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Genetic therapies for hearing loss: Accomplishments and remaining challenges

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Abstract

More than 15 years have passed since the official completion of the Human Genome Project. Predominantly due to this project, over one hundred genes have now been linked to hearing loss. Although major advancements have been made in the understanding of underlying pathologies in deafness as a consequence of these gene discoveries, biological treatments for these conditions are still not available and current treatments rely on amplification or prosthetics. A promising approach for developing treatments for genetic hearing loss is the most simplistic one, that of gene therapy. Gene therapy would intuitively be ideal for these conditions since it is directed at the very source of the problem. Recent achievements in this field in laboratory models spike hope and optimism among scientists, patients, and industry, and suggest that this approach can mature into clinical trials in the coming years. Here we review the existing literature and discuss the different aspects of developing gene therapy for genetic hearing loss.

Keywords

Inner ear; Cochlea; Hair cells; Gene editing; Deafness; Gene therapy

1. The genetic landscape of deafness

Hearing loss affects 1 in 500 newborns and over 466 million people worldwide [1,2]. The condition can be congenital or acquired later in life and varies in severity, with an estimated 40–50% of cases caused by genetic variants [3,4]. Approximately twenty percent of genetic hearing loss cases are syndromic, and other organs and systems may also be affected [5]. There is great heterogeneity within the condition. Genetic hearing loss can be inherited in recessive or dominant modes and transfer can be autosomal, X-linked, or mitochondrial [6]. The genetic alterations that lead to hearing loss can be chromosomal alterations, such as large deletions and inversions, but other possible causes include microdeletions, copy-number variations (CNV), point mutations, several base-pair deletions, substitutions, and splice alterations [7].

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Historically, deafness-associated regions in the genome were named either DFNA, for autosomal dominant forms of deafness, or DFNB, for autosomal recessive forms of deafness. The first gene in which variants leading to hearing loss were identified was *GJB2*. Defects in this gene lead to the most common cause of autosomal recessive non-syndromic hearing loss (ARNSHL), termed DFNB1B, which accounts for 50% of ARNSHL cases in many populations [8–11].

Since the emergence of next-generation sequencing (NGS) technologies, deafness genes have been identified on all chromosomes and over one hundred genes are currently known to be associated with hearing loss [4]. In addition to *GJB2*, the most prevalent gene worldwide, each population is represented by a set of more common genes associated with deafness. For example, in Israel, these include *TMC1*, *SLC26A4*, *MYO6*, *MYO7A*, *MYO15A* and *TECTA* [12].

Because hearing loss has such a diverse genetic background, it is not surprising that the physiological processes affected, and the underlying pathological mechanisms are also varied. Certain deafness genes, such as *PCDH15*, affect mechanotransduction, the ability of hair cells (HC) to translate mechanical stimuli to electrical stimuli [13,14]. Other deafness genes, such as *POU4F3* (DFNA15), are involved in the transcriptional regulation of hair cells [15]. Deafness genes may also affect cell types other than hair cells. Examples of this type are *TECTA* (DFNB21, DFNA8/12), which encodes alpha-tectorin, an extracellular-matrix protein in the tectorial membrane [16] and the *KCNQ1* and *KCNE1* genes, which are affected in Jervell and Lange-Nielsen syndrome and encode potassium channels in the stria vascularis and myocytes [17].

Hearing loss also differs in the age of onset. Progressive conditions, such as those associated with DFNA36, might have wider time-windows for intervention, while congenital cases are likely to require prenatal intervention. This issue is of course greatly dependent on whether the affected cells have already degenerated at the time of diagnosis. A recent study demonstrated complete restoration of hearing in *Otof* knockout mice even after the age of hearing onset, when these mice are already profoundly deaf [18]. In contrast, mice harboring mutations in *Slc12a2*, involved in Kilquist syndrome, present severe developmental abnormalities, such as Reissner's membrane collapse and neuronal migration defects, which means that in this case, gene therapy is likely to be more challenging and will require some sort of prenatal intervention [19].

To summarize, hearing loss is an extremely heterogeneous condition and it is not likely that there is one strategy, one vector, or one approach that will suit all. The potential for rescue needs to be examined carefully for every defect. However, as a result of recent achievements in genetic therapies for hearing loss in mouse models (Table 1), the future now looks promising.

