Michael Morgan, Juliane W. Schott, Axel Rossi, Christian Landgraf, Athanasia Warnecke, Hinrich Staecker, Anke Lesinski-Schiedat, Brigitte Schlegelberger, Hildegard Büning, Bernd Auber, and Axel Schambach*

Gene therapy as a possible option to treat hereditary hearing loss

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Abstract: The process of hearing involves a series of events. The energy of sound is captured by the outer ear and further transferred through the external auditory canal to the middle ear. In the middle ear, sound waves are converted into movements of the tympanic membrane and the ossicles, thereby amplifying the pressure so that it is sufficient to cause movement of the cochlear fluid. The traveling wave within the cochlea leads to depolarization of the inner ear hair cells that, in turn, release the neurotransmitter glutamate. Thereby, the spiral ganglion neurons are activated to transfer the signals via the auditory pathway to the primary auditory cortex. This complex combination of mechanosensory and physiological mechanisms involves many distinct types of cells, the function of which are impacted by numerous proteins, including those involved in ion channel activity, signal transduction and transcription. In the last 30 years, pathogenic variants in over 150 genes were found to be linked to hearing loss. Hearing loss affects over 460 million people world-wide, and current treatment approaches, such as hearing aids and cochlear implants, serve to improve hearing capacity but do not address the underlying genetic cause of hearing loss. Therefore, therapeutic strategies designed to correct the genetic defects causative for hearing loss offer the possibility to treat these patients. In this review, we will discuss genetic causes of hearing loss, novel gene therapeutic strategies to correct hearing loss due to gene defects and some of the preclinical studies in hearing loss animal models as well as the clinical translation of gene therapy approaches to treat hearing loss patients.

Keywords: hearing loss, genetic variants, sequencing, gene therapy, genome editing

***Corresponding author: Axel Schambach,** Institute of Experimental Hematology, Hannover Medical School, Carl-Neuberg-Str.1, D-30625 Hannover, Germany; and REBIRTH Research Center for Translational Regenerative Medicine, Hannover Medical School, Hannover, Germany; and Division of Hematology/Oncology, Boston Children's Hospital, Harvard Medical School, Boston, MA 02115, USA, e-mail: schambach.axel@mh-hannover.de
Michael Morgan, Juliane W. Schott, Axel Rossi, Institute of Experimental Hematology, Hannover Medical School, Hannover, Germany; and REBIRTH Research Center for Translational Regenerative Medicine, Hannover Medical School, Hannover, Germany
Christian Landgraf, Brigitte Schlegelberger, Bernd Auber, Department of Human Genetics, Hannover Medical School, Hannover, Germany
Athanasia Warnecke, Anke Lesinski-Schiedat, Department of Otolaryngology, Hannover Medical School, Hannover, Germany; and Hearing4all Cluster of Excellence, Hannover Medical School, Hannover, Germany
Hinrich Staecker, Department of Otolaryngology Head and Neck Surgery, University of Kansas School of Medicine, Kansas City, USA
Hildegard Büning, Institute of Experimental Hematology, Hannover Medical School, Hannover, Germany; and REBIRTH Research Center for Translational Regenerative Medicine, Hannover Medical School, Hannover, Germany; and German Center for Infection Research (DZIF), partner site Hannover-Braunschweig, Braunschweig, Germany

Introduction

Genetic diagnosis of hearing loss allows prognostic disease assessment and provides the opportunity for early therapeutic intervention. Identification of genes associated with hearing loss has led to deeper insight into the underlying biology of normal hearing and pathology of hearing loss. Coupled with our increased understanding of genetics and advances in molecular biology techniques, this information has greatly facilitated design of novel medical strategies to treat hereditary hearing loss, such as gene therapy approaches. Indeed, the first clinical trial testing gene therapy in hearing loss patients was initiated and there are several gene transfer modalities available for further evaluation and optimization.

From genetic causes to novel therapeutics

Hearing is dependent upon the coordinated interaction of several cell types and their responses to mechanical and physiological stimuli. Especially cells of the sensory epithelium, including the inner and outer hair cells (HC) as

well as spiral ganglion neurons (SGN) of the inner ear, play key roles in the hearing process (Figure 1). As with other human diseases, genetic aberrations that impact cellular functions can also lead to hearing loss.

