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## Deafness: from genetic architecture to gene therapy

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### Abstract

Progress in deciphering the genetic architecture of human sensorineural hearing impairment (SNHI), also commonly referred to as sensorineural hearing loss (SNHL) and multidisciplinary studies of mouse models have led to the elucidation of the molecular mechanisms underlying auditory system function, primarily in the cochlea, the mammalian hearing organ. These studies have provided unparalleled insights into the pathophysiological processes involved in SNHI, paving the way for the development of inner ear gene therapy based on gene replacement, gene augmentation or gene editing. The application of these approaches in preclinical studies over the past decade has highlighted key translational opportunities and challenges for achieving effective, safe and sustained inner ear gene therapy to prevent or cure monogenic forms of SNHI and associated balance disorders.

### [H1] Introduction

The tremendous improvements in interindividual communication based on the sense of hearing during the course of *Homo sapiens* evolution have culminated in the development of language and music. Hearing has, thus, become the primary sense for human communication, with its potential correlate of enhanced sociability. Sensorineural [hearing impairment](#) (SNHI) results from defective sound processing in the auditory sensory system, which can occur anywhere from the cochlea (FIG. 1) to the primary auditory cortex and associated auditory cortices. The causal defects can

occur at any time, from early development *in utero* through to middle age and beyond in forms known as age-related hearing loss (ARHL) or presbycusis.

SNHI is the most frequent sensorineural disability and a major public health problem in terms of both impact and frequency. A permanent bilateral elevation of the hearing threshold >40 decibels hearing level (dB HL) in the better ear (in individuals aged  $\geq 15$  years), is usually considered to constitute a disabling hearing impairment<sup>1</sup>. Hearing impairment of this magnitude affects approximately 1.33 in every 1,000 neonates in developed countries, as demonstrated by neonatal hearing screening<sup>2</sup>. Profound congenital or prelingual SNHI (hearing threshold  $\geq 90$  dB HL) impedes spontaneous oral language development and learning acquisitions; school difficulties also affect children with moderate-to-severe SNHI. ARHL, which may appear as early as the age of 40 years and initially manifests as an impairment of hearing in noisy environments, is by far the most prevalent form of SNHI; its frequency increases exponentially with age, affecting 15.4% of people aged in their 60s<sup>3</sup>. It disrupts social links and can cause depression, loss of autonomy and cognitive decline<sup>4</sup>. Hearing impairment beginning during and after middle age is now recognized as the major modifiable risk factor for dementia<sup>5-7</sup>.

SNHI can be caused by genetic and environmental factors, and ageing. Environmental risk factors include prenatal so-called TORCH (toxoplasmosis, rubella, cytomegalovirus (CMV) and herpes simplex virus) infections and some drugs (for example, aminoglycoside antibiotics, platinum chemotherapy agents and opioids). Smoking and comorbidities such as diabetes mellitus and hypertension are also major risk factors for ARHL. However, overexposure to loud sound in occupational or recreational settings is the most harmful environmental factor<sup>8</sup>, with over 1 billion people between the ages of 12 and 35 years at risk of permanent hearing impairment due to unsafe listening practices<sup>3</sup>.

Over the past three decades, efforts to decipher the genetic architecture of human SNHI have elucidated the molecular mechanisms underlying the development and function of the auditory system, leading to the identification of hundreds of monogenic forms of SNHI<sup>9</sup>. In particular the cochlea had eluded all previous attempts at molecular characterization, owing to the paucity of its cell types rendering biochemical and classic molecular genetic approaches largely ineffective. The use of mouse models of hereditary deafness has proved essential for deciphering the functional role of various deafness genes<sup>10</sup>, with the vast majority of models

reproducing the characteristics of the hearing defect caused by orthologous deafness genes in humans. In-depth studies of these models with multidisciplinary approaches have uncovered the molecular structures and mechanisms underlying the key functions of the cochlea.

SNHI is currently an incurable disorder. Prostheses such as [hearing aids](#) and [cochlear implants](#) are the only corrective options available for mild-to-severe and profound hearing impairment, respectively. However, speech perception with these prostheses remains poor in noisy environments — the prevailing condition in everyday life — and cochlear implants also have a low performance for music perception. Moreover, hearing aids, which work principally as sound amplifiers, fail to provide a satisfactory understanding of speech even in quiet environments for some deafness forms<sup>11</sup>. These limitations have driven the search for preventive and curative approaches to SNHI. Advances in our understanding of the pathophysiological mechanisms underlying monogenic forms of deafness, mostly based on mouse models, have naturally fostered research aiming to treat their root causes by gene therapy. This field is currently booming, spurring hopes for clinical trials in the near future.

In this Review, we first discuss studies that have deciphered the genetic architecture of human SNHI, including monogenic and polygenic forms of deafness, and review resulting insights into the molecular physiology of the cochlea in health and disease. We then focus on recent advances in the development of inner ear gene therapy for monogenic deafness forms, discussing approaches based on gene replacement/augmentation and genome editing strategies. We also emphasize the specific challenges and unresolved issues that must be addressed for inner ear gene therapy to meet high-quality standards for efficacy and safety worldwide.

### **[H1] Genetic architecture of human SNHI**

Over 50% of cases of congenital hearing impairment in developed countries are hereditary<sup>2</sup>. Isolated (non-syndromic) hearing impairment accounts for about 70% of these hereditary cases. These deafness forms are almost exclusively [monogenic](#). Congenital (or prelingual) severe-to-profound deafness displays mostly autosomal recessive inheritance (DFNB forms). By contrast, forms with an onset from late childhood onwards are generally progressive and less severe and display mostly autosomal dominant inheritance (DFNA forms).

## **[H2] Monogenic forms of deafness**

The first steps towards identifying the causal genes for isolated deafness were taken in the early to mid-1990s. At the time, only a few families with SNHI had been reported. Genetic linkage analysis to define the shortest genomic localization interval of a SNHI gene, a prerequisite for identifying the causal gene at the time, was often compromised. Indeed, the transmission of several deafness genes within individual affected families in developed countries was not unlikely, owing to the high frequency of unions between young deaf people usually attending the same specialized schools as well as between normal-hearing children of deaf parents. Together with the absence of reliable clinical criteria for discriminating between the various forms of isolated deafness, this impeded intrafamilial segregation analyses of causal deafness genes.

Profound hearing impairment is exclusively sensorineural, whereas less severe hearing impairment may be either sensorineural or **conductive**. There was evidence to suggest that inherited profound congenital, prelingual forms of SNHI were mostly DFNB forms, hence the rationale for concentrating genetic studies on profoundly deaf families living in countries with high consanguinity rates. Moreover, the likelihood of several deafness genes segregating within the same family could be minimized by focusing on consanguineous deaf families living in geographic isolation, as such communities are generally founded by only a few individuals. This strategy led to the identification of the first DFNB locus, *DFNB1*, in individuals from northern Tunisia<sup>12</sup>, using the then recently completed short-tandem repeat linkage map of the human genome<sup>13</sup> and **homozygosity mapping**. The *DFNB1* locus comprises two genes that encode protein subunits of gap junction channels: *GJB2*, which encodes connexin 26<sup>14</sup>. Harmful variants in *GJB2* lead to DFNB1, (this deafness form being named after the locus), which is by far the most frequent form of congenital severe-to-profound inherited SNHI worldwide (with the exception of some sub-Saharan African countries), with a prevalence of up to 50% of these cases in some regions<sup>15</sup>. Other DFNB forms were also identified in large consanguineous families from Indonesia (Bali)<sup>16</sup>, Pakistan<sup>17-19</sup> and the Middle East<sup>20,21</sup>.

Causal human SNHI genes were then identified via two major approaches. In the first, genes preferentially expressed in the cochlea were expected to play an essential role in this organ and were thus considered as candidate deafness genes.

They were selected from cDNA subtraction libraries and evaluated by matching with human deafness-associated loci<sup>22-25</sup>. In the second, human orthologues of mouse deafness genes located in chromosomal syntenic regions were tested as candidate genes<sup>26-29</sup>. In addition, a few human genes responsible for isolated deafness were identified by direct sequencing of a small candidate genomic region<sup>30</sup> or based on their involvement in syndromic deafness<sup>14,30,31</sup>. Deafness genes have rarely been identified on the basis of functional indications, the most notable exception being myosins, which were recognized early on to be encoded by several mouse and human deafness genes, suggesting a key role for tension forces in cochlear development and physiology<sup>26,27,32,33</sup>. By 2001, the year in which the first draft of the human genome sequence was released, 12 causal genes for DFNB forms had been identified. These genes are now known to account for about three-quarters of unrelated DFNB cases from consanguineous families around the Mediterranean Sea<sup>34</sup>. Some pathogenic variants have particularly high carrier frequencies, estimated at 1.5% worldwide and up to 3% in some countries for the 35delG variant of *GJB2*, for example<sup>15,35,36</sup>. A selective advantage for heterozygous carriers of pathogenic *GJB2* variants was suggested, specifically a role in resistance to skin and gut epithelial infections, as both keratinocytes and enterocytes express connexin 26, a role subsequently supported by clinical studies<sup>37,38</sup>.

The rapid development of next-generation sequencing (NGS) technology, and of whole-exome sequencing in particular, greatly facilitated and sped up the identification of deafness genes causing rare SNHI forms, in particular when only small families could be studied. Today, 77 DFNB-causing genes are known.

With respect to DFNA-causing genes, the *DFNA1* locus responsible for post-lingual progressive hearing loss was identified in a large kindred in Costa Rica<sup>39</sup>, and today, 52 DFNA-causing genes are known. The genes initially identified as responsible for DFNB forms are increasingly also being recognized as being responsible for DFNA forms. In addition, five chromosome X-linked genes, one chromosome Y-linked gene, five mitochondrial genes and one modifier gene of a DFNB gene have been reported. To date, a total of 130 genes causal for early-onset forms of deafness has been identified, according to the [Hereditary Hearing Loss resource](https://hereditaryhearingloss.org/) (<https://hereditaryhearingloss.org/>). About 30% of DFNB cases in non-consanguineous families remain unresolved<sup>34</sup>; this percentage is higher for DFNA cases<sup>40</sup>.