2. The inner ear - delivery approaches

To date, researchers have used three surgical approaches to apply gene therapy in the inner ear: round-window membrane (RWM) injection into the perilymph of the scala tympani and

scala vestibuli; cochleostomy and injection into either the perilymphatic or endolymphatic spaces; and canalostomy, injection into the posterior semicircular canal (PSCC) in mice. While RWM diffusion may be adequate for the delivery of small molecules, large particles such as viruses do not penetrate the RWM easily, rendering this approach less suitable for gene therapy at this time. The RWM and canalostomy approach can be combined as well.

All three approaches have been widely used successfully in animal models [20–22]. In brief, the skin posterior to the auricle is cut and the soft tissues beneath it are dissected to expose the tympanic bulla covering the cochlea or the semicircular canals. A glass pipette is then typically used to penetrate the membrane or cartilage and to inject the therapeutic agent. In adult rodents, a drill is usually required to penetrate the tympanic bulla and bony labyrinth. There have been several reports that described these approaches in great detail [23–26]. One study that compared cochleostomy and RWM injection found little difference, if any, in efficacy and safety [27]. Recent evidence suggested that a combination of RWM injection and PSCC fenestration could achieve higher transduction rates of sensory cells, probably due to enhanced diffusion of viral particles through the bony labyrinth [28]. In another recent study, the authors used the RWM injection approach to successfully deliver viral particles into the cochlea of a non-human primate, suggesting this approach could potentially be used in humans too [29].

Delivery into the developing organ of Corti of mice *in utero* has also been achieved [30,31]. This approach allows rescue in models where sensory cells fail to develop or suffer early damage. It will be extremely important to establish this mode of delivery in non-human primates and eventually also in humans, since the onset of auditory function in humans differs from that in animals such as mice. A distinct auditory brainstem response (ABR) is only observed at about two weeks of age in mice, while humans begin to respond to auditory stimuli in the first trimester of pregnancy [32,33]. Prenatal intervention might therefore prove necessary in order to enable the translation of the exciting preliminary results achieved in mice to the clinic, and to develop therapies for additional conditions of congenital hearing loss.

3. Targeted cell populations

3.1. Hair cells

The highly specialized epithelial hair cells are able to translate sound waves to electrical signals. The inner hair cells (IHC) release neurotransmitters to the spiral ganglion neurons (SGN) from intracellular synaptic vesicles, while the outer hair cells (OHC) contract to amplify the deflection of the basilar membrane [34,35]. There are a number of genetic defects in genes expressed in hair cells that may differ in the cellular processes they impair. Gene delivery into hair cells has been extensively studied using AAV8, Anc80l65, AAV2 quadY-F, AAV9 PHP.B, Cas9-gRNA RNPs, and ASOs, among others, with major to complete recovery of hearing thresholds reported [18,20,29,36–40].

3.2. Supporting cells

Supporting cells (SC) comprise essentially all the non-sensory cells of the organ of Corti (Fig. 1). Like hair cells, they are specialized and can be divided into many sub-populations, including Hensen cells, Dieter's cells, and phalangeal cells. While these cells are clearly important for the normal function of the inner ear, many of their roles remain elusive. A relatively well-characterized function of supporting cells is their regulation of ion concentrations in the endolymph via various ion channels such as ENaCs and intercellular gap-junctions [41]. Gap junctions in the inner ear are primarily heterodimers of connexin 26 and connexin 30, encoded by *Gjb2* and *Gjb6*, respectively. As already discussed, variants in *GJB2* are the most prevalent genetic defects in deafness and are therefore a major target for gene therapy in the inner ear [8,9].

Targeting the supporting cells of the organ of Corti remains challenging, since they are transduced by some of the widely used AAV vectors only at very low rates [42]. A recent study reported improved auditory function in *Gjb2* conditional-knockout mice following early postnatal injection of AAV5 bearing the coding sequence of *Gjb2* [43]. Treated ears had approximately 20 dB-SPL lower ABR thresholds compared to untreated ears. Other studies reported gap function restoration but no auditory function improvement following *Gjb2* delivery mediated by AAV1 and bAAV [44,45]. Transuterine injection of *Gjb6* expression vectors, followed by electroporation, was able to rescue hearing and restore endocochlear potential in *Gjb6*-knockout mice [46]. Hearing thresholds of treated ears were not significantly different than wild-type ears. The numerous genetic variants that affect the function of various subpopulations of supporting cells indicate that there is a great need for efficient methods for supporting cell gene delivery.