Advances in sequencing technologies led to more rapid identification of genetic aberrations linked to hearing loss. Currently, pathogenic variants (PV) in more than 150 genes are known to be associated with hearing loss

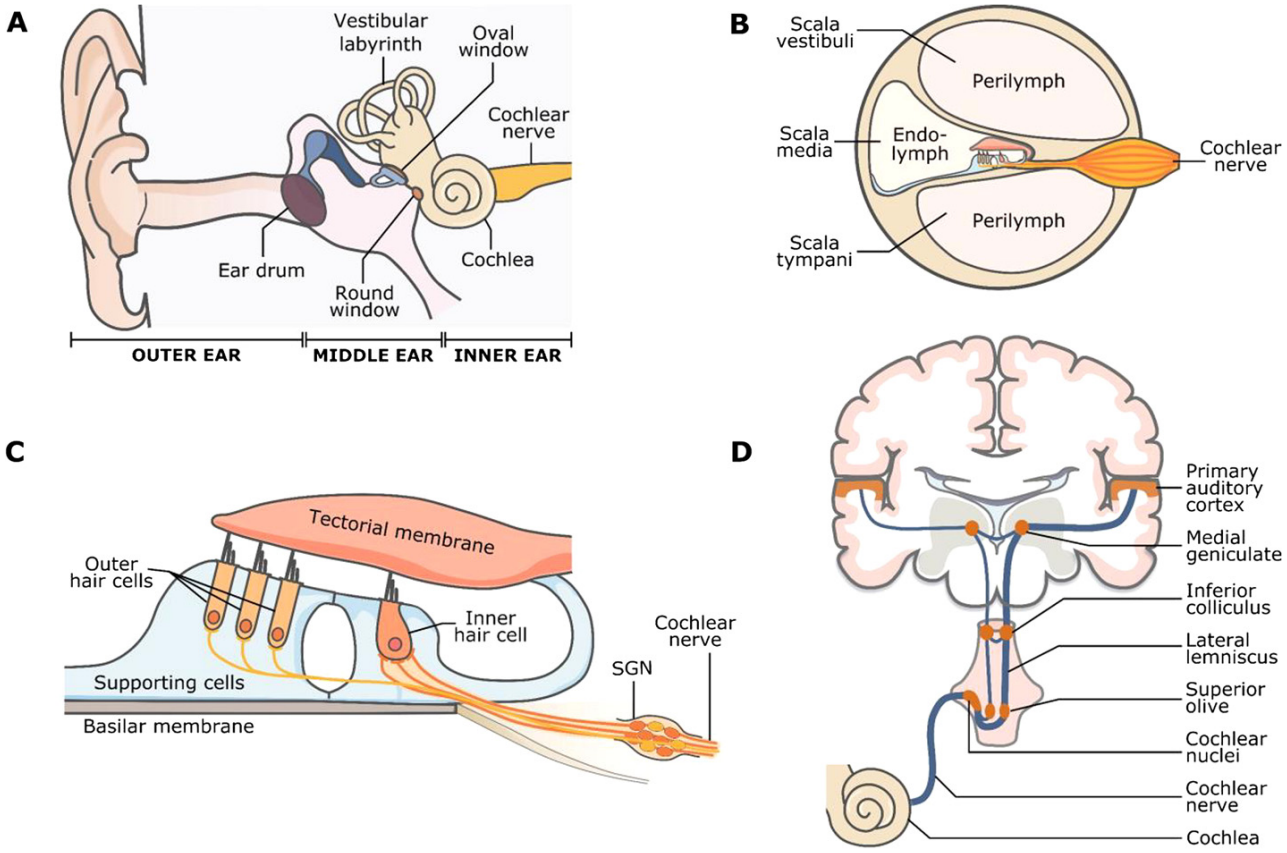

Figure 1: Anatomy of the auditory system and the process of hearing. (***A***) When the air pressure of a sound wave pushes the eardrum, the stimulus is transformed into mechanical motion of the three ossicles in the middle ear and transmitted to the oval window. The pressure applied to the oval window membrane causes movement of the fluid that fills the cochlea. This induces activation of sensory neurons, and the signal is projected to the brain via the cochlear nerve. (***B***) The cochlea is a bony snail-shaped structure containing three fluid-filled compartments (*scalae*). The *scala vestibuli* and *scala tympani* are filled with Na^+-rich and K^+-low perilymph and are continuous through a hole in the separating membrane at the apex of the cochlea. The *scala media* is a separate compartment filled with endolymph, which is low in Na^+ and high in K^+. Here, the *stria vascularis* is responsible for the ion content by actively pumping Na^+ and K^+ against their concentration gradients. The difference in ionic concentration between endo- and perilymph creates an electrical potential known as endocochlear potential. The *scala media* contains the organ of Corti. (***C***) The organ of Corti consists of epithelial sensory cells called hair cells, the rods of Corti as well as supporting cells, and is attached to the top of the basilar membrane, which is flexible and separates the *scala media* and *scala tympani*. The stereocilia on top of the hair cells extend into the endolymph ending either in (outer hair cells) or just below (inner hair cells) the tectorial membrane, a gelatinous acellular membrane protruding into the *scala media* above the organ of Corti. Bending of the basilar membrane resulting from motion of the fluid inside the cochlea leads to flexing of the stereocilia, which induces the opening or complete closure of K^+ channels (depending on the direction of bending) and subsequent de- or hyperpolarization of the hair cells, resulting in neurotransmitter release or cessation of the release, respectively. Hair cells form synapses on spiral ganglion neurons (SGN), whose axons enter the cochlear nerve and fire action potentials to the brain. While outer hair cells amplify the movement of the basilar membrane through motor proteins, most of the signal transmitted to the brain is derived from inner hair cells. (***D***) The auditory nerve projects into the ipsilateral cochlear nuclei (dorsal and ventral). Although different pathways exist through nuclei of the brain stem for projection to the auditory cortex, one of particular importance is through the ventral cochlear nucleus to the superior olive on both sides of the brain stem, and from there via the lateral lemniscus to the inferior colliculus of the midbrain, where all ascending auditory pathways converge. Axons from neurons in the inferior colliculus innervate the medial geniculate in the thalamus, which projects to the primary auditory cortex, where these impulses are deciphered.

