in hair bundles disrupt the process of converting mechanical information into electrical signals. For a number of animal models with mutations in genes involved in mechanotransduction, successful therapy by viral and nonviral methods has been described. Among those are genes of transmembrane channel-like proteins 1

and 2 (TMC1 and TMC2; Askew et al. 2015), whirlin (Chien et al. 2016; Isgrig et al. 2017), harmonin (Pan et al. 2017), and TMHS (György et al. 2017). Because hair cells are nondividing, postmitotic cells arranged in a compact epithelial layer, they are challenging targets for gene delivery. However, recent progress has revealed a number of suitable vectors that target both inner and outer hair cells along the length of the cochlea at both neonatal and mature stages. As such, vectors are no longer a limiting factor and there are multiple options for the design of gene therapeutic interventions. Rather, the biological limitations, including control of proper expression level and delivery during therapeutically amenable windows, are important challenges that remain and may be unique to each form of genetic deafness. For example, genes expressed early in development or those required for morphogenesis of the hair bundle may have a limited window of opportunity (Frolenkov et al. 2004). Likewise, gene mutations that lead to death of hair cells will limit the potential for therapeutic intervention.

Stria Vascularis

The stria vascularis (SV) is a highly vascularized epithelium which lines the lateral wall of the scala media. It is composed of three cell types: marginal, intermediate, and basal cells, together with capillary endothelial cells (Wangemann and Schacht 1996). It is responsible for the production and maintenance of both the high endolymphatic K^+ concentration for the scala media and the endocochlear potential (EP), +80–120 mV relative to perilymph, both essential for normal inner ear function. The SV plays a critical role in sound detection by hair cells: as a result of hair bundle deflections, K^+ in the endolymph enters hair cells to depolarize the cells. Eventually, the K^+ exits passively through potassium channels localized along the hair cell basolateral membrane and then is transported into supporting cells and fibrocytes via a network of gap junction channels. From there, K^+ moves to the stria vascularis, which completes the loop by secreting K^+ back into the endolymph (Steel and Barkway, 1989). The process allows for potassium homeostasis in the endolymph and shifts the energy burden from hair cells to the marginal cells of the stria vascularis.

It is not surprising that defects in genes that mediate the K^+ recycling in the inner ear cause inherited deafness. For example, autosomal recessive Jervell and Lange-Nielsen syndrome is caused by mutations in KCNQ1 and KCNE1 genes which are expressed in marginal cells of the SV (Lee et al. 2000). The proteins produced by these two genes work together to form potassium channels that transport

potassium ions out of the SV and into the potassium-rich endolymph and help generate the endocochlear potential.

Chang et al. (2015) injected AAV1 gene therapy vectors into the scala media of a mouse model of JLN syndrome, in which the mice were deficient in KCNQ1. Using exogenous expression of KCNQ1, they demonstrated successful gene therapy treatment and partial recovery of ABR thresholds. The study was a first for a gene specifically affecting the function of the stria vascularis, suggesting that this inner ear structure may be amenable to clinical intervention in humans (Chang et al. 2015).

Supporting Cells

The development, function, and maintenance of inner ear sensory epithelia are heavily dependent upon supporting cells, which are nonsensory cells that reside between and around hair cells (Wan et al. 2013). The supporting cells provide mechanical support to the epithelium and the hair cells. They also help to maintain an environment in the epithelium that enables hair cells to function. Supporting cells are coupled to each other by large numbers of gap junctions (Souter, 2008; Kikuchi et al. 1995). The apical surfaces of supporting cells contact the endolymph (located in the scala media), and its basolateral surfaces contact with perilymph—Na^+-rich fluid located in the scala vestibuli and the scala tympani that bathes the basolateral surface of hair cells and supporting cells. Due to their negative resting potential, supporting cells can absorb Na^+ from the scala media through apical sodium channels (ENaCs), effectively reducing endolymphic Na^+ concentration, which is important for the generation and maintenance of the endocochlear potential. Supporting cells also help to remove K^+ ions from the intercellular spaces of the sensory epithelium after it exits hair cells and thereby maintain the low K^+ environment around the hair cell body, which is necessary for transduction and auditory sensitivity. As discussed above, the gap junction network in supporting cells provides a means to shuttle the K^+ ions away from hair cells preventing local accumulation, depolarization, and death (Gulley and Reese 1976; Wangemann 2006).

Gap junctions are clusters of intercellular channels that allow direct diffusion of ions and small molecules between adjacent cells (Goodenough and Paul 2009). The protein subunits that form gap junction channels are members of the connexin protein family (Souter, 2008). Connexin 26 (Cx26) and Connexin 30 (Cx30) are the primary members of the connexin family that are expressed in the inner ear. Cx26 mutations are among the most common causes of genetic hearing loss in humans (Denoyelle et al. 1997; Kelsell et al. 1997; Estivill et al. 1998). Mutations in *Gjb2* which encodes Cx26 can cause autosomal-dominant or autosomal-recessive deafness. Recently, it was shown that hearing loss in *Gjb2* knockout mice might be restored by delivering normal copies of the *Gjb2* into the inner ear using AAV delivering approach. Yu et al. (2014) managed to reestablish intercellular gap junction networks in mutant mouse cochleas by *Gjb2* gene therapy.