For monogenic syndromic deafness, hundreds of forms have been described, and about 300 causal genes have been identified, many of which have since also been reported to cause isolated deafness<sup>41</sup>. The causal genes for [Usher syndrome](#) (USH) warrant particular attention, as this syndrome is the most frequent monogenic multisensory disorder. Its most severe form (USH1), which combines congenital profound SNHI, balance defects and retinitis pigmentosa leading to blindness, is caused by defects of five USH1 genes. Studies of these genes provided the first insight into the key components of the cochlear sensory cells, the hair cells (HCs), which were found to be major constituents of the mechanoelectrical transduction machinery (Fig. 1)<sup>23,30,42–45</sup>.

Molecular diagnostics for early-onset monogenic forms have been integrated into everyday clinical settings in some countries. As a result, *de novo* mutations have recently been reported to contribute to a substantial fraction of SNHI cases<sup>46</sup>.

With respect to gene–environment interactions, a few monogenic forms of noise-induced hearing loss have been identified in both humans and mice<sup>47–51</sup>.

## **[H2] Polygenic forms of deafness**

Genetic factors account for roughly half the phenotypic variance of ARHL<sup>52</sup>. Following initial studies of association with candidate genes selected on the basis of their biological plausibility, [genome-wide association studies](#) (GWAS) were performed with dense [SNP](#)-based human genetic maps, assuming that ARHL is polygenic, like other common disorders<sup>53</sup>. To date, about 80 candidate ARHL-predisposing genes<sup>53–55</sup> have been reported, establishing the polygenic component of ARHL inheritance. The advent of gene therapy, which is particularly suitable for monogenic disorders, has encouraged the search for monogenic forms of ARHL. Pathogenic monoallelic variants of genes responsible for juvenile DFNA forms have been detected in 20–25% of familial and sporadic cases of ARHL characterized by onset of hearing impairment at about 50 years of age and an absence of comorbidity and environmental risk factors<sup>56</sup>. Despite its high frequency, our genetic knowledge of ARHL remains limited, particularly regarding the expected contribution of GWAS-associated variants to the elucidation of pathophysiological mechanisms<sup>57</sup> and the prediction of ARHL risk.

Genetic variants associated with noise-induced hearing loss have been identified mostly through association studies with candidate genes in humans<sup>58</sup> and



by GWAS on panels of recombinant inbred strains in mice<sup>59</sup>. One of the main findings emerging from these genetic studies is the existence of a continuum across the various forms of SNHI, based on evidence for the involvement of some causal or susceptibility genes in several monogenic and/or polygenic forms of SNHI, early and late isolated forms<sup>54,56</sup>, syndromic forms (for instance, four of the five USH1 genes are also responsible for isolated deafness), noise-induced hearing loss<sup>60</sup> or other inner ear disorders, such as Meniere's disease<sup>61</sup>. These findings suggest that these genes may be candidate hub genes for SNHI pathogenic pathways.

### ***[H2] Genetic approaches to decipher cochlear molecular physiology***

Multiprotein complexes that form key cochlear structures and act in molecular pathways essential to cochlear functions have been identified by interdisciplinary studies of mouse models of monogenic forms of human deafness and analyses of the proteins encoded by deafness genes, including their interaction protein networks. The auditory mechano-electrical transduction machinery is one of the best examples (FIG. 1f); all its known components were identified as the products of genes responsible for USH1 or isolated forms of deafness (Supplementary Table 1). Further examples are provided by the composition and hitherto unrecognized critical roles of the various sets of fibrous links interconnecting the stereocilia within the hair bundle, the sound-reception structure of the hair cells<sup>62</sup>, or attaching the stereocilia to the overlying tectorial membrane (Fig. 1d), or the molecular assemblies underlying the ultra-fast glutamate exocytosis of inner hair cells (IHCs)<sup>63,64</sup> (Fig. 1e), which are the genuine auditory sensory cells (see FIG.1 legend). Prestin, a voltage-sensitive membrane motor protein of the outer hair cells (OHCs) underlying their key function in the amplification of sound-evoked stimulation, is a notable exception<sup>65</sup>, as it was identified solely by cDNA subtraction. Finally, microRNAs are known to lead to transcript destabilization and translation inhibition. Genetic approaches identified miR-96 as responsible for a progressive post-lingual DFNA form of deafness in humans and progressive hearing loss in the diminuendo mouse mutant<sup>66,67</sup>, through hair cell defects<sup>68,69</sup>.

The cochlear cell types, subcellular compartments and structures that express deafness genes — or contain their encoded proteins and/or were recognized as targets of gene defects in mouse mutants — are compiled in Supplementary Table 1. Seven classes were defined based on the cell types or structures affected: the hair



bundles of IHCs and OHCs in class 1; the nucleus, cytoplasmic soma and cell–cell junctions of IHCs and OHCs in class 2; IHC-auditory neuron synapses and other subcellular compartments of auditory neurons and associated glial cells in class 3; hair cell-supporting cell junctions and other subcellular compartments of the supporting cells in class 4; **stria vascularis**, spindle and root cells, spiral ligament and spiral limbus (fibrocytes) and endolymphatic sac in class 5; and extracellular matrices in class 6. Class 7 contains ubiquitously distributed or unknown defects. The distribution of deafness genes between these classes reveals that deafness forms often affect a single cochlear cell type, mostly hair cells, or a single extracellular matrix, such as the tectorial membrane (FIG.1d). However, some forms affect several cell subtypes from the same type of cochlear cells, such as various supporting cells or, for example, supporting cells, stria vascularis epithelial cells and fibrocytes. About ~30% of these deafness forms are associated with vestibular defects (Supplementary Table 1) . Such forms are overrepresented in class 1, as cochlear and vestibular hair bundles share morphological and physiological characteristics.

We have defined five major groups of cochlear functions that are each defective in several deafness forms; they account for more than half of all deafness forms (67/125): *(i)* mechano-electrical transduction by the hair bundle; *(ii)* transcriptional regulation and post transcriptional modifications; *(iii)* actin cytoskeleton dynamics and associated proteins; *(iv)* ion homeostasis; and *(v)* energy and redox homeostasis, inflammation and immunity (Table 1, Supplementary Table 1). Only one category of sensorineural deafness, auditory neuropathies or auditory neuropathy spectrum disorder (ANSD)<sup>70</sup>, is currently distinguishable from other deafness forms on the basis of clinical characteristics (Table 1 for causal deafness genes and Supplementary Table 1 for the cells targeted).

In principle, all these inherited forms of deafness could benefit from inner ear gene therapy. Such therapies may be the only way to deal with genetic defects of structural proteins. For several of these inherited forms, especially those involving ion homeostasis, energy and redox homeostasis, inflammation and immunity, pharmacological approaches may be an alternative (particularly for forms with occasional acute phases). Cell therapy approaches for genetic defects leading to irreversible cochlear cell damage (in hair cells, primary auditory neurons, cochlear blood–labyrinth barrier in the stria vascularis, or spiral ligament fibrocytes) may benefit from cell regeneration using transplanted autologous mesenchymal stem

cells<sup>71</sup>, and treatment of hair cells may also benefit from *in situ* transdifferentiation of supporting cells<sup>72</sup> with mitotic regeneration<sup>73</sup>, in association with gene therapy to treat the newly generated cells.

### **[H1] Towards inner ear gene therapy**

Mouse models of various monogenic forms of human deafness have successfully provided proof of concept for positive effects of inner ear gene therapy, as reported in several recent reviews<sup>74–80</sup>. For an accurate appreciation of the potential transferability of the results of these studies to humans, we first highlight some of the features of the auditory system, focusing particularly on those that differ between mice and humans and, thus, merit particular attention.

### **[H2] Challenges posed by inner ear therapy**

The mouse and human auditory systems display major [heterochrony](#). The timing of inner ear interventions in mouse models for congenital profound deafness is therefore problematic. In humans, hearing onset occurs *in utero*, at about 4.5 months of gestation<sup>81</sup> whereas mice are born deaf and do not begin to hear until postnatal day 12 (P12). The maturation of the human cochlea is well-advanced at mid-gestation and complete at birth, with only subtle refinements of some structures occurring thereafter<sup>82</sup>. By contrast, the mouse cochlea is morphologically and functionally fully immature at birth, with most cochlear cell types being at early stages of differentiation. The mouse cochlea is considered to be mature at around P20<sup>83</sup>, even though the hair cell-primary auditory neuron synapses do not reach full maturation until about P28<sup>84</sup>. The first clinical trials of inner ear gene therapy for congenital profound deafness will probably involve postnatal interventions (as fetal interventions require the development of dedicated expertise and entail some risk). It follows that, for proof-of-concept studies to be performed at a similar stage of cochlear maturation in mouse models (that is, in the fully mature cochlea), inner ear interventions would need to be performed from approximately P20 onwards.

The period of auditory cortex plasticity determines the upper limit of the therapeutic window in congenitally deaf children. Before hearing onset, spontaneous bursts of auditory neuron electrical activity assist the assembly of the central neuronal circuits, including those underlying the [tonotopic](#) (frequency) maps in the various relay nuclei of the central auditory pathway and auditory cortex<sup>85</sup>. At hearing

onset, a period of plasticity (also known as the critical period) opens, driven by the acoustic environment; this plasticity is particularly marked in the auditory cortex, which displays representational maps of the various features of sound<sup>86</sup>. During this period, the neuronal microcircuits adapt to environmental sounds through sequential windows of plasticity, each tuned to different sound features, beginning with a robust effect on the organization of cortical tonotopic maps<sup>87,88</sup>. Hearing rehabilitation with cochlear implants in children affected by congenital profound deafness is highly instructive: the development of hearing in children fitted with cochlear implants before the age of 7 years allows spontaneous oral language acquisition, with higher quality of hearing perception and language skills reported for children receiving an implant before the age of 3 years<sup>89,90</sup>. These findings suggest that the auditory critical period begins to close at about this age. Cochlear implantation in congenitally profoundly deaf children performed after 7 years results in poorer auditory perception and speech skills. These conclusions would apply to gene therapy approaches. In mice, the period of auditory cortex plasticity for tonotopic maps extends around hearing onset from P11 to P14<sup>88</sup>, suggesting that, even if cochlear function is fully restored (as assessed by *in vivo* electrical cochlear responses), for interventions beyond P14 auditory perception may not reach the level of performance observed in wild-type mice.