3.3. Stria vascularis

The stria vascularis (SV) is a three-layered epithelial structure lining the lateral wall of the scala media (Fig. 1), which houses a rich network of capillaries and is an active participant in cochlear homeostasis [47]. A key role of the SV is the production of the high concentration of potassium in the endolymph and the related endolymphatic potential (EP) of +80–120 mV [48]. Defects in the *KCNQ1* and *KCNE1* genes that encode the potassium channels involved in this homeostatic process of the SV, cause Jervell and Lange-Nielsen syndrome [17]. AAV1 was used to deliver the coding sequence of *Kcnq1* into the inner ears of *Kcnq1*-knockout mice, and resulted in both morphological and complete auditory rescue, as well as reestablishment of EP [49]. This was the first gene therapy study to rescue the function of the SV.

4. Strategy - replace, silence or edit

Deafness variants cause hearing impairment through a wide range of molecular mechanisms and biological pathways. For this reason, it is crucial to devise an appropriate strategy for each individual condition, and more than one approach may be suitable for a given case. Figure 2 summarizes the modes of inheritance and molecular mechanisms, as well as possible strategies and appropriate modes of action.

4.1. Gene replacement

The most common type of genetic manipulation in gene therapy experiments is where the objective is to deliver a wild-type copy (typically only the coding sequence) of a gene whose function has been abolished by a genetic variant. Since the gene is delivered in trans, it is also necessary to supply regulatory elements, such as promoters, enhancers, and poly-A sequences, to drive expression. To date, all gene therapy studies in the inner ear have used general strong promoters and enhancers, such as CMV, and WPRE. Data about inner ear-specific promoter sequences are sparse, but there are a number of identified sequences that can be used to enhance the specificity of transgene expression and increase the safety and efficacy of these treatments. A sequence several kilobases upstream of *Pou4f3* has been shown to drive hair cell-specific EGFP expression [50]. A 118 bp region upstream of *Myo7a*, together with the first intron of *Myo7a*, can drive hair cell-specific expression of GFP [51]. Identifying these regulatory elements will be of paramount importance for the further development of safe and effective genetic therapies for deafness.

Gene replacement is typically most relevant for hearing loss caused by loss-of-function or splicing variants that result in recessive inheritance, or dominant inheritance in cases of haploinsufficiency. It is likely to be less effective in other cases, where the genetic variant leads to the production of a misfolded protein that creates toxic aggregates, or a dysfunctional protein with a dominant-negative effect.

4.2. Gene silencing

Gene silencing is achieved by disrupting the genomic sequence of the mutated allele using CRISPR/Cas9, Transcription activator-like effector nucleases (TALEN), or Zinc Finger Nucleases (ZFNs), or by preventing translation of the aberrant sequence with anti-sense oligonucleotides (ASOs), siRNA, or other types of complementary sequences that mediate the degradation of mRNA. ASOs are synthesized strands of either DNA or RNA that are designed to bind to a specific mRNA and thereby inhibit translation or mediate degradation via recruitment of enzymes such as RNase H. The use of ASOs in the inner ear has recently been reviewed [52]. Of note, ASOs can also be used to target splice junctions and alter the splicing pattern of genes, which can have a therapeutic effect, as shown for the recently FDA-approved drug for spinal muscular atrophy Spinraza (previously known as Nusinersen) [53]. An example of this therapeutic potential was demonstrated for a form of Usher Syndrome [37,54] where ASOs were used to correct the defective splicing pattern of the *Ush1c* gene in a mouse model carrying a c.216G>A variant. The results indicated near complete rescue of both auditory and vestibular function following peritoneal injection of the ASO. Another form of gene silencing involves the use of siRNA to mediate RNA interference (RNAi). For this purpose, double-stranded RNA containing a sequence complementary to the target gene is synthesized and delivered to the cell where it activates the RNAi pathway, including Dicer and the RISC complex, leading to cleavage of the target mRNA and gene knockdown. This technique was used to alleviate hearing loss in a mouse model of *TMC1* autosomal dominant hearing loss [39,55]. An AAV coding for a synthetic microRNA designed to target the dominant allele was injected into the cochlea of Beethoven mice, which carry a dominant variant in *Tmc1* [56]. The results show a variable degree of success, with some mice presenting near wild-type ABR thresholds. This technique has been