Spiral Ganglion Neurons

The auditory afferent neurons, also known as spiral ganglion neurons (SGNs), receive synaptic input from auditory hair cells, encode the information into trains of action potentials, and transmit auditory information via the eighth cranial nerve to the cochlear nucleus in the brainstem. Two populations of SGNs have been described. Type I SGNs account about 90 % of the entire population and innervate exclusively IHCs. Type II SGNs account for 5–10 % of the population and innervate numerous OHCs (Spoendlin, 1981; Ota and Kimora 1980). For normal function, SGNs require both neurotrophin 3 (NT-3) and BDNF (Ernfors et al. 1995; Pirvola et al. 1997). BDNF and NT-3 are expressed by the sensory hair cells in the developing organ of Corti and by supporting cells. Loss of SGNs can occur directly through damage to the afferent synapses/spiral ganglion cell bodies or as a secondary consequence through lack of adequate trophic support.

In the case when death of neurons occurs because of the lack of key trophic factors, these factors can be delivered into inner ear to promote SGN survival. Hence, several animal studies showed that in vivo direct delivery (viral and nonviral) of both BDNF and NT-3 into the cochlea (scala tympani and supporting cells) rescued auditory neurons from target deprivation-induced neuronal death (Lalwani et al. 2002; Miller et al. 1997). Moreover, not only neurotrophic factors expressed in the inner ear may protect SGN's survival. Delivery of glial cell-derived neurotrophic factor (GDNF) into the inner ear of guinea pigs (ototoxic model) promoted the survival of SGNs (Kuang et al. 1999; Yagi et al. 2000; Kanzaki et al. 2002).

Preserving SGNs from degeneration is of paramount importance for cochlear implant patients, since the implants require SGNs to convey the auditory information from the periphery to brainstem nuclei (Pfingst et al. 2011). Furthermore, the use of growth factors that promote survival, protection, or regrowth of IHC-SGN synapses may be of benefit for patients with hidden hearing loss due to synaptopathy (Kobel et al. 2016; Hickox et al. 2017; Liberman and Kujawa 2017).

GENE THERAPY STRATEGIES

Gene Replacement

The basic principle of gene replacement is to supplement a nonfunctional mutant gene by delivering the correct coding sequence of functional cDNA for a gene of interest into targeted cell types. Ideal candidates for this form of gene therapy are inherited disorders caused by loss-of-functional mutations, such as recessive diseases. The absence of factors that would negatively affect a delivered cDNA in the target cell is critical for gene replacement, for example dominant-negative proteins, or even protein fragments that might outcompete the wild-type therapeutic sequence. The time of transgene delivery is also critical. If gene expression begins during prenatal development, the absence of a functional gene may lead to significant developmental consequences which may not be recoverable by delivery of the functional sequence at later stages. In other words, for gene replacement to be successful, there must be a viable clinical window available for intervention. In addition to an available window, if the target gene is expressed into adulthood, expression of the exogenous sequence must be maintained for extended periods of time, perhaps a lifetime. Alternatively, although not yet attempted, it may be possible to reintroduce the therapeutic sequence at multiple time points. Gene replacement strategies are less likely to be successful for overcoming the influences of dominant mutations. For these forms of genetic deafness, alternate approaches may be better suited.

Gene Silencing

Gene silencing is a form of gene regulation that may be used to "switch off" expression of a mutant gene. The approach may be useful for dominant mutations in heterozygous animals. In this scenario, silencing of the mutant allele while allowing expression of the wild-type allele may be sufficient to overcome the consequences of the dominant hearing loss. Gene silencing can be accomplished at either the genomic, transcriptional, or post-transcriptional levels.

At the transcriptional level, it may be achieved by inducing modification in histone protein or by changing the environment for binding of transcriptional complexes such as RNA polymerase, transcription factors, etc. In such cases, mRNA is not transcribed. The most popular tools to implement this approach are catalytically inactive Cas9 endonuclease and engineered zinc finger transcription factors, as well as their fusion complexes with effector enzymes that introduce required epigenetic modifications. The simplest example is the gRNA-dCas9 DNA recognition complex that spatially interferes with transcriptional elongation,

RNA polymerase binding, or transcription factor binding (Qi et al. 2013). Fusion complexes of KRAB (Kruppel-associated box repressor) with dCas9 and zinc finger domains draw a diverse group of histone modifiers that cooperate to form heterochromatin. Targeting of the dCas9-KRAB and ZF-KRAB to distal regulatory elements effectively represses them and leads to silencing of the controlled genes (Thakore et al. 2015; Chen et al. 2014b, Luo et al. 2017). A complex of dCas9 with methyltransferase 3A efficiently methylates promoter regions of targeted genes, decreasing their transcription (McDonald et al. 2016). Zinc finger domains fused to the catalytic domain of histone methyltransferases affect local methylation of H3K9 and consequently repress gene expression (Snowden et al. 2002).