Studies of animal models of later-onset deafness forms (which, like affected humans, initially hear normally and become progressively deaf) have suggested that, after normal development, the auditory cortex becomes disorganized, with a reorganization of the established tonotopic maps and a decrease in the temporal processing acuity of sound<sup>91</sup>. The restoration of cochlear neuron output signals may partly fade these cortical changes<sup>88</sup>. Evidence from cochlear implantations in adults who become profoundly deaf also indicates that some cortical plasticity (albeit less than during the critical period) persists in adulthood<sup>92</sup>. At first glance, proof-of-concept studies for positive effects of gene therapy in the corresponding mouse models might thus be easier to transfer to humans. However, additional factors may come into play in both species, such as genomic variants affecting cochlear ageing or susceptibility to environmental factors such as noise.

Finally, contrary to a widespread misconception, the inner ear is not an isolated organ. In particular, observations that recombinant adeno-associated virus (rAAV) injected into the perilymphatic compartment guinea pig cochlea was

detectable in the contralateral cochlea<sup>93</sup> have long raised questions about the safety of inner ear gene therapy. Indeed, therapeutic agents injected into the cochlea can diffuse out of the sensory organ into the cerebrospinal fluid via the cochlear aqueduct, with a potential risk of these agents entering the brain parenchyma. In humans, the patency (permeability) of the cochlear aqueduct is generally more limited and decreases with age<sup>94</sup>. This consideration should guide the choice of delivery routes for inner ear gene therapy and the design of gene therapy agents (see below). Similarly, contrary to the immune privilege initially thought to prevail in the cochlea, the presence of resident or colonizing macrophages and their roles in cochlear tissue maintenance and injury responses are now well established<sup>95</sup>.

### ***[H2] A decade of preclinical gene therapy studies***

We adopt here the US Food and Drug Administration (FDA) definition of gene therapy as “a technique that modifies a person’s genes to treat or cure disease”<sup>96</sup>, thus excluding transcriptional and post-transcriptional modifications. We, therefore, focus here on proof-of-concept studies demonstrating positive effects on hearing in mouse models of human deafness obtained by gene replacement, augmentation or editing (Fig. 2). The outcomes of RNA-targeting approaches are briefly mentioned, as they may contribute to the interpretation of gene therapy results.

The selection of gene therapy methods for early- or late-onset forms of SNHI is guided by the nature of the causal variants and their functional impact. Most known causal variants are involved in early-onset forms of SNHI. About 82% of them are single-nucleotide variants (SNVs), the others being deletions (~10%), insertions (~5%) or duplications (~3%)<sup>79</sup>. Variants of genomic regulatory sequences affecting the expression of causal deafness genes and variable numbers of tandem repeats have only very rarely been reported, but molecular diagnosis, increasingly including whole-genome sequencing, should ensure they no longer escape detection<sup>69</sup>. Pathogenic variants may be loss-of-function, whereby they disrupt the production or activity of the gene product, gain-of-function, whereby they result in an altered gene product with a new molecular function, a new pattern of expression, or dominant-negative variants. Loss-of-function variants can be compensated by gene replacement or corrected by gene editing. Alleles carrying gain-of-function or dominant-negative variants can be inactivated or corrected by gene editing, or their effects countered by increasing wild-type protein production through gene

augmentation to interfere with the protein encoded by the mutant allele. Gene editing and replacement may need to be combined if the gene with the allele to be inactivated is a haploinsufficient gene; this sensitivity to gene dosage remains incompletely documented for many deafness genes.

Forty-one proof-of-concept studies for positive effects of gene therapy approaches in deaf mouse mutants (41 of 46 reported studies; interventions using the same gene therapy agent at different time points in the same mouse model being counted as a single proof-of-concept) have already been obtained (Table 2 and Supplementary Table 2). These proofs of concept concern 32 mouse models, 29 for DFNB forms and 3 for DFNA forms, involving 21 deafness genes in total.

### ***[H2] Gene replacement and gene augmentation***

Gene replacement and augmentation involve the transfer of a functional, wild-type version of the coding cDNA sequence derived from the causal SNHI gene (or, occasionally, a [paralogous gene](#)) into target cells. The cDNA can be integrated into the genome at a specific site or may persist in a non-integrated form. Viral and non-viral methods for therapeutic agent delivery to the inner ear are discussed in BOX 1. In all but one of the published proof-of-concept studies for gene replacement/augmentation for inherited forms of SNHI<sup>97</sup>, the therapeutic cDNA was delivered with an AAV vector, resulting in its maintenance as a stable [episome](#) within transduced cells (FIG. 2Aa). Given the diversity of cochlear cell types directly affected in the various deafness forms (Supplementary Table 1), AAV capsids with various cell tropisms are required; this has led to the development of AAV capsid engineering (BOX 1). One disadvantage of AAV vectors is their low packaging capacity, with cargos limited to 4.8 kb, but the longer sequences of many deafness genes can be accommodated with dual-AAV strategies (FIG. 2Ab and BOX 1). The therapeutic cDNAs with a poly A tail were either restricted to the coding sequence or also included the 5' untranslated region (UTR) and/or 3'-UTR sequences<sup>98</sup> (Supplementary Table 2). The promoters used drove ubiquitous expression of therapeutic cDNA and were therefore expected to yield high, non-regulated levels of therapeutic agent expression in transduced cells, with possible toxicity. Other concerns include the presence of pre-existing antibodies and T-cell immunity to AAVs, and the innate immune response to AAVs, which may limit the outcomes of AAV-mediated gene therapy<sup>99</sup> (see also BOX 1). The delivery routes for therapeutic

agents affect the efficiency of cochlear cell transduction, the tonotopic distribution (FIG. 1) of the effects and the safety of the intervention with results that may differ between animal species and cochlear development/maturation stages<sup>100</sup>. Several routes for injecting therapeutic agents directly into the mouse inner ear have been developed (see below and FIG. 3).

The 34 positive proof-of-concept studies, based on gene replacement (31 studies) and gene augmentation (3 studies), concerned 18 deafness genes, and the vast majority addressed class 1 and 3 cell defects (29/34), with the others dealing with class 2 and 5 defects (Supplementary Table 1). Most of these studies involved injections through the round window membrane (RWM) (FIG. 1c,d and FIG. 3); cochleostomy has given highly variable results<sup>101,102</sup>. Two routes of injection into the vestibule — **canalostomy** and direct injection into the utricle — were recently reported to increase cochlear transduction efficiency<sup>103,104</sup> (see FIG. 3 and the legend for the methods proposed in humans). All these proofs of concept were obtained in mice carrying recessive causal variants modelling human DFNB forms. All were due to loss of gene function variants, with the exception of the *Tmc1*<sup>Bth/Bth</sup> mouse mutant, which carries the Beethoven (Bth) mutation of *Tmc1*, a semi-dominant gain-of-function variant (Table 2). *Tmc1* encodes the transmembrane channel-like 1 (TMC1) protein, a component of the mechano-electrical transduction channel pore of both IHCs and OHCs<sup>105–107</sup>. All mouse models were congenitally deaf except the *TgAC1;Cln1*<sup>-/-</sup> mutant, a model for USH3A, which displays delayed-onset, progressive hearing loss<sup>98</sup>.

One key feature emerging from these studies is that successful interventions are restricted to a short time window. For all mouse models, positive effects have been reported for inner ear interventions only when performed during the early neonatal period, mostly on P0–P2, occasionally extending to P7 (Table 2). Given the timing of these interventions, these proof-of-concept studies were therefore assessing the prevention, but not the cure, of hearing impairment (see above). However, certain cochlear defects (histological or electrophysiological abnormalities) detected before therapeutic gene expression have been shown to be alleviated or corrected in a few studies<sup>101,116,123,127,133</sup>. Interventions performed shortly before hearing onset (from P8 to P11) (4/34) were ineffective, even for gene therapy agents that showed preventive effects following early neonatal interventions in the same mouse models via the same delivery routes.

As discussed above, for proof-of-concept of a cure for SNHI to be transferable to humans, positive effects would need to be observed for interventions performed from ~P20 onwards. To date, genuine cures of a hearing impairment have been obtained in proof-of-concepts for only two mouse mutants: *Vglut3*<sup>-/-</sup> (also known as *Slc17a8*<sup>-/-</sup>) mice (the orthologous human gene causes DFNA25, with no DFNB form reported to date)<sup>102</sup>, and *Otof*<sup>-/-</sup> mice, a model for DFNB9<sup>112,113</sup>. Hearing restoration has even been reported for interventions on P30 in *Otof*<sup>-/-</sup> mice<sup>112</sup> and on P35, P56 and P140 for *Vglut3*<sup>-/-</sup> mice<sup>114</sup>. These congenitally profoundly deaf mouse mutants are the only two in which SNHI is caused by IHC synaptic vesicle protein defects preventing neurotransmitter (glutamate) release; they notably present only minor morphological abnormalities of the ribbon pre-synapse (FIG. 1e), which are partly corrected by gene therapy.

Another key issue regarding the potential clinical transfer of proof-of-concept studies is the durability of the effects obtained. Firstly, data for the long-term durability of positive effects are often lacking, as the treated mice were frequently followed for no more than a few weeks or months after the intervention. Half the proof-of-concept studies reported a decline or disappearance of the positive effects on hearing within 2 months of the intervention, although technical improvements have driven better results in recent years. In eight of the 34 proof-of-concept studies involving gene replacement/augmentation, positive effects on hearing persisted for at least 4 months, albeit often with a progressive elevation of hearing threshold for certain sound frequencies<sup>98,99,104,107,123,127,131,132</sup>. Remarkably, in the *TgAC1;Clrn1*<sup>-/-</sup> mouse model, which presents a delayed-onset progressive DFNB form of deafness, strong and stable hearing improvement persisted at the final hearing assessment, 5 months after the neonatal intervention<sup>100</sup>.