described in further detail [57]. It is important to note that while delivering oligonucleotides requires repetitive delivery, an siRNA delivered in an AAV is presumably a one-time treatment. This approach can also be an advantage of oligonucleotides, since the treatment may be reversible in case of adverse effects.

4.3. Gene editing

Gene editing can be achieved by the introduction of gene editing agents, such as CRISPR/Cas9, TALEN, and ZFNs. In a sense, gene editing is the holy grail of gene therapy. While editing the genomic sequence of humans is perilous, 'correcting' the genomic sequence would potentially avoid any toxic effects generated by random integration, by expression of the transgene in off target cells, or even by misregulated expression in the right cells. A wild-type sequence in its native genomic neighborhood should be subject to regulation by endogenous regulatory elements, resulting in appropriate and timely expression and would bear the closest resemblance to the wild-type state. On the other hand, off-target events generated by promiscuous binding of nucleases bears the potential to damage cells and even lead to carcinogenic mutagenesis. Recent reports on unexpected rearrangement events following double-strand breaks (DSB) induced by CRISPR-Cas9 are likely to hamper the adaption of this technique for therapy (in its current state) [58]. A variety of genome-editing techniques and applications of genome editing techniques to hearing research, such as for modeling genetic variants, have been reviewed [59,60].

Two studies used CRISPR/Cas9 systems in order to introduce indels and silence a dominant allele in a deafness mouse model of *Tmc1* [36,61]. A commercial transfection reagent, Lipofectamine 2000, was used to deliver Cas9-guide RNA ribonucleotide-protein complexes (RNP) and Anc80L65 was used to deliver the coding sequence of SaCas9-KKH into hair cells of Beethoven mice in order to specifically target the mutated allele, resulting in gene silencing and partial rescue of hearing. The relatively modest degree of rescue of less than 20 dB-SPL in most frequencies in the case of the lipofectamine 2000 can be attributed to limited transfection efficiency and relatively poor specificity to the mutant allele. An important distinction to be made is that gene editing for the purpose of gene silencing leads to complete abolishment of target gene expression, effectively a 'knockout', in successfully edited cells, while ASOs and siRNAs lead to partial to near-complete abolishment, termed 'knockdown'. Another distinction to be made is that silencing achieved by genome editing potentially requires only one cleavage event, making it irreversible. It might therefore be favorable to deliver genome editing agents such as protein or mRNA in order to limit the potential off-target events.

A recently developed fusion of Cas9 and cytidine-deaminase enables 'base editing', conversion of one base to another, without the need to introduce a DSB [62]. This approach is suitable for genome editing in non-dividing cells where a single base-pair variant leads to disease. An example in deafness is the p.Thr5Met substitution in *GJB6* [63].

5. Viral vectors

5.1. AAV- The perfect vector?

Adeno-associated virus (AAV) is a replication-deficient member of the Parvoviridae family discovered in the 1960s [64]. The genome consists of a single DNA molecule flanked by two inverted terminal repeats (ITRs) at both ends. These form secondary structures that are recognized by packaging proteins and are believed to promote primase-independent second strand synthesis. ITRs are also important for the integration of AAV genomes into a specific site on chromosome 19 [65]. Recombinant AAVs (rAAVs) are generated by providing the host cell with the structural (cap) and packaging (rep) genes in trans, thereby creating safe delivery vectors capable of packaging up to 4.8kb of transgene [66]. rAAVs have been widely used for genetic manipulations in research and are very promising candidates for gene therapy in humans. Key features of AAVs that account for their popularity, include their broad range of tropism towards various tissues and cell types, their ability to produce long-term expression of transgenes without integrating into the genome, and the fact that they elicit little to no immune response. Table 2 summarizes the reported transduction efficiencies of different AAV capsids used in the inner ear.