For post-transcriptional gene silencing, turning off gene expression occurs at the mRNA transcript level by RNA interference (RNAi) (Zamore et al. 2000; Elbashir et al. 2001). Gene silencing by RNAi was first described in 1998 by Andrew Fire and Craig Mello (Fire et al. 1998). Since that time, this endogenous mechanism has found wide application in biology, including gene therapy. Conceptually, RNA interference is a natural biological process in which noncoding RNA molecules regulate gene expression by preventing mRNA translation. The central role in RNAi is played by two types of short complementary small RNA—microRNA (miRNA) or small interfering RNA (siRNA). These two small RNAs can be designed to bind specific mRNA sequences and either increase or decrease their activity, for example by preventing an mRNA from producing a protein.

A main advantage of this method is that RNAi is a natural target-specific method for gene regulation. Its sequence specificity makes it particularly adept for silencing dominant mutations (Maeda et al. 2005; Shibata et al. 2016), without affecting wild-type sequences or off target sequences.

Gene Editing

Precise direct manipulation of genome sequences in the inner ear (Zou et al. 2015) is becoming an emerging reality with the technologies of gene editing. The main instruments of gene editing are engineered nuclease-based enzymes able to find a target genome sequence and to introduce single- or double-strand DNA, which stimulate innate DNA repairing machinery. The repair proceeds via two major pathways—nonhomologous end-joining (NHEJ) or homology-directed repair (HDR). NHEJ results in nonspecific breakage healing with stochastic small insertions or deletions (indels), which may lead to shifts of reading frame, premature stop sequences, or truncated proteins. HDR relies on the presence of

homologous template (like sister chromatid or exogeneously supplied template) and leads to specific repair of DNA breakage. This pathway can be exploited to precisely manipulate a native gene sequence. Moreover, recent technologies allow directed mutagenesis without DNA breakage (Komor et al. 2016).

There are several main instruments for gene editing developed up to date. Zinc finger nucleases represent chimeric nucleases constructed from FokI restriction endonuclease cleavage domain and tandems of DNA-binding domains from zinc finger transcriptional factors, mainly murine transcriptional factor Zif268 (Klug 2010). TALENs are derivatives from the TALE proteins from Xantomonas proteobaceria that have conservative amino acid repeats binding a single DNA base pair (Chen and Gao 2013). Meganucleases are re-engineered homing endonucleases of the LAGLIDADG family able to target custom sequences (Silva et al. 2011). Chimeric proteins containing domains from zinc fingers, TALENs, and meganucleases are designed to take advantage of all these tools to enhance binding affinity and cleavage specificity (Kleinstiver et al. 2012; Wolfs et al. 2014). Nucleases from CRISPR/Cas and /Cpf1 endonucleases were adapted from prokaryotic immune systems for resistance to phages and plasmids. It is considered as the most pervasive and easy-to-use system with multiple applications. Cas9 and Cpf1 can be programmed to find a desired DNA target by a single guide RNA molecule which is partially complementary to the target sequence. Both Cpf1 and Cas9 require the presence of a protospacer adjacent motif (PAM) immediately following the DNA target sequence, although the PAM sequences matching Cpf1 and Cas9 are different. To date, much effort has been directed toward the design of CRIPSR nucleases with altered PAM specificities and diminished off target activities allowing CRISPR/Cas approaches with even more precision (Kleinstiver et al. 2015a, b; Hirano et al. 2016).

PRELIMINARY RESULTS WITH GENETIC HEARING LOSS

Genetic forms of sensorineural hearing loss account for almost half of all cases of congenital deafness with more than 300 genetic loci implicated [hereditaryhearingloss.org]. HL due to genetic factors can have different modes of inheritance and may be delayed in onset with different progression rates. Many "deafness" genes have key roles in inner ear development, structure, or function, and some of them may be suitable targets for gene therapy. Although a number of deafness genes will likely not be good targets for gene therapy, given their temporal

expression pattern, requirement for cell survival, or key role during a critical window of development, several have already been the target of proof-of-principle studies in animal models. These cutting-edge gene therapy studies have targeted *Vglut3*, *Tmc1*, *Whirlin*, *Ush1-c*, *Clarin-1*, and *GJB2* and will be reviewed in the following sections.

Vglut3 (SLC17A8)

Mutations in *Vglut3* (SLC17A8) causing autosomal dominant hearing loss occur in approximately 1.6 % of hearing loss patients in a diverse representative sample (Sloan-Heggen et al. 2016). AAV-mediated delivery of the *Vglut3* cDNA into *Vglut3* knock-out (KO) mouse cochleas was the first example of successful gene therapy in mammalian hair cells (Akil et al. 2012). *Vglut3* encodes a vesicular glutamate transporter (VGLUT3) which is essential for transporting the neurotransmitter glutamate into synaptic vesicles. In humans, missense mutations in this gene lead to progressive high-frequency autosomal dominant hearing loss (DFNA25) (Ruel et al. 2008). *Vglut3*-KO mice are profoundly deaf with early degeneration of some cochlear ganglion neurons (Seal et al. 2008). Hearing loss in these rodents is a consequence of lack of glutamate release by inner hair cells, which results in the absence of auditory neuron depolarization in response to sound. Akil et al. (2012) used AAV2/1 to deliver *Vglut3* cDNA into neonatal (postnatal days 1 to 12) KO mouse cochleas. Based on data from auditory brainstem response (ABR) and acoustic startle reflexes, they demonstrated that auditory function in injected ears recovered within 2 weeks. It should be noted that in spite of using vectors that contain a promoter with broad activity, chicken b-actin, the *Vglut3* transgene was only expressed in the targeted auditory inner hair cells. Partial recovery of the morphological features of inner hair cell-associated afferent ribbon synapses was also reported in this study (Akil et al. 2012). The data revealed the potential for gene therapy to reconstitute molecular and cellular components required for the normal function of the auditory system. The success of *Vglut3* gene therapy motivated the search for other gene therapy targets for more common forms of genetic deafness.