The effect of gene replacement/augmentation on the vestibular defects associated with hearing defects (Table 1) has not been systematically reported. However, in all studies with a positive effect on hearing lasting at least 4 months that were associated with vestibular deficits (5 of 8 studies), the neonatal inner ear gene therapy interventions fully prevented the development of balance defects, with this effect declining little, if at all, right up to the last assessment (up to 15 months after the intervention in one study<sup>110</sup>). Moreover, in another proof-of-concept study, gene replacement intervention on P14 and P30 effectively restored balance despite having no positive effect on hearing<sup>118</sup>. These results highlight the contrasting effects of



inner ear gene replacement on mouse cochlear and vestibular defects. Phenotype correction is much more effective in the vestibule than in the cochlea, with a much wider therapeutic window for intervention and a longer persistence of therapeutic effects.

Finally, an important limitation of gene replacement strategies is the production of different protein isoforms from some causal deafness genes. Transfer of the isoform with the most crucial role has been shown to provide a *bona fide* solution<sup>111,119,120</sup>.

## **[H2] Gene editing**

In gene editing, the endogenous gene is modified within its native genomic context, preserving its normal spatiotemporal pattern of expression and pre-mRNA splicing<sup>121–124</sup>. Gene editing can be applied to any gene, regardless of the size of the transcription product(s), and the resulting genome modifications should be permanent.

## **[H3] CRISPR-Cas9 genome editing**

The CRISPR–Cas system, an RNA-guided adaptive immunity system present in many bacteria and archaea<sup>125</sup>, has provided a highly versatile, effective and easy-to-use gene-editing technology<sup>126,127</sup> with the potential for curing genetic diseases. Programmable DNA endonucleases such as CRISPR–Cas generate DNA double-strand breaks (DSBs), which are highly recombinogenic. The type of DSB repair mechanism — homology-directed repair (HDR) or non-homologous end-joining (NHEJ) — expressed by transduced cells determines the outcome of CRISPR–Cas activity (Fig. 2Ba). HDR<sup>128,129</sup>, which resolves DSBs with an endogenous or exogenous homologous DNA donor template (sDNA or dsDNA), is a high-fidelity repair pathway that can correct mutations or integrate large DNA fragments (gene replacement). However, the highly accurate HDR is restricted to mitotic cells, functioning principally during the S and G2 phases of the cell cycle, whereas the error-prone NHEJ operates throughout the cell cycle and is the principal DSB repair mechanism even in dividing cells. At birth, cochlear cells are post-mitotic in mice<sup>130</sup> (with the exception of the tympanic border cells, FIG. 1)<sup>131</sup> and humans<sup>82,132</sup>, theoretically restricting postnatal gene editing of these cells by CRISPR–Cas9 to NHEJ-mediated loss-of-function edits, which are aimed at silencing targeted alleles.

Liu and coworkers were the first to achieve proof-of-concept for CRISPR–Cas9 gene editing in the mouse cochlea<sup>133</sup> and then for editing a deafness gene in a mouse model of a progressive form of human deafness (DFNA36)<sup>134</sup>. Since then, four additional studies in which pathogenic alleles affecting hair cells (class 1 and 2 defects) were silenced using CRISPR–Cas9 gene editing have reported positive effects on hearing in mouse models of progressive DFNA forms of deafness<sup>105,108,126,128</sup>. All involved early neonatal interventions (P0–P3), thus assessing the prevention or alleviation of hearing impairment. Three studies analysed *Tmc1*<sup>Bth/+</sup> mutants<sup>134,137,138</sup>, a mouse model of DFNA36 (caused by the p.Met418Lys variant in the orthologous human gene<sup>139</sup>), in which hearing impairment begins after hearing onset. In *Tmc1*<sup>Bth/+</sup> mice, the p.Met412Lys variant is responsible for the progressive hearing-loss phenotype, which is first detected at 3–4 weeks. Progressive hearing loss results from decreased expression of *Tmc2*, a paralogue of *Tmc1*, from 1 week after birth, leading to the disappearance of its compensation of the *Tmc1* mutation. In the initial proof-of-concept study, the authors injected a ribonucleoprotein complex (RNP) complex of purified *Streptococcus pyogenes* Cas9 (SpCas9) protein and *Tmc1* single guide RNA (sgRNA) into the cochlea (FIG. 3, Table 2 and Supplementary Table 2) of P2 *Tmc1*<sup>Bth/+</sup> mice<sup>125</sup>. One month after the cochlear intervention, selective disruption of the *Bth* allele with insertion/deletions (indels) was observed, together with a slowing of both hair-cell degeneration and progression of hearing impairment, particularly for mid-range sound frequencies (8–23 kHz). The second study used a *Staphylococcus aureus* Cas9 (SaCas9) variant, SaCas9-KKH, in AAV2/Anc80L65, which transduces IHCs very efficiently and OHCs less efficiently (Table 2 and BOX 1). This approach achieved 10-fold higher levels of *Tmc1* gene editing compared with the first study<sup>125</sup> and almost completely prevented hearing impairment at low- and some mid-range frequencies (5–15 kHz)<sup>137</sup>. The third proof-of-concept used SpCas9 and AAV2/9-PHP.B, which has a higher OHC transduction efficiency than AAV2/Anc80L65, preventing hearing impairment at low- and mid-range frequencies<sup>138</sup>. The duration of the positive effects differed between the studies, decreasing strongly 2 months after intervention for the RNP-based study<sup>134</sup>, but persisting, almost unchanged, for all frequencies but the highest, up to 6 months (the last day of hearing exploration) for the two AAV-based studies<sup>137,138</sup>.

Two other proof-of-concept studies with positive effects obtained by CRISPR–Cas9 editing were performed in mice carrying either a semi-dominant mutation in the

IHC- and OHC-expressed myosin 6-encoding gene (*Myo6*<sup>C442Y/+</sup>) or a dominant mutation in the OHC-expressed Kv7.4 voltage-gated potassium channel subunit gene (*Kcnq4*<sup>W276S/+</sup>)<sup>135,136</sup>. A post-hearing onset of deafness was documented only for *Myo6*<sup>C442Y/+</sup>. The *Myo6*<sup>C442Y</sup> allele was silenced by rAAV2/9-PHP.B, encoding SaCas9 and sgRNA, whereas the *Kcnq4*<sup>W276S</sup> allele was silenced by a dual rAAV2/Anc80L65 vector encoding split-intein SpCas9 and sgRNA. However, the effect had decreased considerably at 5 months for *Myo6*<sup>C442Y/+</sup> and no longer persisted after 7 weeks for *Kcnq4*<sup>W276S/+</sup> (Table 2 and BOX 1).

Owing to the genotoxicity of the DSBs, cells have evolved multiple pathways for their repairs. Those allowing to correct defective genes in post-mitotic cells would considerably extend the therapeutic potential of CRISPR-Cas9 for post-natal gene therapy intervention for deafness, provided the DSB repair pathway is active in post-mitotic cochlear cells. These pathways are being actively explored, the only pathway well explored thus far is based on HDR but up to recent data<sup>140</sup>, was sought to be active only in S and G2 phases. As a result, the microhomology-mediated end-joining (MMEJ) (in some respects related to end-joints DNA DSB repair systems, the classical NHEJ (cNHEJ)), being active without DNA template and throughout the cell cycle<sup>141</sup> that is including in post-mitotic cells, although being prone to errors was considered as a possible alternative option. Taken into account in Delphi's prediction for gRNA proposed to have the highly efficient correction, selected gRNAs were shown to promote mutation correction and duplication suppression<sup>142</sup>. Interestingly, mutation correction by CRISPR-Cas9 gene editing through MMEJ repair has already produced encouraging results in retinal post-mitotic cells in a mouse model of inherited retinal degeneration<sup>143</sup>. Furthermore, efficient repair of frameshift mutations by NHEJ-mediated CRISPR-Cas9 acting without template but with selected guide RNAs was recently demonstrated *in vivo*, in post-mitotic hair cells<sup>144</sup>. An inner ear intervention on P2 in *Pcdh15*<sup>av3J/av3J</sup> mice carrying a biallelic single-nucleotide insertion leading to a frameshift mutation in the gene encoding PCDH15, a component of the mechano-electrical transduction machinery, was shown to restore the coding frame in about half the major edit products; the effect on hearing threshold was modest, albeit significant, but was strong on the balance defects<sup>144</sup>.

[H3] Base editing and prime editing

Two strategies expanding CRISPR–Cas9-based gene-editing technologies have recently been developed: base-editing<sup>145,146</sup> and prime-editing<sup>147</sup>. Both aim to correct pathogenic variants without generating DSBs, thereby avoiding all the uncontrolled editing outcomes. They are efficient in post-mitotic cells.

Cytosine base editors (CBEs) use a catalytically dead SpCas9 (dCas9, which lacks nuclease activity) fused to a single-stranded DNA-specific cytidine deaminase domain, acting as an editor, and a sgRNA complementary to the target genomic DNA<sup>145,146</sup> (FIG. 2Bb). The CBE converts cytosine to uracil, which base pairs like thymine. The cytidine deaminase domain has also been replaced by a laboratory-evolved single-stranded DNA adenosine deaminase, acting as an adenosine base editor (ABE)<sup>148</sup>. This enzyme converts adenosine within the editing window into inosine, which base pairs like guanine. The resulting heteroduplexes are resolved by cellular DNA mismatch repair mechanisms installing pairing based on the complementary DNA strand, resulting in the conversion of the C•G pair into a T•A pair<sup>145</sup> and of the A•T pair into a G•C pair<sup>148</sup> for CBE and ABE, respectively. Overall, 47% of pathogenic or likely pathogenic variants causing hearing impairment are transition SNVs (according to the ClinVar database) and could, therefore, potentially be corrected by base editing (79% with an ABE and 21% with a CBE)<sup>79,149</sup>. Inner-ear gene-base editing was first reported in 2017, with the aim of achieving hair cell regeneration<sup>150,151</sup>. A single base-editing approach addressing an inherited form of SNHI has been reported to date<sup>152</sup> (Table 2). The effect of the CBE was investigated in the *Baringo* mouse mutant (*Tmc1*<sup>Y182C/Y182C</sup>), which carries a biallelic loss-of-function variant of *Tmc1* (c.545A>G; p.Tyr182Cys). *Baringo* mutants have been reported to be profoundly deaf by the age of 4 weeks. Injections of the gene therapy agent on P1 had a modest effect on hearing thresholds, for all frequencies, in 4-week-old mice; this effect had almost completely disappeared within 2 weeks<sup>152</sup>.