5.2. Synthetic AAV

Due to the limitations of natural serotypes, efforts have been made to develop novel AAV capsids. These are often referred to as synthetic AAVs and are generated by introducing changes in the sequences of capsid proteins. One prominent example is an AAV named Anc80L65, which was generated by in-silico reconstruction of ancestral AAVs. Anc80L65 transduces sensory cells with immensely improved efficiency, transducing virtually all IHC, and a substantial portion of OHC, both *ex vivo* and *in vivo* [67]. The construct has already been used to rescue hearing in *Tmc1*-knockout and *Ush1c* mice [20,68]. Near complete recovery of auditory function, as well as morphological and electro-physiological rescue, was achieved in a mouse model of Usher syndrome type 1C following RWM injection of AAV2/Anc80L65.CMV.harmonin-b1 [20].

AAV9-PHP.B is a pseudotype of AAV9 that was recently developed by cre-dependent evolution designed to produce a vector for efficient transduction of neurons [69]. PHP.B could transduce the majority of IHC and nearly half the OHC in mice [29]. Even more importantly, there was near complete transduction of both types of hair cells in an injected non-human primate, as well as considerable expression in spiral ganglion neurons and the lateral wall. In contrast to the promising transduction efficiency, injection of AAV9-PHP.B-CBA-Clrn1 in a mouse model of Usher syndrome 3A resulted in partial restoration of hearing that was only slightly superior to that achieved with AAV8 [38]. Despite these results, the high transduction efficiency of HC and safety profile of AAV9-PHP.B mark it as a promising candidate for gene therapy in hearing loss resulting from HC defects.

AAV2.7m8 is a synthetic AAV developed for retinal gene delivery by in-vivo screening of AAV libraries with modified capsid proteins [70]. In a recent study, AAV2.7m8 was shown to transduce both IHC and OHC at high rates *in vivo*; 84.1 and 83.1% on average, respectively [71]. These rates are reported to be higher for OHC than the rates achieved by

Anc80L65 delivered at a similar titer. More importantly, AAV2.7m8 was shown to transduce LGR5-positive supporting cells, which are thought to possess some regenerative capacity and are therefore a desired target for gene delivery [72].

5.3. Exo-AAV

Exosomes are lipid-based extracellular vesicles involved in physiological processes such as intercellular communication in response to metabolic changes or exercise [73]. They have been studied in recent years as potential carriers of small molecules, proteins, nucleic acids and others, and have been shown to enhance AAV-mediated gene delivery [74]. Exosome-associated AAV1 (exo-AAV1) was shown both *in vitro* and *in vivo*, to transduce mouse HCs at rates nearly twice those of standard AAV1 (~90% of IHC and ~30% of OHC when administered via RWM injection) [75]. The exo-AAV1 demonstrated the potential to rescue hearing and vestibular function in a *Lhfp15* knockout mouse model exhibiting profound deafness and circling behavior, enabling mice that would otherwise be profoundly deaf present ABR responses at approximately 80 dB-SPL.

It is not entirely clear why exosome-associated AAV are able to transduce cells at higher rates. One proposed mechanism is that the lipid envelope provided by exosomes enhances the stability of the vector and protects against antibody-mediated degradation [76]. Another possibility is that surface proteins that naturally appear on the vesicle surface enhance endocytosis and facilitate entry into target cells [77]. Full characterization of these properties is very important since they may then be modified to further increase efficiency and specificity. In this context, a recent study demonstrated that overexpression of CD9 increases exosome production and improves transduction efficiency [78]. Due to safety considerations, exosome-associated AAV for clinical use would preferably rely on synthetic exosomes rather than those derived from cancer cell lines, such as HeLa or HEK293T, and would require precise reconstruction of exosome composition.

To summarize, exosome-associated AAV seems to be a promising improvement to existing methods of AAV production, although a more detailed characterization of their biochemical properties, mechanism of action, and safety profile will be required for their translation into clinical use.