[1] (http://www.hereditaryhearingloss.org). Introduction of gene knockouts to model PV genotypes in various animal models has helped to reveal the biological mechanisms through which loss of functional proteins leads to hearing loss and has also led to improved understanding of normal hearing processes (Figure 1). For example, loss of function variants cause disruption of cellular and molecular processes necessary for normal hearing, such as inner ear sensory cell development, control of transmembrane potentials via ion channels, gap junction channels for maintenance of the endocochlear potential, glutamate signaling and alternative gene splicing events.

Identification of PV in hearing loss patients also allows the possibility for personalized medicine approaches such as gene therapy to treat these patients. Knowledge gained from previous experience using gene therapies to treat other human diseases can be exploited to adapt protocols for use in hearing loss patients. Here, some of the most relevant vector technologies available for gene therapy of the inner ear include adenoviral (AdV) vectors, adeno-associated viral (AAV) vectors and lentiviral vectors. Each of these vector systems has unique properties with potential advantages and disadvantages. Importantly, these vector systems are currently used to deliver advanced genome editing tools to correct PV in target cell populations and the relevance of this technology to repair genetic lesions in inner ear cells with the aim to improve hearing is a clinically relevant area to be exploited. In combination with the extensive information acquired from animal models, gene transfer and genomic modification technologies are expected to drive clinical development of novel gene therapy strategies to treat hearing loss patients.

Genetic alterations and clinical consequences

Hereditary hearing loss can be divided into two major subgroups, syndromic – i. e. as a symptom of a superordinate disorder involving further anomalies of the ear and other organ systems – and non-syndromic hearing loss (NSHL), involving only the function of the inner ear. Today, more than 400 distinctive syndromes are well-defined, for which hearing loss is a characteristic mandatory or accessory symptom. Many non-distinctive syndromes and multi-systemic disorders comprise hearing loss as well. Approximately 70 % of genetically determined hearing loss is non-syndromic [2]. The by far most common genetic cause for hereditary NSHL are pathogenic variants affecting the gene *GJB2*, which account for roughly 10–30 % of NSHL [3–5], reaching over 50 % in some ethnic groups [6]. Noteworthy, the majority of NSHL is distributed among more than 100 genes known to be associated with NSHL, and likely more will be identified. Hereditary hearing loss may follow an autosomal recessive (most frequently, about 80 % of NSHL), autosomal dominant, X-linked or mitochondrial inheritance pattern [2].

The underlying molecular changes are variable and comprise substantial numbers of truncating, missense and intronic variants as well as copy number variations (CNVs). While historically the genetic heterogeneity restricted testing to just a few genes, recent studies underline the benefits of comprehensive genetic testing by targeted sequencing panel analysis [7]. Against a background of steadily decreasing costs with continuously improving sequencing coverage as well as increasing reliability in computed CNV detection, it is only a question of time before whole exome sequencing (WES) and soon whole genome sequencing (WGS) will become the preferred method. There is no doubt that the interpretation of a large number of variants remains a major challenge in all of these techniques, as well as the task to handle and appropriately report a multitude of variants of unknown significance (VUS) [8]. The identification of the genetic cause becomes increasingly important regarding personalized prognosis and therapy selection [9]. Regarding the outcome of cochlear implant therapy, recipients with genetic alterations affecting the function of the cochlear sensory organ seem to perform significantly better in terms of speech recognition than patients with PV in genes associated with spiral ganglion neuron function [10].

Thus, all newborns and infants as well as children and young adults with confirmed (syndromic or non-syndromic) hearing loss without strong evidence for an environmental etiology should be seen by a geneticist and offered testing. In adults, a more detailed assessment of the development of hearing loss seems appropriate before recommending genetic counseling, although literature suggests that over 30 % of adult onset progressive hearing loss that results in cochlear implantation has a genetic cause [11]. Once a causative PV is identified, genetic testing should be offered to family members at risk. Genetic counseling may also include assessment of recurrence risk for potential offspring [2]. While performing prenatal as well as preimplantation genetic diagnosis is generally feasible, this should be carefully considered with respect to the Deaf culture [1] and according to country-specific legal aspects. Due to the extreme heterogeneity of genetically determined hearing loss that accompanies the complex challenge of variant interpretation, general population-wide

prenatal or newborn genetic screening is currently not recommended. However, in the future, screening for at least specific genetic alterations should be considered in addition to physiologic newborn screening [12, 13], with particular regards to targeted and potentially curative therapeutic approaches. Determination of genetic aberrations responsible for a patient´s hearing loss can be used to direct personalized approaches like gene therapy.