First reported in 2019<sup>147,153</sup>, prime editing involves a Cas9 nickase (H840A) fused to a reverse transcriptase (the Moloney murine leukemia virus reverse transcriptase), an RNA-dependent DNA polymerase acting as an editor. This enzyme uses a prime-editing guide RNA (pegRNA) as a template for polymerization of a complementary edited DNA strand, which is then introduced into the target DNA (FIG. 2Bc). As in base editing, the heteroduplex is resolved by mismatch repair mechanisms. As prime editing can operate on all SNVs including transversions, it is possible to introduce any type of DNA base-pair substitution. It can also introduce

small insertions (up to 44 bp) and deletions (up to 80 bp), eliminating such indel genomic variants<sup>147</sup> (FIG. 2Bc). No proof-of-concept studies have been reported for prime editing in deaf mouse mutants, probably reflecting the need to improve the efficiency of this gene therapy approach *in vivo*<sup>154,155</sup>. However, according to the ClinVar database, 53% of all pathogenic or potentially pathogenic variants causing monogenic hearing impairment could be repaired only by prime editing (half these variants being transversion SNVs, the other half being small indels<sup>79,149</sup>).

## **[H2] RNA-targeting approaches**

A few proof-of-concept studies for positive effects on hearing have been obtained with RNA-targeting methods in mouse models of human deafness. They used antisense oligonucleotides (ASOs), **small interfering RNA** (siRNA)-related or RNA-editing approaches. One of the earliest studies in a mouse model of human congenital hereditary deafness (*Ush1c*<sup>tm1Bkts/tm1Bkts</sup>) concerned a form of USH1, USH1C, caused by a biallelic exonic cryptic 5' splice donor site variant of the harmonin gene (Table 2 and Supplementary Table 2); in this proof-of-concept, an ASO was used as a steric blocker to mask the cryptic splice site<sup>156</sup>. Interventions on P1 have been reported to improve hearing substantially, with only a slight decline after 6 months, and to abolish associated vestibular defects up to the age of 2 years<sup>157</sup>. By contrast, no effect on hearing was detected for ASO or gene augmentation interventions on P10<sup>111</sup>. These negative results, despite positive effects of these two therapeutic agents for interventions on P0–P2, suggest that the failure to achieve preventive effects is due to the occurrence of irreversible cochlear damage between P2 and P10.

A siRNA complementary to the mutated *Tmc1* transcript of the *Tmc1*<sup>Bth/+</sup> mutant<sup>158</sup> synthesized from a rAAV-expressing a precursor, a pri-miRNA scaffold (also referred to as artificial miRNA-miTmc1)<sup>158</sup>, prevented the development of hearing impairment following inner ear interventions in P0-P2 *Tmc1*<sup>Bth/+</sup> mutant mice (Table 2)<sup>158</sup>. This effect persisted for up to 8 months, but began to decline at about 5.5 months<sup>158</sup>. As seen above, a CRISPR-Cas9 gene-editing approach silencing the mutant allele of *Tmc1*<sup>Bth/+</sup> mice at the same age also prevented hearing impairment, but with a more pronounced effect on hearing that persisted unchanged for up to six months<sup>137</sup>. Interventions with artificial miRNA-miTmc1 performed shortly after hearing onset at P15/P16 resulted in only a modest effect on hearing impairment, restricted to

low frequencies, and interventions at 2 months led to no recovery or slowing of the progression of hearing loss<sup>159</sup>. Results for CRISPR–Cas9 interventions performed after hearing onset in the same mouse model should help to determine whether irreversible cochlear cell damage has already occurred by these timepoints.

Finally, several RNA-editing approaches for mRNA degradation or correction have emerged<sup>160</sup>. Among them, an RNA-guided RNA endonuclease acting with a sgRNA to cleave complementary mRNA was recently shown to partially prevent hearing loss after P1–P2 intervention in *Tmc1*<sup>Bth/+</sup> mutants<sup>161</sup>.

## **[H2] Moving from mouse to human**

Determining the genetic architecture of SNHI and advances in gene therapy technology have naturally led to gene therapy emerging as a very attractive approach for treating inherited forms of deafness. The vast majority of proof-of-concept studies have reported positive effects on hearing only for neonatal gene replacement/augmentation interventions performed no later than P7 (that is, before normal hearing onset at P12) in mouse models of human congenital forms of deafness, thereby establishing the feasibility of hearing impairment prevention, but not cure. Moreover, given the major heterochrony between the auditory systems of mice and humans, these gene therapy interventions for preventing congenital deafness in humans would need to be performed *in utero*. Remarkably, two exceptions have emerged: the curing of hearing impairment in *Otof*<sup>-/-</sup> mice<sup>112,113</sup>, a model of the DFNB9 form of human congenital deafness, and in *Vglut3*<sup>-/-</sup> mice, a model of DFNA25<sup>102,114</sup>.

These results are particularly promising and should shed light on the difficulties encountered in attempts to cure SNHI in mouse models of other human forms of congenital deafness. These difficulties might be simply explained by inner ear therapeutic interventions after P7 being highly traumatic. However, the success achieved with the same delivery routes in *Otof*<sup>-/-</sup> mice and *Vglut3*<sup>-/-</sup> makes this assumption highly unlikely. The most straightforward explanation would be the existence of irreversible damage to the cells directly affected by the gene defect before P8, with any loss of cochlear cells occurring at later time points in the mouse models of human deafness tested<sup>116,118,138</sup>. Consistent with this hypothesis, *Otof*<sup>-/-</sup> and *Vglut3*<sup>-/-</sup> mice are the only known mutants with SNHI caused by IHC synaptic vesicle defects affecting release of the neurotransmitter glutamate. Morphological

defects are also minimal in these mutants and are at least partly reversed by gene therapy. Furthermore, most mouse models of congenital forms of human deafness display abnormalities *in utero*, whereas the defective exocytosis of *Otof*<sup>-/-</sup> mice occurs postnatally<sup>162</sup>. The absence of rescue of the hearing phenotype for interventions performed after P7 in mouse models in which vestibular defects were effectively cured even by interventions on P30, is also consistent with the occurrence of irreversible cochlear damage<sup>118</sup>. However, indirect effects of damaged target cells on their neighbouring cells — which cannot be reversed by treating only the target cells — may also contribute to the failure to cure hearing impairment in other mouse models. Both *Otof*<sup>-/-</sup> and *Vglut3*<sup>-/-</sup> mice also escape this possibility to at least some extent: the partial postsynaptic recovery of primary auditory neurons suggests that the post-synapse remains sufficiently plastic to respond to the restoration of presynaptic function<sup>112,114</sup>. Another possible explanation is differences in the impact of defective IHC glutamate release (in *Otof*<sup>-/-</sup> and *Vglut3*<sup>-/-</sup> mice) and impaired mechano-electrical transduction activity (overrepresented in proof-of-concept studies to date) on subtype specification of the primary auditory neurons, occurring during the postnatal prehearing period<sup>163</sup>. Certain pathophysiological manifestations known to be stronger in the cochlea than in the vestibule, such as inflammation or oxidative stress, may be involved<sup>95</sup>. Finally, the early postnatal environment of pups may exacerbate the consequences of genetic defects in some mutant mice. Some of these causes may be of no relevance to humans: for instance, cochlear stimulation (via air or soft-tissue conduction) may aggravate some cochlear abnormalities in mice, which are highly vocal and produce very loud sounds from birth to weaning at about P20.

The maintenance of hearing improvement is another critical issue for the translation of preclinical studies into clinical trials. Long-term effects have rarely been described. Long-lasting positive effects have been reported for the cure of hearing impairment by gene replacement in *Otof*<sup>-/-</sup> and *Vglut3*<sup>-/-</sup> mice, and for the prevention (interventions on P0 to P2) of two forms of progressive hearing loss with post-hearing onset, *Tmc1*<sup>Bth/+</sup> and *TgAC1;Clrn1*<sup>-/-</sup>, through CRISPR–Cas9-mediated mutated allele silencing and gene replacement, respectively<sup>100,137,138</sup>. Plausible explanations for the decline of positive effects over time include the development of adverse effects, such as inflammatory and immune responses or toxic accumulations of the proteins encoded by some therapeutic genes (with expression driven by strong ubiquitous



promoters in gene replacement/augmentation strategies). The long-term persistence of positive effects if the initial preventive or curative effect is sufficiently extensive to counter the pervasive negative effect of untreated defective cells has been proposed as a unifying explanation. This hypothesis is supported by experimental data<sup>137</sup>; achieving full initial recovery may, therefore, be seen as a major objective.

The current difficulties in curing hearing impairment by gene therapy in mouse models of human congenital deafness highlight the need to improve our understanding of the pathophysiological mechanisms operating throughout the cochlea in the various forms of deafness and over long time periods. In parallel, given our current inability to cure these deafness forms within a therapeutic window transferable to babies or young children, we must be prepared for the probable need to associate complementary therapeutic adjustments with gene therapy to ensure the successful cure of these forms of hereditary deafness. The results currently available also highlight the need for models of human hereditary deafness in mammals with auditory systems more closely resembling those of humans in terms of both developmental timing and physiological characteristics (especially with respect to sound frequency range detection, the perception of low-frequency sound (<2 kHz) being particularly crucial for speech understanding, whereas such frequencies are not detected by mice); such models are currently scarce<sup>164,165</sup>.

By contrast, attempts to develop gene therapy for later-onset, generally progressive DFNA forms are gaining momentum<sup>56</sup>. Gene therapy for monogenic DFNA and DFNB forms with a postlingual onset of hearing impairment (in children to middle-aged adults (early age-related hearing loss)) should, indeed, have several advantages. It should benefit from the existence of a broad therapeutic time window for interventions, taking into account both the timing of the onset of cochlear defects and the time at which auditory cortical maps and phonological representations become established. The time window for interventions for such forms would limit dissemination of the gene therapy agent into the brain parenchyma, as there is a progressive occlusion of communication between the cochlear perilymph and cerebrospinal fluid in humans. Several POCs have already been obtained for positive effects in mouse models of some DFNA forms with a post-hearing onset. However, the interventions in these POC studies were performed during the early neonatal period, the effects therefore being preventive but not curative, and transfer to humans would imply in utero intervention. Future studies extending these results by

demonstrating a preventive effect of gene therapy interventions performed after hearing onset in mutant mouse models are therefore required for the development of postnatal gene therapy interventions in humans. However, the success of gene therapy in this context will depend on diagnosis being made as close as possible with respect to the first signs of hearing impairment. This will be easiest for familial cases but will undoubtedly require the development of highly sensitive clinical tests, which will also be essential for assessments of the benefits of gene therapy. Interestingly, recent results have revealed that some deafness forms hitherto classified as congenital display an overlooked progressivity; moreover, some cases escape detection in neonatal auditory screening tests, suggesting a possible postnatal onset<sup>166</sup>.