5.4. Dual-AAV

Due to its relatively low packaging capacity of 4.8 kb, several approaches have been developed to split the desired transgene between two AAV vectors [79]. One such method utilizes the cell's splicing machinery by delivering the 5' half of the coding sequence followed by a splice donor sequence and a recombination sequence in one vector, with a recombination sequence followed by a splice acceptor sequence and the 3' half of the coding sequence in the second vector. This approach was recently used to rescue hearing in a mouse knockout for *Otof*, which is a model for DFNB9 [18]. In this case, the coding sequence of *Otof* was split into two AAV2 quadY-F vectors, an AAV developed for retinal gene delivery [80], and injected into mice through the RWM at several time points before and well after the onset of auditory function (P2, P10, P17, P30). While untreated knockout mice exhibited profound deafness with no observable ABR waves in response to stimuli of any frequency or

level, the hearing thresholds of treated mice did not differ from wild-type animals at any of the frequencies tested. Click-ABR tests performed 30 weeks post-injection gave the same results. Of note, mice injected at P30 still improved to wild-type levels, demonstrating a wide time-window for intervention in this model.

The study described above demonstrated the feasibility of delivering large transgenes using dual-AAV and highlighted the potential therapeutic relevance. An earlier study on *Otof* knockout mice also provided proof-of-principle for this approach using AAV6 [81]. However, since several large deafness genes, such as *MYO7A* and *CDH23*, are also expressed in OHC, and since OHC are not easily transduced at this time, the rescue of these forms of deafness is likely to prove more challenging [82].

5.5. Adenovirus

Adenoviruses (AdV) are icosahedral viruses of the Adenoviridae family [83] where serotypes 2 and 5 are the best-characterized for human gene delivery. These viruses can infect a wide variety of cells and tissues at high efficiencies and can produce robust transgene expression, in several cell types in the inner ear.

A major advantage of AdV is the large packaging capacity, which is approximately 36 kb genome in third generation AdV produced by introducing the genome of the natural AdV in trans or by packaging cell lines. This process also results in a lower immunogenic response [84]. A major limitation in the use of AdV is their association with human disease. Elevated ABR thresholds were reported in mice whose otocyst was injected with a second-generation AdV, suggestive of ototoxicity [85]. Another limitation of AdV is the relatively limited (weeks to months) duration of transgene expression, which is a consequence of the lack of integration into the host genome [86]. These characteristics render AdV more appropriate for applications involving otoprotection and regeneration and less for rescue of genetic hearing loss, where long-term expression of transgene is typically required.

A recent study tested the transduction efficiency of sensory epithelial cells of three AdV vectors injected into the scala media in mice [87]. The results indicated considerable transduction of supporting cells but very low transduction rates of sensory cells. In contrast, there was efficient transduction of both sensory and non-sensory cells in human vestibular organotypic explants and a recent study even demonstrated the ability to use AdV to regenerate hair cell-like cells in human vestibular epithelia exposed to gentamicin [88]. In conclusion, further improvements and modulations of tropism are required, but there is definite potential for the use of these vectors for human inner ear gene delivery.

5.6. Lentivirus

Lentivirus (LV) is a member of the Retroviridae family and is derived mainly from human or feline immunodeficiency viruses. It is comprised of enveloped capsids encapsulating a single stranded DNA genome capable of carrying transgenes of approximately 8kb in advanced recombinant generations. The recently developed integration-deficient LV has an enhanced safety profile for potential clinical use [89,90]. Although initial concerns about the safety of LV in the inner ear seemed to limit its relevance for inner ear gene therapy, the ease of production, large cargo capacity, and wide tropism prompted testing of LV in hearing

applications. There was no impact on hearing in wild-type rats injected with LV carrying *Atoh1* [30,91]. In addition, several subsequent studies demonstrated the ability of LV to transduce non-sensory cells in the inner ear of animal models. Transgene expression in SGN, supporting cells and perhaps a few fibrocytes was reported following in-vitro transduction of rat cochlear organotypic explants [92]. However, very limited transgene expression was found in cells lining the perilymphatic spaces following in vivo injection by cochleostomy. Similar results were reported following injection through the RWM. Of note, little to no expression of transgene was seen in sensory cells in any of the reports [93].

The limited tropism towards appropriate cell populations has made LV an infrequent choice for inner ear application. However, considering the dramatic improvements in vector efficiency achieved by synthetic evolution and pseudotyping of AAV, it is possible that future modifications to existing LV vectors could generate viable candidates for use in inner ear gene delivery. Other less frequently used viral vectors for inner ear gene therapy were discussed in a recent review [94].