Vector options to introduce gene therapeutics into the inner ear

Generally, different concepts are available for gene therapy of hereditary hearing loss. To substitute for the function of a defective gene, an intact copy can be introduced into the relevant cells of the inner ear. Gene suppression, for example through expression of an shRNA that targets the transcript of the mutated gene to prevent its translation, can serve to eliminate dominant-negative effects that may interfere with proper cellular function even if an intact gene copy is provided. Finally, gene correction utilizing gene editing based on designer nuclease systems allows the specific removal of PV, thereby also keeping the natural regulation of gene expression via the physiologic promoter and chromatin environment.

Common to all different gene therapy strategies is the requirement for efficient transfer technologies to equip the target cells with expression units for the intact gene or miRNA, for shRNAs, or for the gene editing components. The complex 3D architecture and defined arrangement of the specific cell types inside the cochlea (see Figure 1) excludes *ex-vivo* cell manipulation and restricts treatment options to *in vivo* delivery systems. This is in contrast to other organ systems, such as the hematopoietic system, where stem cells can be extracted and re-infused into the patient upon *ex-vivo* gene therapy. Viruses have evolutionarily co-evolved with their hosts and, as such, have developed specialized mechanisms to enter their target species and cell type(s). Therefore, viral vectors appear to be ideal vehicles to deliver genetic information to the cochlea. Furthermore, the different compartments in the cochlea are filled with lymph, which allows for the distribution of injected viral vectors throughout the cochlea via this intracochlear fluid, while spread to other organs is theoretically limited to the enclosed organ system of the inner ear.

Several parameters are important for the success of viral vector-based gene therapy approaches in the cochlea: **(1)** The vector volume that can be administered is limited. The outer wall of the inner ear is rigid, so that injection of too high vector volumes would increase the pressure and cause hydraulic trauma. Standard injection volumes are 1 µL in mice and are estimated to be 10–30 µL in humans. Thus, high-titer vector preparations are required to allow delivery in a small volume. One advantage for gene therapy application to the inner ear is that the total number of cells present in the cochlea is low as compared to other gene therapy-relevant organ systems, so that a comparably low number of vector particles should suffice to achieve clinical benefit. **(2)** The endocochlear potential as a result of the different ion compositions of perilymph and endolymph is an important prerequisite for proper functioning of the hearing cascade. Thus, the buffer used to deliver vector preparations should be compatible with inner ear fluids and cell types. **(3)** Optimal delivery routes to administer viral vectors to the cochlea need to be investigated (Figure 2), and vector distribution and dissemination from the site of injection need to be characterized. **(4)** Pre-existing immunity to vector components, such as the capsid, or to transferred genes might limit gene transfer and/or expression efficiency, or cause local inflammation.

Currently, three main viral vector systems have emerged for inner ear gene therapy: **(1)** lentiviral (LV) vectors, **(2)** adenoviral vectors (AdV), and **(3)** adeno-associated virus (AAV) vectors. Each of these were tested in *in vitro* transduction experiments using cell lines, disso-

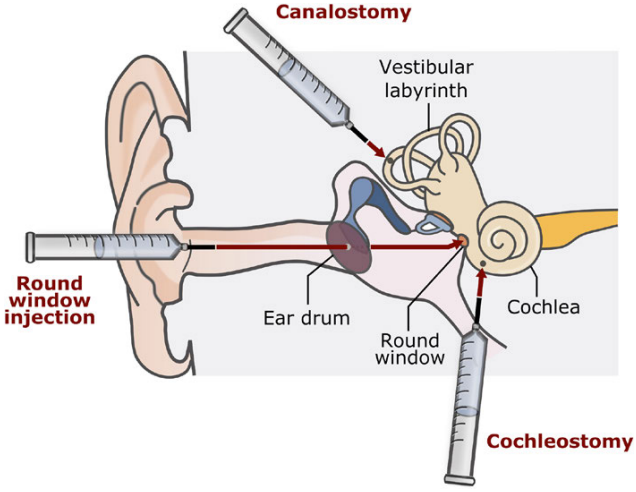

Figure 2: Possible routes for delivery of gene therapy vectors into the inner ear. For in-human cochlear gene therapy, viral vectors can be administered passing through the middle ear by injection through the round window membrane into the cochlea. Alternatively, in rodent models, vector preparations can be introduced by cochleostomy, i. e. direct injection into the cochlea, or by canalostomy, i. e. direct injection into one of the semicircular canals of the vestibular labyrinth. In both cases, surgical access to the inner ear is necessary.

ciated primary tissue and cochlear explants and were also characterized *in vivo* in rodent models. Due to space limitations, we will primarily focus on LV and AAV vectors.