Tmc1

TMC1 encodes the TMC1 protein, a component of the hair bundle transduction machinery (Kawashima et al. 2011; Pan et al. 2013) which is located at the apical pole of hair cells (Kurima et al. 2015). Mutations in *TMC1* causing hereditary hearing loss occur in approximately 2.3 % of all hearing loss patients in a diverse representative sample, including autosomal dominant (3.2 %) and autosomal recessive (2.2 %) forms (Sloan-Heggen et al. 2016) Mutations in

human TMC1 can account for 4 to 8 % of genetic deafness in some populations (Kalay et al. 2005; Santos et al. 2005; Kitajiri et al. 2007; Hilgert et al. 2008; Sırmacı et al. 2009). It has been implicated in the pathogenesis of both dominant and recessive nonsyndromic HL, DFNA36 and DFNB7/11, respectively (Kurima et al. 2002a, b).

In a recent report, Askew et al. (2015) described AAV2/1-mediated gene transfer of *Tmc1* coding sequence into the mouse inner ear. Two strains of *Tmc1* mutant mice were used: *Tmc1-KO* mice which served as a recessive loss-of-function model for human DFNB7/11, and *Beethoven* (*Bth*) mice, a model of human DFNA36, which carry a dominant point mutation. Both animal models show several physiological deficits in hair cell mechanosensory transduction. In vivo injection of the AAV2/1-*Tmc1* vectors at P0–P2 restored mechanotransduction in inner hair cells but not outer hair cells of *Tmc1-KO* mice. In spite of the fact that AAV2/1 was capable of driving exogenous gene expression in vitro, OHCs were not efficiently targeted in vivo. The small number of OHCs transduced was not sufficient to restore distortion product otoacoustic emissions (DPOAEs). However, because IHCs were transduced with 80–90 % efficiency, there was recovery of ABR responses with elevated thresholds. Furthermore, the recovery was sufficient to drive acoustic startle responses, a behavioral measure of auditory function. In addition, AAV2/1-*Tmc2* vectors were capable of preserving auditory function and IHCs survival in *Bth* mice, which supports the hypothesis that *Tmc1* and *Tmc2* perform somewhat redundant functions and can partially substitute for each other, at least in IHCs. However, *Bth* mice AAV2/1-*Tmc2* vectors did not recover startle responses, suggesting that alternate approaches for overcoming the influence of dominant mutations may be required.

Using an alternate approach, Shibata et al. (2016) developed artificial miRNA designed to inhibit the progressive hearing loss phenotype in *Bth* mice. The mouse carries the semidominant *Tmc1* c.1235T>A (p.M412 K) allele, which is orthologous to a human *TMC1* mutation, c.1253T>A (p.M418 K) (Zhao et al. 2014). Moreover, natural progression of hearing loss in humans with p.M418K closely mimics the phenotype of the *Bth*-heterozygous mice. Artificial miRNA carried in an AAV vector was delivered into inner ears of *Bth* mice by single intracochlear injection at early developmental stages. Treated mice showed improved hair cell survival compared to untreated mice. This procedure was able to delay the onset of hearing loss for up to 35 weeks. This was substantially longer than the recovery of function demonstrated by *Vglut3* gene delivery in postnatal mice, which deteriorates by 7 weeks (Akil et al. 2012), as well as in utero injection

of rAAV2/1-MsrB3 to rescue MsrB3−/− mice which deteriorates by 4 weeks (Kim et al. 2016).

WHRN (Whirlin)

WHRN is one of the causative genes of Usher syndrome type 2, the most common clinical type of the disease, characterized by congenital moderate to severe hearing loss (Yang et al. 2012). Mutations in *WHRN* causing autosomal recessive hearing loss occur in approximately 0.5 % of hearing loss patients in a diverse representative sample (Sloan-Heggen et al. 2016). The gene is expressed in hair cells and encodes the cytoskeletal PDZ-scaffold protein whirlin. Two major isoforms of whirlin (long and short) have distinct locations in stereocilia and also across hair cell types. Lack of both isoforms causes abnormally short stereocilia and profound deafness (Ebrahim et al. 2016). Whirlin appears to operate as a quaternary complex with other Usher 2 proteins, USH2A, GPR98, and PDZD7, and is localized at the ankle link region of the hair bundle (Chen et al. 2014a). Abnormal expression or mutations of any of these genes leads to disorganization and gradual degeneration of hair bundles in mice (Chen et al. 2014a). In addition, interaction of whirlin with myosin-XVa is critical for proper whirlin delivery to the tips of stereocilia (Belyantseva et al. 2005).