One unexpectedly robust feature emerging from proof-of-concept studies is the amenability of congenital inherited vestibular defects to gene therapy. Whatever the strategy used — gene therapy or RNA-based therapy — congenital vestibular defects associated with deafness are much more efficiently prevented than cochlear defects and can even be cured. The cochlea was derived from the vestibule during evolution, subsequently acquiring characteristics potentially accounting for this differential response to gene therapy. Notably, the vestibular hair cells can spontaneously regenerate, at least in embryonic and newborn mice<sup>167,168</sup>, whereas cochlear hair cells have lost this capacity<sup>169,170</sup>. The higher-frequency responses of cochlear cells than of vestibular hair cells, together with the specific tonotopically tuned structure and function of cochlear cells, may translate into more stringent requirements for functional restoration than those of impaired vestibular hair cells. The results obtained in mouse models, with highly effective long-term effects lasting the entire lifetime of the animal, and a wide therapeutic window for curative interventions<sup>118</sup>, suggest that gene therapy providing a permanent solution to genetic vestibular disorders in humans is within reach. Surprisingly, the impact of balance disorders on health is largely underappreciated<sup>171</sup>. Balance disorders of vestibular origin are a common clinical problem in the elderly and may compromise independence, as they increase the risk of falls and injury and are associated with considerable morbidity. Moreover, balance disorders at any age, even in the young, are markedly aggravated when associated with visual impairment, as in USH1.

## **[H1] Conclusions**

With the very promising preclinical results obtained with gene replacement approaches in a mouse model *Otof<sup>-/-</sup>* of DFNB9, we are rapidly approaching clinical trials of gene therapy for this congenital deafness form. Later onset progressive forms of deafness, whether DFNA or DFNB forms, appear to be particularly favourable for future development of gene therapy. As discussed above, other opportunities should emerge once the timing of irreversible direct or indirect cochlear damage has been determined for various inherited deafness forms and their underlying causes clarified for correcting strategies being associated. For optimizing gene therapy procedures, additional knowledge will also be required, to broaden and deepen our view of the underlying pathophysiological processes, to decipher the regulatory sequences controlling the expression of the therapeutic agents for preventing the possible deleterious effects of their ectopic or uncontrolled expression through the design of specific promoters, and to characterize the signalling pathways operating in the various types of cochlear cells to ensure their long-term survival. In this respect, the ongoing development of cochlear single cell transcriptomic atlases and inner ear organoids should be particularly instructive. Such knowledge will also greatly benefit the development of other therapeutic approaches for SNHI.

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**Table 1. Physiological defects of human monogenic forms of isolated early-onset sensorineural deafness**

Gene symbol	Protein name	Deafness form	OMIM identifier
<b>Auditory mechano-electrical transduction machinery<sup>a</sup></b>			
<i>CDHR15</i>	Cadherin-related 15	DFNB23 USH1F	605514
<i>CDHR23</i>	Cadherin-related 23	DFNB12 USH1D	602092
<i>CIB2</i>	Calcium and integrin-binding family member 2	DFNB48	605564
<i>CLRN1<sup>#</sup></i>	Clarin-1	USH3A	606397
<i>CLRN2</i>	Clarin-2	DFNB117	619174
<i>LHFPL5</i>	LHFPL tetraspan subfamily member 5 protein	DFNB66/67	609427
<i>LRTOMT</i>	Transmembrane O-methyltransferase	DFNB63	612414
<i>MYO7A<sup>*</sup></i>	Unconventional myosin-VIIa	DFNB2/DFNA11 USH1B	276903
<i>TMC1</i>	Transmembrane channel-like protein 1	DFNB7/11/DFNA36	606706
<i>TMIE</i>	Transmembrane inner ear expressed protein	DFNB6	607237
<i>USH1C</i>	Harmonin	DFNB18 USH1C	605242
<i>USH1G<sup>#</sup></i>	Scaffold protein containing ankyrin repeats and SAM domain	USH1G	607696
<b>Transcriptional regulation and post-transcriptional modifications<sup>a</sup></b>			
<i>BDP1</i>	Transcription factor TFIIIB component B double prime homolog	DFNB112	607012
<i>ESRP1</i>	Epithelial splicing regulatory protein 1	DFNB109	612959
<i>ESRRB</i>	Steroid hormone receptor ERR2	DFNB35	602167
<i>EYA4</i>	Eyes absent homolog 4	DFNA10	603550
<i>GRHL2</i>	Grainyhead-like protein 2 homolog	DFNA28	608576
<i>LMX1A</i>	LIM homeobox transcription factor 1-alpha	DFNA7	600298
<i>MIR96</i>	miRNA96	DFNA50	611606
<i>POU3F4</i>	POU domain, class 3, transcription factor 4	DFNX2	300039

<i>POU4F3</i>	POU domain, class 4, transcription factor 3	DFNA15	602460
<i>REST</i>	RE1-silencing transcription factor	DFNA27	600571
<i>SIX1</i>	Homeobox protein SIX1	DFNA23	601205
<i>TRRAP</i>	Transformation/transcription domain-associated protein	DFNA75	603015
<i>WBP2</i>	WW domain-binding protein 2	DFNB107	606962
<b>Actin cytoskeleton dynamics and associated proteins<sup>a</sup></b>			
<i>ACTG1</i>	Actin, cytoplasmic 2	DFNA20/26	102560
<i>DIAPH1</i>	Protein diaphanous homolog 1	DFNA1	602121
<i>DIAPH3</i>	Protein diaphanous homolog 3	AUNA1	609129
<i>ELMOD3</i>	ELMO domain-containing protein 3	DFNB88	615427
<i>EPS8</i>	Epidermal growth factor receptor kinase substrate 8	DFNB102	600206
<i>EPS8L2</i>	Epidermal growth factor receptor kinase substrate 8-like protein 2	DFNB106	614988
<i>ESPN</i>	Espin	DFNB36	606351
<i>HOMER2*</i>	Homer protein homolog 2	DFNA68	604799
<i>MYH9</i>	Myosin-9	DFNA17	160775
<i>MYH14</i>	Myosin-14	DFNA4A	608568
<i>MYO3A</i>	Myosin-IIIa	DFNB30/DFNAi	606808
<i>MYO6</i>	Unconventional myosin-VI	DFNB37/DFNA22	600970
<i>MYO7A*</i>	Unconventional myosin-VIIa	DFNB2/DFNA11 USH1B	276903
<i>MYO15A</i>	Unconventional myosin-XV	DFNB3	602666
<i>PLS1</i>	Plastin-1/Fimbrin	DFNA76	602734
<i>RDX</i>	Radixin	DFNB24	179410
<i>RIPOR2</i>	Rho family-interacting cell polarization regulator 2	DFNB104/DFNA21	611410
<i>TPRN</i>	Taperin	DFNB79	613354
<i>TRIOBP</i>	TRIO and F-actin-binding protein	DFNB28	609761
<i>WHRN</i>	Whirlin	DFNB31 USH2D	607928
<b>Cochlear ion homeostasis<sup>a</sup></b>			
<i>ATP2B2</i>	Plasma membrane calcium-transporting ATPase 2	DFNA82	108733
<i>BSND</i>	Barttin	DFNB73	606412
<i>CLIC5</i>	Chloride intracellular channel protein 5	DFNB103	607293
<i>GJB2</i>	Gap junction beta-2 protein	DFNB1A/DFNA3A	121011
<i>GJB3</i>	Gap junction beta-3 protein	DFNA2B	603324
<i>HGF</i>	Hepatocyte growth factor	DFNB39	142409
<i>HOMER2*</i>	Homer protein homolog 2	DFNA68	604799
<i>KCNQ4</i>	Potassium voltage-gated channel subfamily KQT member 4	DFNA2A	603357
<i>MET</i>	Hepatocyte growth factor receptor	DFNB97	164860
<i>P2RX2</i>	P2X purinoceptor 2	DFNA41	600844
<i>SLC12A2</i>	Solute carrier family 12 member 2	DFNA78	600840
<i>SLC22A4</i>	Solute carrier family 22 member 4	DFNB60	604190
<i>SLC26A4</i>	Pendrin	DFNB4	605646
<i>SLC26A5</i>	Prestin	DFNB61	613865