In contrast to AdV and AAV vector platforms, LV vectors stably anchor their genomic information into the host cell's genome. While this feature is of great advantage when targeting dividing cells – guaranteeing stable, long-term gene addition and transmission to daughter cells – non-integrating vectors have a superior safety profile. Many of the specialized and treatment relevant otic cell types, such as hair cells (HC) and spiral ganglion neurons (SGN), are post-mitotic and thus compatible with non-integrating vector systems. Nevertheless, although naturally integration-competent, LV vectors can be rendered integration-deficient, e. g. upon catalytic inactivation of the viral integrase enzyme, creating so-called non-integrating LV vectors. Although so far only the integrating LV vectors have been tested in the context of otic gene therapy settings.

Generally, LV vector systems harbor several inherent advantages. Their coding capacity is extraordinarily high, with up to and beyond 10 kb of space for transgene incorporation. With that, also large genes, such as otoferlin, can be easily transferred using standard vector designs. Furthermore, LV particles are quite versatile in terms of pseudotyping accepting the envelope glycoproteins from several related and non-related viruses. Another advantage is that LV particles can be concentrated by several hundredfold upon ultracentrifugation to yield high-titer preparations, which is important for preparation of vector particles for clinical application.

As a prelude to future application in gene therapy settings, LV vectors designed to deliver marker genes (e. g. EGFP) were shown to be safe upon *in vivo* injection into normal hearing rodents. Independent studies reported no spread to the brain [14] or dissemination to other organs [15]. Moreover, cochlear morphology was normal and intact, and the number of HC was unaltered upon LV vector injection [16, 17]. Functional testing using auditory brainstem response (ABR) measurements revealed no negative impact on auditory functions [16, 17]. While differences in auditory functions pre- versus post-injection were observed in one study, this effect was equally present in the control group injected with artificial endolymph, suggesting that the observed effects on hearing were caused by the delivery strategy rather than features of the LV vector [18]. However, *in utero* delivery resulted in a mild hearing loss specifically for the LV vector-injected group [19]. Thus, further characterization of the effects of cochlear LV vector injection is required for future studies. In terms of potential immunogenicity, only a very mild immune response was observed upon round window membrane (RWM) injection of a LV EGFP reporter vector into P11–13 mice [15], and cochlear sections presented few inflammatory cells [15] or were even free of inflammation [20].

Beyond safety, efficiency is a crucial parameter that determines the success of a gene therapy setting. Upon *in vivo* injection of LV vector in rodents, marker gene expression revealed gene transfer to supporting cells [16] and structural lining cells of the perilymphatic space [15, 20], to marginal cells of the *stria vascularis* and intermediate cells [17, 18, 21], and to the basement membrane [21]. Transduction of these cell types allows treatment of genetic hearing disorders in which particularly these cells are affected, or – alternatively – the secretion of gene products into the lymphatic space. While LV transduction of HC and SGN was observed in few reports [16, 18], a general lack of sufficient marker gene expression in these cell types was revealed in most previous studies [15, 17, 20, 21]. In particular, LV vector-mediated gene transfer to and expression in HC and SGN needs to be further optimized as these cells represent central cell types of the sensory epithelium, and PV in genes specifically relevant for their function are causative for many forms of SNHL. LV transduction of primary cochlear explants led to modest gene transfer of 1–3 % and approximately 4 % in HC [20, 22]. As LV vector administration to the cochlea has thus far been accomplished through cochleostomy or injection into the perilymphatic space through the RWM, the exploitation of alternative delivery routes might be of interest.

In the future, once this promising system has been optimized, it will be interesting to make the move beyond marker gene expression in hearing rodents to the expression of therapeutic genes in models of hereditary hearing loss. The LV platform fulfills important safety criteria for future clinical translation as preclinical studies demonstrated the absence of inflammation and immunogenicity as well as no auditory dysfunction upon LV vector injection.

With three marketing approvals [23] and 170 human clinical trials (clinicaltrials.gov), AAV vectors are currently considered as the delivery system of choice for *in vivo* gene therapy. In contrast to LV, AAV vectors do not possess an integrase activity and are therefore primarily maintained as episomes or concatemers in the cell nucleus. Efficient and long-term (maybe even life-long) transgene expression is achieved in post-mitotic cells or tissues with a slow turnover rate. The viruses from which AAV vectors are derived are non-pathogenic and the viruses and vectors exhibit low immunogenicity. Besides vectors derived from naturally occurring serotypes, which differ in tropism and epitopes recognized by the immune system, AAV vectors

with engineered capsids have been developed and represent highly potent assets to the AAV vector toolbox. So far, AAV vectors demonstrated an excellent safety profile, even when applied in high doses such as $2x10^{14}$ per kg body weight in spinal muscular atrophy (SMA) patients.