Recovery of hearing with gene therapy for whirlin mutations has thus far met with mixed success. Whirlin−/− mice treated with AAV8-Whirlin cDNA recovered stereocilia morphology but did not recover normal auditory function, perhaps due to insufficient length and number of regenerated stereocilia, low virus infectivity rate, or isolated isoform delivery (Chien et al. 2016). However, in a follow-up study, some recovery of hearing and balance function was demonstrated following AAV8-whirlin injection via the posterior semicircular canal (Isgrig et al. 2017). Higher transduction efficiency or delivery of both whirlin isoforms may further enhance recovery.

Ush1c (Harmonin)

Type I Usher syndrome is characterized by congenital onset deafness, vestibular dysfunction, and retinitis pigmentosa. The severity of the phenotype depends on the specific *Ush1c* mutation, which includes point mutations, expansions of exons, and multiexon continuous deletions (Ouyang et al. 2005; Lentz et al. 2013; Bitner-Glindzicz et al. 2000; Verpy et al. 2000). Mutations in *Ush1c* causing autosomal recessive hearing loss occur in approximately 0.3 % of hearing loss patients in a diverse representative sample (Sloan-Heggen et al. 2016). In the mouse inner ear, *Ush1c* was found to be expressed in hair cells only. The

Ush1c product, harmonin, is a multi-PDZ domain-containing protein synthesized in three isoforms. All the isoforms contain an N-terminal helical domain, two PDZ domains, and a coiled-coil domain. Harmonin-b is localized in stereocilia and forms a stable tertiary complex with unconventional myosin VIIa through the adaptor protein Sans. Besides, harmonin-b also interacts with cadherin-23 and protcadherin-15 (Yan et al. 2010). These Ush 1 proteins form a scaffold which facilitates development and maintenance of stereocilia into highly organized hair bundles. Therefore, mutations affecting structure or production of harmonin lead to hair bundle disorganization resulting in deafness (Boëda et al. 2002). Harmonin-a is localized at hair cell synapses where it is thought to be required for calcium channel localization and proper synaptic transmission (Gregory et al. 2011, 2013).

For one of the *Ush1c* mutations, c.216G>A which leads to formation of defective mRNA, correction of splicing was achieved by using antisense oligonucleotides (Lentz et al. 2013). Due to their high sequence specificity, antisense oligonucleotides have tremendous therapeutic potential for gene expression modification related to alternative splicing defects (Kole and Sazani 2001). Delivery requires efficient localization to pre-mRNA splicing sites in cell nuclei and overcoming cellular barriers, including the vascular endothelial barrier, reticuloendothelial system, renal clearance system, and blood-brain barrier (Juliano 2016). Traversing the blood-brain barrier using systemic administration has been met with limited success; however, antisense oligonucleotides can reach the inner ear via the reticuloendothelial system and vasculoendothelial system using chemical modifications and cellular processes. The addition of phosphorothioate backbones, neutral backbones, 2′ modifications, neutral siRNAs, and polymer RNAs to antisense oligonucleotides increases their cellular affinity in endocytosis (Juliano 2016). Most of the currently used modified antisense oligonucleotides in clinical trials contain 2′-deoxynucleotide-phosphorothioate backbones (Tamm et al. 2001). The primary mechanism of cellular uptake and transport of antisense oligonucleotides is via accumulation in late endosomes and multivesicular bodies (Juliano 2016). The transport to the cell nuclei is then regulated by the multiprotein complex ESCRT, recently found to be critical to antisense oligonucleotide effectiveness in disease therapy (Henne et al. 2011; Wagenaar et al. 2015). Through this mechanism, antisense oligonucleotides can modify gene expression in the inner ear. In a mouse model, a single intraperitoneal injection of antisense oligonucleotide (300 mg per kg body weight) increased correct harmonin expression and improved hair cell stereocilia organization, low-frequency hearing (8–16 kHz), and vestibular function. It is of note that improvements in vestibular function were observed for mice injected with antisense oligonucleotide on up to postembryonic day 13 (P13), whereas injection on P16 did not bring any improvement suggesting the window of clinical intervention may be limited. However, the therapeutic effects were sustained for 3 months following treatment, indicating good potential of this approach (Lentz et al. 2013). The study suggests that delivery of antisense oligonucleotides may suppress the consequences of specific point mutations and may be a viable approach for treatment of some forms of genetic hearing loss.

How antisense oligonucleotides introduced via IP injection make their way across the blood-labyrinthine barrier and into the targeted cell type has not been clarified.

In a recent study, gene replacement was successfully implemented to correct the pathological phenotype due to the c.216G>A mutation in the same mouse model of *Ush1c* discussed above (Pan et al. 2017). Two splice forms of harmonin (harmonin-a1 and harmonin-b1), under the control of CMV promoter, were delivered with AAV2/Anc80 vector into the inner ear via the RWM at early postnatal stages. To demonstrate successful targeting of the proteins in vitro and in vivo after the RWM injection, AAV vectors bearing fluorescently tagged harmonins were used. It was shown that the tagged harmonin-b was correctly localized to the distal end of stereocilia near the tip-link insertions (Boëda et al. 2002; Lefèvre et al. 2008; Grillet et al. 2009) and the tagged harmonin-a localized to the synapse, which was consistent with the previous localization work (Gregory et al. 2011, 2013).