6. Non-viral delivery

Gene delivery into cell lines is performed routinely in almost any molecular biology laboratory usually by using commercially available cationic-lipid (CL) formulations without any involvement of viral vectors. These formulations are easy to use, generally elicit no immunogenic response, and are potentially safe for use in humans, since unlike viral vectors, they are produced synthetically and do not require any involvement of biological systems. In addition, non-viral delivery systems can also be used for the delivery of RNA and protein, a feature that expands their potential uses beyond those available with viral vectors. However, despite these advantages, with a number of exceptions discussed below, non-viral delivery methods are not common in inner ear gene therapy studies.

Lipofectamine 2000 is a commercially available CL widely used for gene delivery in-vitro. While concerns about its potential ototoxicity exist, it was demonstrated to be capable of delivering Cre-recombinase and Cas9-guide-RNA ribonucleotide-protein complexes (RNP) into OHC *in vivo* [95]. More recently, the therapeutic potential of this approach was demonstrated [36] when Lipofectamine 2000 was used to deliver RNP complexes into the inner ears of Beethoven mice in order to mediate genome editing of a *Tmc1* dominant-negative allele.

Other examples of non-viral delivery formulations, such as dendrimers, polyamine non-liposomal transfection reagents, and cell-penetrating peptides, have also been tested for inner ear gene delivery, but have not been used for therapy to date [21,96].

Electroporation is another form of non-viral gene delivery that is carrier-independent and instead relies on the application of high-voltage electrical pulses generated by electrodes to disrupt the integrity of the cellular membrane and permit internalization of nucleic acids. Among other advantages are the ability to deliver large transgenes and the fact that the method does not require a packaging cell line and expensive reagents. The major disadvantages of electroporation are poor specificity, relatively low efficiency, and potential

toxicity. Nevertheless, several studies have utilized this approach to deliver genes into the inner ear *in vivo* [31,46,97,98]. In a study by Wang et al, the authors demonstrated expression of EGFP in the hair and supporting cells of mice whose otocyst was injected with a plasmid and electroporated at E11.5. Further investigation of the efficiency and safety of *in vivo* electroporation is required.

7. Conclusions: preliminary successes alongside remaining challenges

Gene therapy for deafness and hearing loss is currently coming-of-age. Recent developments in viral-vector technology have led to a dramatic increase in manuscripts reporting successful rescue in animal models of human hearing loss. Advancements in gene editing and gene regulation are likely to further enhance the efficacy and specificity of these treatments in the near future. While several challenges still exist, the enthusiasm among the scientific community is evident, and for good reason.

This exponential growth in gene therapy literature has not gone unnoticed by the pharmaceutical industry, and almost 50 biotechnology and pharmaceutical companies are currently developing therapeutics for inner ear and central hearing disorders [99]. AGTC, Akouos, and Rescue Hearing Inc. are three companies currently engaged in pre-clinical testing of AAV to treat genetic hearing loss. Similarly, Casebia Therapeutics, a joint venture between Bayer and CRISPR Therapeutics, is conducting preclinical testing of CRISPR/Cas9 for the treatment of genetic deafness. In addition, important progress in the clinical translation of technologies to treat other forms of hearing loss is expected from a number of prominent companies, such as Decibel Therapeutics, who are currently working on various forms of inner ear therapy and specifically on regeneration strategies. Potential inner ear therapeutics currently in preclinical stages or in clinical trials have been recently reviewed [99,100].

A major challenge in treating genetic hearing loss is its heterogeneity. Since there are over one hundred genes in which variants have been shown to lead to deafness, and because every gene tells a ‘different story’ with respect to protein function, therapeutic time windows, and cell populations to target, the optimal approach to be taken is still under debate. Will precision medicine efforts enable us to target each variant separately, even if only a few patients are involved? Will a “proof-of-concept” success be relevant for variants affecting less than 1000 patients worldwide, or will the costs required for therapy prohibit such work unless large profits are anticipated? It is important to note that although rare variants affect only a handful of patients individually, collectively they are the most common cause of deafness. Will approaches to deliver a wild-type copy of a gene, irrespective of the specific variant involved, be the method of choice or will tailor-made gene editing be preferred? The answers to these questions will naturally differ from case to case and at this time, there are more questions than answers. But what is clear is that there have been breakthroughs towards the path to gene therapy for hearing impairment, as described above and in the literature referred to in this review, and the future appears to hold great promise for the ability to treat and cure deafness using genetic tools.