AAV vectors are already established tools in gene therapy of eye diseases. Different cell types and/or delivery routes have been explored and vectors were tailored to serve respective needs by capsid engineering. Also, the challenge of transgenes exceeding the packaging capacity was addressed, e. g. by the split vector strategy or the more recently described intein technology [24]. HC (outer as well as inner HC), supporting cells (SC) and/or SGN represent potential therapeutic targets. To reach these cells, several delivery routes have been investigated with vector application via the RWM as the preferred strategy. The serotype(s) best suited for delivering gene therapy to the inner ear remain(s) to be determined as current studies have explored different AAV serotypes, as well as various delivery routes, doses and experimental kinetics. However, inner ear cell types can, in principle, be transduced by AAV vectors, although outer HC and SC seem to be more refractory compared to the inner HC and SGN for the majority of the tested serotypes [19, 25–28].

To improve efficiency and/or specificity of cell transduction, capsid engineering was explored based on the impressive results obtained for other cell types and tissues in the past [29]. First, Landegger and colleagues reported improved transduction of HC, including outer HC, with a "synthetic" AAV termed Anc85L60 [30, 31]. The capsid of this particular AAV is an *in silico* lineage reconstruction of a hypothetical ancestor of AAV serotypes 1, 2, 6, 8 and 9 [30–32]. This AAV vector was discussed widely as its synthetic capsid is antigenically different from the "descendant" capsids, which allows evasion from potentially preexisting antibodies in the human population and is, therefore, likely to be applicable to patients seropositive for naturally occurring serotypes. Next, exosome-associated AAV1 (exoAAV) was described to outperform conventional AAV1 vectors in transducing HC, including outer HC, *in vitro* and *in vivo* [33]. ExoAAV are isolated from the supernatant of the producer cells (*e. g.* HEK-293T), which are used to generate conventional AAV vectors, and are associated with extracellular vesicles [34]. In contrast to conventional AAV, exoAAV escape from recognition of anti-AAV antibodies [35] and show – in addition to improved transduction of HC – improved transduction efficiencies for other refractory cells, such as neurons and astrocytes. ExoAAVs can be produced for different serotypes (*e. g.* AAV1, 2, 8 and 9) with acquisition of new features, such as crossing the blood-brain barrier or a more widespread distribution after local injection in the brain. More recently, a capsid variant selected by a directed evolution approach designed to target photoreceptor cells, AAV7m8 [36], was tested for its ability to transduce inner ear cells *in vivo* and showed an extraordinary ability to transduce both outer HC and SC without any negative impact on the ABR measurement. Equipping AAV vectors with a cell-penetrating-like peptide also showed promise as it conferred AAV-DJ, an AAV shuffled capsid, with the ability to cross the mesothelial cell layer. The most recent addition to the AAV toolbox for inner ear gene therapy is the capsid variant ieAAV [37]. This variant outperformed Anc80L65 in all inner ear cell types, including SC. The great potential of this vector was demonstrated by inducing trans-differentiation of SC into an immature stage of HC via overexpression of Atoh1 in SC. These examples clearly show that a plethora of technologies are available to adapt AAV vectors for efficient transduction of all cell types relevant for inner ear gene therapy. In addition to tailoring the vector, changing the delivery strategy might be a promising way to advance this concept as exemplified by the recent report of Yoshimura *et al.*, who combined the conventional RWM injection with a semicircular fenestration technique [38] (Figure 2).

Gene editing technologies for correction of hearing loss

Progress in genome editing technologies is bringing the possibility of precision medicine for genetic diseases, such as some types of hearing loss, closer to clinical reality. The most commonly used designer nucleases include zinc-finger nucleases (ZFN), transcription activator-like effector nucleases (TALEN) and the clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 (CRISPR-associated protein 9) systems. These genome engineering methods each employ enzymes to cleave specific DNA sequences, but via different mechanisms of functional genome targeting. While ZFN- and TALEN-approaches incorporate protein-DNA interactions to target genes, the CRISPR-Cas9 method utilizes an RNA guide sequence to direct DNA sequence editing. Thus, the CRISPR-Cas9 system has become the editing tool of choice due to the relative simplicity of designing guide RNA sequences tailored to invoke efficient targeted editing [39]. In addition to the guide RNA and Cas9 nuclease, a DNA sequence called the protospacer-adjacent motif (PAM), located 2–6 nucleotides from the targeted DNA sequence, is required

for the DNA scission activity of Cas nucleases, with a distinct PAM sequence for each type of Cas nuclease.

While these nuclease-driven genome editing techniques have great promise for correction of genomic aberrations in clinical settings, the mechanism of DNA editing with induction of double-strand DNA breaks that can be repaired by the cellular machinery via two processes, namely non-homologous end joining (NHEJ) and homology directed repair (HDR), creates a potential risk of adverse events such as unwanted DNA recombination. To overcome this limitation, a modified version of the Cas9 protein that only cleaves a single DNA strand (i. e. a nickase) was recently generated and fused to a reverse transcriptase [40]. A prime editing guide RNA directs the modified Cas9 to the precise DNA target site to be cleaved and also encodes the genomic edit to be introduced. It is predicted that this technique could allow correction of almost 90 % of genetic variants currently known in human diseases [40], including point mutations known to be associated with hearing loss.