RWM injection of AAV2/Anc80.CMV.harmonin-b at P0–P1 restored mechanotransduction as well as auditory and vestibular function in deaf and dizzy mice to unprecedented levels. ABR thresholds were as low as 25 dB in some *Ush1c* injected mice. The rescue was associated with recovery of mRNA expression encoding for wild-type harmonin. Scanning electron microscopy revealed decreased hair cell loss and preserved hair bundles with normal staircase morphology in rescued ears (Pan et al. 2017).

ABR and DPOAE thresholds in mice co-injected with harmonin-a1 and harmonin-b1 vectors were similar to those injected with harmonin-b1 alone. Thus, co-injection did not provide further improvement, suggesting that harmonin-a1 may be dispensable for auditory function. Along with rescue of audiological function, the use of viral-mediated gene delivery restored vestibular function: mice injected with AAV2/Anc80.CMV.harmonin-b1 and those co-injected with harmonin-a1 and harmonin -b1 vectors

maintained balance function consistent with control mice (Pan et al. 2017).

USH3A (Clarin-1)

Mutations in *USH3A* are the only currently identified cause of Usher syndrome type 3, which usually has postlingual onset (mostly during the first decade) and is primarily characterized by progressive deafness, blindness, as well as variable vestibular balance disorder (Adato et al. 2002). The *USH3A* gene is expressed in the organ of Corti and spiral ganglion cells of the mouse ear and encodes the protein clarin-1, a transmembrane protein of the tetraspanin superfamily. Clarin-1 is an essential hair bundle protein and acts as a modulator of mechanotransduction activity and presynaptic ribbon assembly (Ogun and Zallocchi 2014; Geng et al. 2012; Gopal et al. 2015). Clarin-1 is apparently indispensable for survival of hair cells, since ribozyme-mediated decay of clarin-1 mRNA leads to pronounced apoptosis of the cells in mouse inner ear. Although the mechanism is unclear, clarin-1 deficiency-mediated hair cell death is one of the factors that causes the clinical manifestations of Usher syndrome, type 3 (Aarnisalo et al. 2007).

Ush3A gene therapy has not been reported but remains an attractive target, due to its later onset and short sequence. However, an alternate approach was recently reported to mitigate progression of Usher syndrome, type 3 invoked by the N48K mutation in clarin-1. This mutation leads to elimination of an N-linked glycosylation site and failure in clarin-1 plasma membrane expression by preventing its engagement with the endoplasmic reticulum calnexin folding cycle and/or imposing a chaperone-independent energetic folding defect of clarin-1 (Lukacs et al. 2016). High-throughput screening identified a regulatory molecule called BioFocus 844 (BF844) that stabilizes clarin-1^{N48K} and restores its plasma membrane expression. It thereby attenuates hearing loss in *Ush3A* mutant mice. Although the molecular targets of BF844 have not been identified, it appears to influence the proteostasis network preventing ERAD-mediated degradation of the mutated clarin-1 (Alagramam et al. 2016).

Gjb2 (Connexin26)

Mutations in the *gap junction protein beta 2* (*GJB2*) gene are among the most common causes of genetic HL worldwide. Mutations in *Gjb2* causing autosomal recessive hearing loss occur in approximately 21.5 % of hearing loss patients in a diverse representative sample, including autosomal-dominant (1.6 %) and autosomal-recessive (25.3 %) forms (Sloan-Heggen et al. 2016). The *GJB2* gene encodes the transmembrane protein Cx26 which together with other connexins form channels that allow rapid transport of ions or small molecules between cells. Cx26 is expressed in an inner ear network of supporting cells that connect the organ of Corti through the basilar membrane to the cells of the stria vascularis (Wangemann 2006). 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. *GJB2* is involved in both autosomal-dominant (DFNA3) and autosomal-recessive (DFNB1) forms of deafness (Richard et al. 1998; Maeda et al. 2005, 2007). Despite great interest in the *GJB2* gene as a potential target for gene therapy, it has remained a difficult task because germline mutations in mouse *Gjb2* result in death in utero. One solution to this problem was the creation of mouse models with inducible or cell-specific deletion of *Gjb2*.

The restoration of Cx26 function to recover hearing has been met with partial success (Ahmad et al. 2007). Maeda et al. (2007) used RNAi to suppress the expression of a dominant-negative mutation (p.R75W) in the *Gjb2* gene. They transfected cochleas of adult mice in vivo with a plasmid vector expressing *GJB2*-R75W-eGFP and showed that this allele variant causes hearing loss by a dominant-negative effect. This pathological phenotype was corrected by delivering RNAi that specifically silenced *GJB2*-R75W expression. It was confirmed that siRNA was potent enough to modify the phenotype caused by the expression of *GJB2*-R75W-eGFP, decreasing the expected ABR threshold shift (Maeda et al. 2007).