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Highlights:

- Hearing restoration is being demonstrated in an increasing number of human deafness models
- Novel AAV capsids improve transduction efficiencies and safety
- Gene-editing technologies hold great promise but pose new challenges

Fig. 1. Structure of cochlea and organ of Corti, indicating the surgical routes used for administration of therapeutic materials to the inner ear. SV - stria vascularis, TM - tectorial membrane, IHC - inner hair cell, OHC - outer hair cell, SC - supporting cell, BM - basilar membrane.

Fig. 2.
Different levels of complexity and subsequent strategies in gene therapy for deafness.

Table 1

Gene therapy results in mouse models

Gene	Variant	Inheritance	Strategy	Targeted cells	Delivery	Reference
<i>Tmc1</i>	<i>Tmc1</i> ^{Bth/+}	AD	Silence by editing	HC	Lipofectamine 2000	[36]
<i>Tmc1</i>	<i>Tmc1</i> ^{Bth/+}	AD	Silence by RNAi	HC	AAV9	[39]
<i>Tmc1</i> and <i>Tmc2</i>	KO	AR	Replacement	HC	Anc80L65	[68]
<i>Ush1g</i> (sans)	KO	AR	Replacement	HC	AAV8	[101]
<i>Ush1c</i> (harmonin)	c.216G>A	AR	Replacement	HC	Anc80L65	[20]
<i>Ush1c</i> (harmonin)	c.216G>A	AR	Splicing correction by ASO	HC	Intraperitoneal injection of ASO	[37,54]
<i>Vglut3</i>	KO	AR	Replacement	HC	AAV1	[40]
<i>Otof</i>	KO	AR	Replacement	HC	Dual AAV2 quadY-F	[18]
<i>Otof</i>	KO	AR	Replacement	HC	Dual AAV6	[81]
<i>Gjb2</i>	cKO	AR	Replacement	SC	AAV5	[43]
<i>Gjb6</i>	shRNA mediated knockdown	AR	Replacement	SC	Electroporation in utero	[46]
<i>Kcnq1</i>	KO	AR	Replacement	SV	AAV1	[49]
<i>Msr3</i>	KO	AR	Replacement	HC	AAV1	[102]
<i>Lhfp15</i>	KO	AR	Replacement	HC	Exo-AAV1	[75]
<i>Cln1</i>	KO	AR	Replacement	HC	AAV9-PHP.B	[29]
<i>Cln1</i>	KO	AR	Replacement	HC	AAV8	[38]
<i>Whrn</i>	<i>Whrn</i> ^{wi/wi}	AR	Replacement	HC	AAV8	[103]

* KO - knockout, cKO - conditional knockout, AR - autosomal recessive, AD - autosomal dominant.

Table 2

AAV serotype in-vivo transduction efficiencies

Capsid	Transduction efficiency				Reference
	IHC	OHC	SC	Other	
AAV1	<20	<20	<5	SV	[42,49,67]
AAV2	<20	<40	<5	SG	[42,67,71,104]
AAV5	<29	0	<3		[42]
AAV6	<10	<10	NA		[67]
AAV6.2	0	0	11		[42]
AAV7	<21	0	<4		[42]
AAV8	40–84	24–28	NA	SG	[38,42,67,71,101,104]
AAV9	<22	0	<10		[42]
AAVrh.10	<40	0	<6		[42]
AAVrh.43	<6	0	<15		[42]
bAAV	16 *	28 *	NA		[105]
AAV2.7m8	84	83	61–86 LGR5+ SC		[71]
AAV8BP2	56	44	0		[71]
AAV2quadY-F	78	NA	NA		[18]
Exo-AAV1	90	30	low		[75]
Anc80l65	>90	>90	75 inner pillar cells		[67,71]
AAV9-PHP.B	60–80	30–40	0	SV	[29]

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ex-vivo data only