Allele-specific gene editing via CRISPR-Cas9 was recently shown to successfully prevent hearing loss by disrupting the mutated transmembrane channel-like gene family (*Tmc1*) allele in *Beethoven* mice, which is a model for DFNA36. One study delivered CRISPR-Cas9 by injecting Cas9-guide RNA-lipid complexes into the inner ear of neonatal mice and demonstrated that *in vivo* disruption of the mutant *Tmc1* allele resulted in reduced progressive hearing loss [41]. A second study demonstrated the feasibility of using an AAV vector to deliver a PAM variant of *Staphylococcus aureus* Cas9 (SaCas9-KKH) to selectively disrupt mutated *Tmc1*, while leaving the wild-type allele intact and thus preventing dominant progressive hearing loss [42]. In addition to single genetic edits, CRISPR-Cas9 technology was shown to efficiently and simultaneously disrupt multiple genes associated with hearing loss. The authors used a recently described third generation base editing (BE3) strategy that employs an engineered Cas9 fusion protein (APOBEC1-XTEN-dCas9 (A840H)-UGI) and single guide RNAs to simultaneously disrupt *vGlut3* (*Slc17a8*), otoferlin (*Otof*) and prestin (*Slc26a5*) to generate triple homozygous F0 mouse mutants [43].

Important points that remain to be addressed include (specific) delivery of genome editing molecules to the proper target cell population (i. e. those in which the PV needs to be corrected) at an efficiency high enough to elicit the desired genome editing, while also ensuring lack of unintentional side effects, such as off-target activities that may disrupt other genes.

Ongoing pre-clinical and clinical studies

Hearing loss is developing as a major target for gene therapy since the successful commercial launch of a gene therapy drug for blindness (targeting RPE95) by Spark Therapeutics. Over 150 genes that cause congenital and progressive hearing loss have been identified and, like the eye, the inner ear is an isolated organ that can potentially be locally targeted [1, 44]. This avoids the potential side effects that could be associated with systemic delivery. At present, there has been one human inner ear gene therapy trial (*NCT*02132130). This trial focused on the delivery of the transcription factor Atoh1 to patients with severe to profound hearing loss with the goal of regenerating auditory HC by forcing trans-differentiation of SC into HC [45], the feasibility of which was recently shown in mice as described above [37]. Data collection for this trial is currently ongoing. This trial utilized a replication-deficient AdV vector based on serotype 5 with the rationale that only brief periods of gene expression would be required [46]. Since then, there has been an explosion of publications showing rescue of hearing loss caused by monogenic PV in mouse models that have refocused the field on the treatment of genetic disorders [41, 47–54].

However, there are several major hurdles for translation of animal studies to treatment of human hearing loss. Human hearing matures towards the end of the second trimester, whereas mouse hearing matures at postnatal day 12. Therefore, PV that manifest in the neonatal mouse would represent a human *in utero* onset. Treatment of these disorders would, therefore, require delivery of vector during pregnancy, which is presently impossible due to ethical concerns. Recent animal studies have, however, demonstrated the feasibility of *in utero* gene transfer for hearing loss [55]. There are also differences in transfection/transduction observed in neonatal animals compared to adult animals.

A major current focus of research is the development of optimized factors for use in the inner ear that can be applied to adult animals and that work in several model systems. A novel AAV9.PHP.B vector was compared to Anc80L65 and delivered via the RWM or injected into the utricle in neonatal animals. Analysis of the transduction efficiency clearly showed advantages over earlier generations of engineered vectors, but complete transduction of outer HC was not achieved in older animals [56]. Thus, an optimized vector that achieves transduction of the entire population of adult HC still needs to be developed. There has been an intense focus on delivery to HC due to the complexity of this cell type. PV in components of the mechanotransduction machinery make up many common

causes of genetic hearing loss and have both dominant and recessive forms (reviewed in [57]). Recent studies have shown successful rescue of these PV (*Clrn1*, *Tmc1*) using AAV vector-based strategies [47, 58]. One important concept to emerge from these studies is that rescue of the gene defect has to occur prior to onset of hearing loss, since this is often accompanied by irreversible degeneration of the target tissue. Logistically, this would mean identifying and treating patients prior to the onset of significant hearing loss. For recessive disorders, where there is not a significant family history to trigger a search for potential genetic causes of hearing loss, a delay in identification and treatment may ensue. The most common *GJB2* PV (35delG) results in congenital hearing loss, but there is significant phenotypic variation with different PV in this gene, including congenital hearing loss as well as late onset postlingual hearing loss [59, 60]. In these cases, the endocochlear potential and cochlear homeostasis are disturbed, so significant populations could be targeted with gene therapy to restore endocochlear potential and, thus, preserve cellular structures [59, 61, 62]. This would require the development of vectors and regulatory sequences to ensure that the therapeutic vector is also delivered to the normal nonsensory cells to achieve adequate distribution of the connexin 26 gene product [63].