Yu et al. (2014) reported that scala media delivery of AAV1 that carried the *Gjb2* coding sequence could recover gap junction intercellular networks in *Gjb2*-deficient cochleas and promoted recovery of dye diffusion, as well as reduced cell death and reduced spiral ganglion neuron degeneration. Unfortunately, the approach did not lead to recovery of hearing function (Yu et al. 2014), perhaps due to limited viral transduction efficiency in the targeted cell type (<50 %), or an injection time point (P0–P1) that was beyond the developmental window for functional recovery. However, more recently, Iizuka et al. (2015) applied perinatal injection of the *Gjb2* gene into the cochlea using AAV5. The approach demonstrated preservation of some cochlear structures and improved ABR thresholds in a conditional *Gjb2* knock-out mouse model (Iizuka et al. 2015).

Gene therapy has also been applied to improving spiral ganglion function associated with *Gjb2* mutations. Spiral ganglion cells in the organ of Corti in Cx26 knock-out mice degenerate during development to varying degrees from the cochlear base to the apex (Takada et al. 2014). The degeneration can be prevented by the application of BDNF to spiral

ganglion neurons in *Gjb2* knock-out mice. Delivery of BDNF using adenovirus, primarily in Rosenthal's canal at the base of the cochlea, rescues neuronal function (Takada et al. 2014). This suggests that the potential for combining gene and neurotrophic factor delivery using viral and nonviral gene therapy methods could enhance hearing loss treatment and recovery.

TRANSLATION TO THE CLINIC

Genetic forms of hearing loss in humans present a challenging but unique opportunity for therapeutic intervention (Minoda et al. 2015). Human inner ear gene therapy has the potential to restore or prevent decay of auditory function. Translation of the proof-of-concept success stories from animal models, highlighted above, into gene therapies for humans with genetic deafness has not yet begun. However, in other systems, there are over 1800 clinical trials that have been either completed, are in progress, or approved involving gene therapy (Ginn et al. 2013). Although clinical trials for genetic hearing loss have not been initiated, several genes involved in hearing loss have also been implicated in other conditions, where gene therapy trials have begun. These include syndromic diseases such as Usher's syndrome, a rare disease that presents with blindness, deafness, and balance disorder, and disorders such as cardiovascular, craniofacial development, and neurological conditions. There has been success using gene therapy in the retinal disease Leber congenital amaurosis, with sustained recovery of vision at 3 years, and in hemophilia B with initial improvement in symptoms (Ali et al. 2000; Cideciyan et al. 2009; Maguire et al. 2009; Simonelli et al. 2010; Jacobson et al. 2012, Manno et al. 2006). Lessons from gene therapy in these other systems may help inform the design and implementation of gene therapy development for genetic hearing loss. The emerging field of inner ear gene therapy would be well-advised to heed the lessons learned from other, more advanced gene therapy fields and avoid the pitfalls that have troubled other fields, as reviewed in the next sections.

Safety

The successes using gene therapy in mouse models of genetic hearing loss warrant careful consideration for translation into therapeutic strategies for treating genetic deafness in humans. Since hearing loss has a very low morbidity rate, it will be of utmost importance to adhere to a key principle of the Hippocratic oath: do no harm. Safety concerns must be given full

consideration, including the safety of the injection technique, the gene delivery vector, and the exogenous gene sequence. As discussed in prior sections, the safety of various routes of delivery into the inner ear will need to be evaluated in humans. Damage to the fragile structures of the human ear will need to be minimized in order to optimize outcome. The physical volume delivered will also require consideration. Given the small volume of the inner ear fluids (~100 μL), the volume and rate of delivery of the injected bolus will need to be well-defined. Thorough pharmacology and toxicology studies for each gene therapy strategy and a range of doses will require full and careful analysis. A significant concern is that a single negative outcome could set back the field of inner ear gene therapy while still in its infancy. Safety studies will also need to be designed that consider the consequences of exogenous gene expression in target cells and off target cells. Are there deleterious effects of overexpression or knock-down in the target cell type or off target cell type? For gene silencing or gene editing approaches, are there potential safety concerns associated with off target effects? Are there long-term safety concerns of the exogenous construct in either cell type? What is the biodistribution of the gene therapy reagents? Will vectors injected into the inner ear remain locally within the targeted organ or can they escape, entering other bodily tissues? Is there a potential for leakage into the cerebral-spinal fluid or the brain? Might the vector capsid or the protein product of the therapeutic gene induce an immune response, and if so, to what extent? Some strategies may necessitate a second injection of the therapeutic vector. In this case, does repeated exposure evoke a stronger immune response? Answers to these and perhaps other unforeseen safety questions will need to be addressed for each investigational new gene therapy reagent. While some risks may be inherent in any potential gene therapy procedure, the potential risks and benefits will need to be well-defined and evaluated to enable optimal design of clinical studies, and in the long term, to allow patients and physicians to make the best well-informed decisions.