<i>TMPRSS3</i>	Transmembrane protease serine 3	DFNB8/10	605511
<b>Cochlear energy and redox homeostasis, inflammation and immunity<sup>a</sup></b>			
<i>AIFM1</i>	Apoptosis-inducing factor 1, mitochondrial	DFNX5	300169
<i>CLPP</i>	ATP-dependent Clp protease proteolytic subunit, mitochondrial	DFNB81	601119
<i>DIABLO</i>	Diablo IAP-binding mitochondrial protein	DFNA64	605219
<i>GRXCR1</i>	Glutaredoxin domain-containing cysteine-rich protein 1	DFNB25	613283
<i>GRXCR2</i>	Glutaredoxin domain-containing cysteine-rich protein 2	DFNB101	615762
<i>GSDME</i>	Gasdermin-E	DFNA5	608798
<i>IFNLR1</i>	Interferon lambda receptor 1	DFNA2C	607404
<i>KARS1</i>	Lysine--tRNA ligase	DFNB89	613916
<i>MSRB3</i>	Methionine-R-sulfoxide reductase B3	DFNB74	613719
<i>MTRNR1</i>	Mitochondrially encoded 12S RNA	-	561000
<i>MTTS1</i>	Mitochondrially encoded tRNA serine 1	-	590080
<i>NARS2</i>	Probable asparagine--tRNA ligase, mitochondrial	DFNB94	612803
<i>NLRP3</i>	NACHT, LRR and PYD domains-containing protein 3	DFNA34	606416
<i>OSBPL2</i>	Oxysterol-binding protein-related protein 2	DFNA67	606731
<i>PJVK</i>	Pejvakin	DFNB59	610219
<i>PNPT1</i>	Polyribonucleotide nucleotidyltransferase 1, mitochondrial	DFNB70	610316
<b>Auditory neuropathy spectrum disorders<sup>b</sup></b>			
<i>AIFM1</i>	Apoptosis-inducing factor 1, mitochondrial	DFNX5	300169
<i>CABP2</i>	Calcium-binding protein 2	DFNB93	607314
<i>CLRN1</i>	Clarin-1	USH3A	606397
<i>DIAPH3</i>	Protein diaphanous homolog 3	AUNA1	609129
<i>NARS2</i>	Probable asparagine--tRNA ligase, mitochondrial	DFNB94	612803
<i>OTOF</i>	Otoferlin	DFNB9	603681
<i>PJVK</i>	Pejvakin	DFNB59	610219
<i>ROR1</i>	Inactive tyrosine-protein kinase transmembrane receptor ROR1	DFNB108	602336
<i>SLC17A8</i>	Vesicular glutamate transporter 3	DFNA25	607557
<i>TBC1D24</i>	TBC1 domain family member 24	DFNB86/DFNA65	613577
<i>TMEM43</i>	Transmembrane protein 43	AUNA3	612048

<sup>a</sup>Classes of cochlear physiological defects underlying human monogenic forms of isolated sensorineural deafness. 74 of 125 defective genes can be assigned to one (exceptionally two) of the five classes. <sup>b</sup>Human monogenic forms of isolated sensorineural deafness contributing to auditory neuropathy spectrum disorder. These forms are characterized by an impairment of speech recognition that is more pronounced than predicted from the auditory threshold. They result from a desynchronization of neural conduction in the auditory pathways. For the various causal deafness genes, see their target cells in Supplementary Table 1.



**Table 2. Proofs-of-concept for inner ear gene therapy in mouse models of human deafness [Au: This Table is still very large, so I removed mention of RNA targeting approaches; we can try to see if it will fit in the final layout but may need to reduce size further. ]**

Gene	Mouse models	Age at delivery	Therapeutic agents	Diseased cells targeted	Outcomes <sup>a</sup>	Ref.
<i>Cabp2</i>	<i>Cabp2</i> <sup>-/-</sup>	P5–P7	AAV2/1-hCMV/hBA- <i>Cabp2</i> -P2A-eGFP-WPRE-bGH AAV9-PHP.eB-hCMV/hBA- <i>Cabp2</i> -P2A-eGFP-WPRE-bGH	IHC	Partial prevention of HI for up to 2 months	<sup>172</sup>
	<i>Gjb2</i> <sup>fl/fl</sup> P0-Cre	P0	AAV1-CMV- <i>Gjb2</i> -IRES- <i>hrGFP</i>	SC, fibrocytes of the spiral limbus and spiral ligament	Partial prevention of HI for up to 3 months	<sup>173</sup>
<i>Kcne1</i>	<i>Kcne1</i> <sup>-/-</sup>	P0–P2	AAV1-CB7- <i>Kcne1</i>	SV marginal cells and vestibular dark cells	Substantial prevention of HI up to 5 months, decline thereafter; prevention of vestibular dysfunction up to 6 months	<sup>117</sup>
<i>Kcnq1</i>	<i>Kcnq1</i> <sup>-/-</sup>	P0–P2	AAV1-CB7- <i>Kcnq1</i>	SV marginal cells	Substantial prevention of HI up to 4 months; effect almost entirely absent at 7 months	<sup>115</sup>
<i>Kcnq4</i>	<i>Kcnq4</i> <sup>W276S/+</sup>	P1–P3	Dual-vector <sup>b</sup> : AAV2/Anc80L65-U6-sgRNA-split-N-Cas9-N-intein and AAV2/Anc80L65-C-intein-split-C-Cas9-bGH	OHC	Prevention of HI up to 7 weeks for all frequencies	<sup>135</sup>
<i>Lhfp15</i> (Tm)	<i>Lhfp15</i> <sup>-/-</sup>	P0–	exo-AAV1-CBA-HA- <i>Lhfp15</i> -IRES-	IHC, OHC,	Prevention of	<sup>101</sup>

hs)		P2	<i>GFP</i>	SGN	HI (limited) and of some vestibular dysfunction, up to 4 weeks	
<i>MsrB3</i>	<i>MsrB3</i> <sup>-/-</sup>	E12.5	rAAV2/1-CMV- <i>MsrB3</i> - <i>GFP</i>	IHC, OHC	Near full prevention of HI up to 4 weeks	<sup>174</sup>
<i>Myo6</i>	<i>Myo6</i> <sup>C442Y/+</sup>	P0– P2	AAV-PHP.eB-SaCas9-KKH- <i>Myo6</i> -g2	IHC, OHC	Substantial and partial prevention of HI for low- and mid-range, and high frequencies, respectively; major decrease in effect at 5 months	<sup>136</sup>
<i>Otof</i>	<i>Otof</i> <sup>-/-</sup>	P10; P17; P30	Dual-vector: AAV2-quadY-F-CBA- <i>Otof</i> -Nter-SD and AAV2-quadY-F-SA- <i>Otof</i> -Cter	IHC	Full prevention of HI up to 6 months	<sup>112</sup>
		P6– P7	Dual-vector: AAV2/6-CMV- <i>eGFP</i> -P2A- <i>Otof</i> -Nter-SD and AAV2/6-SA- <i>Otof</i> -Cter-WPRE-pA		Marginal prevention up to 3 weeks	<sup>113</sup>
		P5– P7	AAV9/PHP.B-CMV/hBA- <i>fl</i> - <i>Otof</i> -WPRE-bGH		Partial prevention up to 5.5 weeks	<sup>175</sup>
		P0– P2	Dual-vector: AAV9/PHP.B-CMV-h <i>Otof</i> -n_intein and AAV9/PHP.B-CMV-h <i>Otof</i> -c-signal_intein		Almost full prevention of HI up to 6 months	<sup>176</sup>
<i>Pcdh15</i>	<i>Pcdh15</i> <sup>av3J/av3J</sup>	P0– P2	AAV2/9-m- <i>Pcdh15</i> -gRNA1	IHC, OHC	Marginal prevention of HI up to 4-5 weeks; full prevention of vestibular	<sup>144</sup>

					function up to 2 months	
<i>Pjvk</i>	<i>Pjvk</i> <sup>-/-</sup>	P3	AAV2/8-CB7- <i>Pjvk</i> -IRES-EGFP	IHC, OHC, SGN	Partial prevention of HI for low- to mid-range frequencies up to 3 weeks	49
	<i>Pjvk</i> <sup>G292R/G292R</sup>	P0–P1	Anc80L65-CMV- <i>PJVK</i> -EGFP-WPRE-bGH		Substantial prevention of HI up to 3 weeks with decrease at 1 month; prevention of balance defects up to 4 months	177
<i>Slc17a8</i> ( <i>Vglut3</i> )	<i>Vglut3</i> <sup>-/-</sup>	P1–P3	AAV1-CBA- <i>Vglut3</i> -WPRE-bGH	IHC	Full prevention of HI; effect maintained up to 9 months	102
		P10–12			Full restoration of hearing [Au:OK?]	
		P3	AAV8-CMV- <i>Vglut3</i> -Flag-GFP		Full prevention of HI up to 3 months	114
		P35; P56			Substantial restoration of hearing [Au:OK?]	
P140	Partial restoration of hearing					
<i>Slc26a4</i>	<i>Slc26a4</i> <sup>-/-</sup> <i>Slc26a4</i> <sup>tm1Dontuh/tm1</sup> Dontuh	E12.5	rAAV2/1-CMV- <i>Slc26a4</i> -tGFP-pA	Epithelial cells of the endolymphatic sac,	Prevention of HI (up to 3–5 weeks) with major decline	178

				sensory epithelia of utricle, saccule and SCC, putative spindle and root cells	at 16–21 weeks; instability of hearing phenotype; no prevention of vestibular dysfunction	
	<i>Slc26a4</i> <sup>-/-</sup>	E11.5	pCMV- <i>Slc26a4-Egfp-pA</i>		Efficient prevention of HI and vestibular defects up to 4.5 weeks	97
<i>Slc26a4A5</i>	<i>Prestin knockout</i>	P3	AAVie-K558R-CAG-prestin-WPRE-SV40	OHC	Modest prevention of HI up to 4 weeks	179
<i>Strc</i>	<i>Strc</i> <sup>Δ/Δ</sup>	P1	Dual-vector <sup>b</sup> : AAV9/PHP.B-CMV- <i>Strc-n_intein</i> AAV9/PHP.B-CMV- <i>Strc-c-signal_intein</i>	OHC	Partial prevention of HI up to 4 weeks	180
<i>Syne4</i>	<i>Syne4</i> <sup>-/-</sup>	P0– P1.5	AAV2/9.PHP.B.CMV.3xFLAG. <i>Syne4.bGH</i>	OHC	Near-full prevention of HI up to 4 weeks; effect declining at 3 months	181
<i>Tmc1</i> <i>Tmc2</i>	<i>Tmc1</i> <sup>Δ/Δ</sup> <i>Tmc1</i> <sup>Δ/Δ</sup> <i>Tmc1</i> <sup>Bth/Bth</sup>	P0– P2	AAV2/1-CBA- <i>Tmc1</i> AAV2/1-CBA- <i>Tmc1</i> AAV2/1-CBA- <i>Tmc2</i>	IHC, OHC	Partial prevention of HI up to 2 months	108
	<i>Tmc1</i> <sup>Δ/Δ</sup>	P0– P1, P4, P7, P14	AAV2/Anc80L65-CMV- <i>Tmc1</i> -WPRE AAV2/1-CMV- <i>Tmc1</i> -WPRE AAV2/Anc80L65-CMV- <i>Tmc1</i> -WPRE AAV2/Anc80L65-CMV- <i>Tmc2</i> -WPRE	IHC, OHC, VHC	Modest prevention of HI (P0–P1) decreasing from 6 weeks; Very modest prevention (P4); almost no effect (P7); no effect (P14)	118