Many of the potential recessive targets in the inner ear are too large (>4.5 kb) for delivery by an AAV vector system. A dual AAV strategy, in which the transferred gene is split into two separate vectors that recombine in the target cell [64], was developed and applied to target otoferlin, PV of which cause auditory neuropathy [65]. In this disorder, there is impaired release of neurotransmitter from HC. Rescue of a mouse model using a dual AAV strategy was demonstrated and a human clinical trial using this approach is in the planning stages [66]. Use of triple AAV delivery for very large genes has been attempted, but is currently too inefficient for routine use. There is, therefore, a clear need to develop larger capacity vector systems, such as LV vector systems.

Dominant genes causing sensorineural hearing loss will need to be addressed via RNA inhibition or gene editing approaches. Both of these approaches have been successfully demonstrated in mouse models. The advent of more sophisticated single base editing approaches will significantly extend our capacity to address a broad range of genetic causes of hearing loss [67]. The identification of these patients can be aided by novel artificial intelligence-based analysis of audiograms [68–70].

Outlook

Critical factors that must be considered to advance gene therapy application in hearing loss patients include identification of gene targets most likely to result in improved hearing levels upon their correction, determination of optimal time to apply gene therapy and establishment of suitable patient cohorts. Furthermore, regenerative approaches, such as those involving induced pluripotent stem cells and/or otic progenitors, may one day allow repopulation of the cells required for proper function of the sensory epithelium of the inner ear. To achieve the best clinical outcome, it is important to deliver corrective genes prior to onset of hearing loss. Furthermore, clinical gene therapy structures will need to be established with special consideration of efficacy, safety and delivery to the patient populations.

Conclusion for the clinical practice

- Hearing loss is an attractive model for gene therapy approaches as multiple causative monogenetic defects have been identified.
- The inner ear can be locally targeted with established surgical approaches.
- Gene replacement and gene correction, e. g. gene editing strategies, can be used to treat hearing loss due to recessive and dominant gene variants.
- Gene delivery tools, including LV, AdV and AAV vectors, have demonstrated remarkable proof-of-concept for other monogenetic diseases.
- Systemic effects can be avoided as vectors can be applied directly to the target cells in the inner ear. Low vector amounts should be sufficient as the inner ear is a small, defined target.
- First patients with monogenetic hematopoietic diseases were treated with gene editing using designer nucleases in Europe and the US, which has created a clinical infrastructure that could be adapted to inner ear gene therapy.
- Gene therapy for inner ear diseases is expected to create new therapeutic options for genetic forms of hearing loss.
- The first inner ear gene therapy trial was very recently completed.

Infobox – More information regarding the topic:
http://www.hereditaryhearingloss.org
https://www.isiet.org/

https://www.cdc.gov/ncbddd/hearingloss/genetics.html
https://www.actiononhearingloss.org.uk/hearing-health/hearing-loss-and-deafness/types-and-causes/genetic-hearing-loss-and-deafness/
https://www.hear-it.org/Genetic-hearing-loss

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Michael Morgan
Institute of Experimental Hematology, Hannover Medical School, Hannover, Germany
REBIRTH Research Center for Translational Regenerative Medicine, Hannover Medical School, Hannover, Germany

Juliane W. Schott
Institute of Experimental Hematology, Hannover Medical School, Hannover, Germany
REBIRTH Research Center for Translational Regenerative Medicine, Hannover Medical School, Hannover, Germany

Axel Rossi
Institute of Experimental Hematology, Hannover Medical School, Hannover, Germany
REBIRTH Research Center for Translational Regenerative Medicine, Hannover Medical School, Hannover, Germany

Christian Landgraf
Department of Human Genetics, Hannover Medical School, Hannover, Germany

Athanasia Warnecke
Department of Otolaryngology, Hannover Medical School, Hannover, Germany
Hearing4all Cluster of Excellence, Hannover Medical School, Hannover, Germany

Hinrich Staecker
Department of Otolaryngology Head and Neck Surgery, University of Kansas School of Medicine, Kansas City, USA

Anke Lesinski-Schiedat
Department of Otolaryngology, Hannover Medical School, Hannover, Germany
Hearing4all Cluster of Excellence, Hannover Medical School, Hannover, Germany

Brigitte Schlegelberger
Department of Human Genetics, Hannover Medical School, Hannover, Germany

Hildegard Büning
Institute of Experimental Hematology, Hannover Medical School, Hannover, Germany
REBIRTH Research Center for Translational Regenerative Medicine, Hannover Medical School, Hannover, Germany
German Center for Infection Research (DZIF), partner site Hannover-Braunschweig, Braunschweig, Germany

Bernd Auber
Department of Human Genetics, Hannover Medical School, Hannover, Germany

Prof. Dr. med. Axel Schambach, PhD
Institute of Experimental Hematology, Hannover Medical School, Carl-Neuberg-Str.1, D-30625 Hannover, Germany
REBIRTH Research Center for Translational Regenerative Medicine, Hannover Medical School, Hannover, Germany
Division of Hematology/Oncology, Boston Children's Hospital, Harvard Medical School, Boston, MA 02115, USA
schambach.axel@mh-hannover.de