Efficacy

The majority of inner ear gene therapy studies have evaluated the approach in embryonic, neonatal, and young adult mice. Many reports have suggested that there may be a critical period for gene therapy to be effective in hearing preservation or recovery. In addition to timing of delivery, dosing of gene therapy will be extremely important, given the strong dose-dependent relationship between vector titers and transduction efficiency, and it will be crucial to evaluate and establish dose-response relationships

prior to clinical translation. Percentages of target cells transduced and off target cells transduced will need to be quantified. Furthermore, mechanisms that allow for control over the level of gene expression will be valuable and perhaps necessary for some applications. One such approach for controlling expression level has recently been demonstrated using 4-OH-tamoxifen (Parker et al. 2013). To conditionally express Atoh1, Parker et al. (2013) developed a stand-alone genetic construct that takes advantage of the tamoxifen sensitivity of a mutated estrogen receptor (ER) ligand-binding domain. In this construct, Atoh1-ER-DsRed is translated to ATOH1-ER-DSRED, a fusion protein that remains isolated and inactive in the cytoplasm. With the administration of 4-hydroxy-tamoxifen, the fusion protein ATOH1-ER-DSRED translocates to the nucleus, wherein it binds to the Atoh1 enhancer, and activates downstream Atoh1 signaling pathways. The construct is reversible wherein the removal of tamoxifen leads to downregulation of Atoh1 signaling.

In addition to expression level, the longevity of expression will need to be determined. Maintaining the effects of gene therapy will be important for clinical translation and may require supplementation as is the case for neurotrophic growth factors, including BDNF, particularly in spiral ganglion neurons (Zanin et al. 2014). In this case, adequate BDNF production was ensured by the introduction of stably transfected fibroblasts which secreted BDNF and was able to maintain survival of spiral ganglion cells for at least 30 weeks. Obviously, longer-term studies will be needed.

Other Animal Models

In addition to mouse models, other mammalian model systems will be needed to advance inner ear gene therapy toward clinical application. Since the FDA encourages testing of any experimental new treatment in more than one animal model, additional model systems may be worth establishing for translational inner ear research. The guinea pig is a useful and arguably underutilized mammalian animal model. Konishi et al. (2008a, b) demonstrated early success with a guinea pig model of genetic deafness, demonstrating that AAV vectors could successfully be transferred to guinea pig cochlea (Konishi et al. 2008a, b). The guinea pig was also an important model in a combined cochlear implant/gene therapy study. In this case, neurotrophin gene therapy enhanced cochlear implant device performance by stimulating spiral ganglion neurite regeneration, which could be targeted and concentrated to mesenchymal cells using focused electric fields generated by

different cochlear implant electrodes (Pinyon et al. 2014).

While many animal models lack the power of mouse genetics, they may nonetheless provide a platform for tests of safety, vector targeting, generation of the therapeutic molecule at the nucleic acid and protein levels, protein localization, and other key therapeutic goals. Moreover, with the advent of CRISPR/Cas9 approaches, the ability to generate genetic hearing loss models in other species is possible. Genetic hearing loss models in larger animals, such as pigs or nonhuman primates, which have ears that are anatomically more similar to those of humans, may provide a valuable platform for extending translational proof-of-principle studies.

Human Tissue In Vitro

Translating gene therapy for inner ear disorders from animal models to human patients in clinical trials could also be facilitated by studies of human inner ear tissue in vitro. Human vestibular epithelia harvested during surgical excision of eighth cranial nerve tumors have been used as a source of live human inner tissue (Kesser et al. 2007; Staecker et al. 2014; Talyor et al. 2015; Landegger et al. 2017). In some cases, the human tissue with living hair cells has been maintained in organotypic culture for up to 10 days. While a limited source, cultured human inner ear tissue may be a viable platform for testing gene therapy reagents in vitro. Indeed, Kesser et al. (2007) demonstrated that adenoviral vectors encoding GFP and the potassium channel gene KCNQ4 were able to target and transduce human vestibular hair cells in vitro. Furthermore, the KCNQ4 protein was translated and correctly targeted to the basolateral membrane. Landegger et al. (2017) used human vestibular tissue to test the synthetic AAV vector, Anc80L65, and reported transduction and GFP expression in large numbers of type I and type II hair cells. While efficacy studies for genetic inner ear disorders may not be possible using this experimental paradigm, the approach may be valuable for examining targeting, gene and protein expression, protein localization, cellular toxicity as well as other aspects of gene therapy reagents in live human tissue. Positive outcomes with human tissue in in vitro studies could boost confidence that the gene therapy reagent in question may have similar effects in human tissue in vivo. Human clinical trials are already underway using Ad-Atoh1 gene therapy. While gene therapy for genetic hearing loss has not yet entered clinical trials, recent success with animal models provides hope that gene therapy in the human inner ear gene may be feasible in the not too distant future.

CONCLUSIONS

These are exciting times for inner ear gene therapy. The prospects of translating the successes with animal models to the clinic seem palpable. Increased interest from government funding agencies, private foundations, industry, physicians, and patients is mounting. The coming years are likely to see additional advances. Despite the growing interest and enthusiasm for inner ear gene therapy, a measured and methodical approach is warranted. A large number of unanswered questions still need to be addressed. With over 100 different genes identified that cause genetic hearing loss, each unique cause will need to be characterized and evaluated for its potential to be the target of successful gene therapy intervention. Despite the challenges, there are reasons for optimism as the field of inner gene therapy advances toward the collective goal of developing novel and effective treatments for patients with genetic hearing loss.

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