					<b>[Au: What about P4?]</b>	
	<i>Tmc1</i> <sup>Δ/Δ</sup> <i>Tmc2</i> <sup>Δ/Δ</sup>	P0– P1, P14, P30			Almost full prevention of balance defects up to 6 weeks (P0–P1), partial prevention (P14, P30)	
	<i>Tmc1</i> <sup>Btr/+</sup>	P0– P2; P42	SpCas9: <i>Tmc1</i> -mut3 RNP complex	IHC, OHC	Substantial prevention for low- and mid-range frequencies at 4 weeks (P0–P2 injection), decreasing at 2 months; similar efficacy of editing for P0–P2 (via CO) and P42 (via CA) injections	134
	<i>Tmc1</i> <sup>Btr/+</sup>	P1	AAV2/Anc80L65-CMV-SaCas9-KKH-U6-gRNA-4.2	IHC, OHC	Almost full prevention of HI for low- and mid-range frequencies up to 6 months	137
	<i>Tmc1</i> <sup>Y182C/Y182C</sup> <i>Tmc2</i> <sup>Δ/Δ</sup>	P1	Dual-vector <sup>b</sup> : AAV2/Anc80L65-Cbh-spCas9-AID.BE3.9max-sgRNA1	IHC, OHC	Partial prevention of HI for low frequencies up to 4 weeks, followed by rapid decline	152

	<i>Tmc1</i> <sup>Δ/Δ</sup>	P1; P7	AAV2/9-PHP.B.CMV- <i>Tmc1</i> - WPRE  Dual-vector: AAV2/9-PHP.B-CMV-SpCas9 AAV2/9-PHP.B-U6-gRNA15- CMV-GFP	IHC, OHC	Substantial prevention of HI for low- and mid-range frequencies up to 4 weeks; effect stable for 3 months; very modest prevention for P7 injection	138
	<i>Tmc1</i> <sup>Y182C/Y182C</sup>				Substantial prevention of HI for low- and mid-range frequencies up to 4 weeks	
	<i>Tmc1</i> <sup>Btm/+</sup>				Prevention of worsening of HI for low- and mid-range frequencies from 4 to 24 weeks	
Ush1c	<i>Ush1c</i> <sup>tm1Bkts/tm1Bkts</sup>	P0– P1	AAV2/Anc80L65.CMV.EGFP:har monin-b1	IHC, OHC, VHC	Substantial prevention of HI (P1 injection) up to 3 months; low frequency responses persisting up to 6 months Prevention of balance defects for up to 6 weeks	111

Ush1g	<i>Ush1g</i> <sup>-/-</sup>	P2.5	AAV2/8-CAG- <i>Sans</i> -IRES-GFP-bGH		Partial prevention of HI up to 4 months; restoration of balance up to 15 months	110
Ush3a	<i>Cln1</i> <sup>ex4<sup>-/-</sup></sup> <i>Cln1</i> <sup>ex4fl/fl</sup> <i>Myo15-Cre</i>	P1-P3	AAV2/8-CAG- <i>Cln1</i> -IRES-GFP	IHC, OHC	Modest prevention of HI Full prevention of HI for all frequencies; effect declining from 8.5 weeks	182
	<i>Cln1</i> <sup>-/-</sup>	P0-- P1	AAV9-PHP.B-CBA- <i>Cln1</i> -WPRE-bGH		Modest prevention of HI for low frequencies at 4 weeks	183
	<i>Cln1</i> <sup>-/-</sup> <i>TgAC1</i> ; <i>Cln1</i> <sup>-/-c</sup>	P1– P3	AAV2-CBA- <i>Cln1</i> ±UTRs AAV8-CBA- <i>Cln1</i> ±UTRs		Prevention of progressive HI (+UTRs) up to 3 months	98
	<i>TgAC1</i> ; <i>Cln1</i> <sup>-/-c</sup>	P1	AAV-S-CBA-opti <i>Cln1</i> -bGH	IHC, OHC, SGN	Complete prevention of HI for low- and mid-range frequencies up to 5 months	100
<i>Whrn</i>	<i>whrn</i> <sup>w/wtd</sup>	P1-P5	AAV2/8-CMV- <i>Whrn</i> -GFP	IHC, OHC, VHC	Modest prevention of HI up to 4 months. Almost full prevention of balance defects up to	116

					4 months	
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Proof-of-concept for inner ear gene therapy based on gene replacement (blue), gene augmentation (green) and gene editing (CRISPR–Cas9 (red), base editing (orange)) in mouse models of human forms of deafness. The nomenclature for therapeutic agents is that used in the corresponding publications. <sup>a</sup>Outcomes are based on auditory thresholds analysed by determining auditory brainstem responses and with vestibular tests. The maintenance of the effects is evaluated by the determination of auditory thresholds in mice until the end point of the study. <sup>b</sup>Dual AAV using a split-intein delivery system. <sup>c</sup>*TgAC1*: a transgene that delays the onset of hearing impairment due to a biallelic loss-of-function *Cln1* variant in *TgAC1;Cln1*<sup>-/-</sup> mice. <sup>d</sup>Short and long isoforms of whirlin are absent. AID: activation-induced cytidine deaminase; bGH: bovine growth hormone polyadenylation signal; Bth: Beethoven mutation; CA: canalostomy; CBA: CMV immediate-early enhancer and chicken β-actin promoter, and CB7 and CAG derivatives (CB7 is identical to CBA but with a shortened CMV early enhancer element; CAG contains the CMV immediate-early enhancer and the chicken β-actin promoter, first exon and first intron followed by the rabbit β-globin splice acceptor site); CBE: cytosine base editor; Cbh: chicken β-actin hybrid; CMV: cytomegalovirus; CO: cochleostomy; eGFP: enhanced GFP; EUGO: electroporation-mediated transuterine gene transfer into otocysts; fl-Otof: full-length Otoferlin cDNA; fl/fl: flox/flox; GFP: green fluorescent protein; HA: hemagglutinin; hBA: human beta actin; hair cell: hair cell; hCMV: human cytomegalovirus, also called human beta herpes virus 5; HI: hearing impairment; hOtof: human Otoferlin; hrGFP: humanized *Renilla reniformis* GFP; IHC: inner hair cells; IRES: internal ribosome entry site; JLNS1: Jervell and Lange-Nielsen syndrome type 1; JLNS2: Jervell and Lange-Nielsen syndrome type 2; KKH: E782K/N968K/R1015H; ND: not documented; OHC: outer hair cells; pA: polyadenylation signal; PSCC: posterior-semicircular canal/canalostomy; RNP: ribonucleotide protein; RWM: round window membrane; SA: splice acceptor site; SaCas9: *Staphylococcus aureus* Cas9; SC: supporting cells; SCC: semicircular canal; SD: splice donor site; SGN: spiral ganglion neuron; SM: scala media; SpCas9: *Streptococcus pyogenes* Cas9; SV: stria vascularis; TM: tympanic membrane; tGFP: turbo GFP; VHC: vestibular hair cells; WPRE: woodchuck hepatitis virus post-transcriptional regulatory element.



**Fig. 1. Schematic representation of the structure and functions of the inner ear.**

a. Representation of the human ear, consisting of the outer, middle and inner ear. The outer ear collects sound waves, inducing tympanic membrane vibrations. The ossicular chain of the middle ear transfers these vibrations to the cochlea, a fluid-filled spiral tube organ. b. The mammalian inner ear contains the cochlea, the hearing organ, and the vestibule, the balance organ, consisting of two otolithic organs, the saccule and the utricle, and three semicircular canals. The cochlea analyses sound frequencies; high and low sound frequencies are processed at the base and apex of the organ, respectively. The relationship between sound frequency and detection position along the longitudinal cochlear axis results in a spatially distributed cochlear frequency map, the tonotopic map. c. The cochlea has three fluid compartments or scalae: the scala media (or cochlear duct), the central compartment filled with endolymph, and the scalae vestibuli and tympani filled with perilymph. The organ of Corti, houses the auditory sensory cells — the inner hair cells (IHCs) and the outer hair cells (OHCs) (red) — and their supporting cells (blue). d. The organ of Corti lies on the basilar membrane and is covered by the tectorial membrane. The hair cells of the organ of Corti are connected at their apical surface by tight junctions, as well as with their flanking supporting cells (blue), forming a physiological barrier that extends to the marginal cells of the stria vascularis and the Reissner's membrane, isolating the endolymphatic compartment (ionic composition similar to that of the intracellular medium: high  $K^+$  and low  $Na^+$ ,  $Ca^{2+}$  and  $Mg^{2+}$  concentrations) from the perilymph (ionic composition resembling extracellular fluids, very similar to cerebrospinal fluid). The hair cells are crowned by the hair bundle, the mechanoreceptive structure to sound (boxed); the different cell types include supporting cells (1); root cells (2); spindle cells (3); fibrocytes of the spiral limbus (4); fibrocytes of the spiral ligament (5); and tympanic border cells (6). e. IHC-primary auditory neuron synapse with synaptic vesicles (with the transmembrane proteins Otoferlin and VGLUT3) tethered to a presynaptic electron-dense ribbon. f. The stereocilia, stiff microvillus-like actin-filled processes, form the hair bundle. A cilium, the kinocilium, is associated with the stereocilia during hair bundle development (not shown). The stereocilia are organized into three rows of increasing height. Sound stimulation induces shearing between the tectorial and basilar membranes, causing the hair bundle to deflect around the insertion point of the stereocilia into the apical hair cell surface. The resulting hair bundle oscillations modulate tip-link (TL) tension. The TL is an extracellular fibrous link connecting the tip of each stereocilium from the short and middle rows to the shaft of the immediately adjacent stereocilium in the taller row. The lower end of each TL is tethered to the mechano-electrical

transduction channels. Sound-induced hair bundle deflection by tensioning the TL opens the mechano-electrical transduction channel, leading to  $K^+$  and  $Ca^{2+}$  influx from the endolymph. In response to the resulting hair cell depolarization, the IHCs release the neurotransmitter glutamate, and the OHCs actively amplify their mechanical response (a process essential for the high sensitivity of hearing). The proteins of the mechano-electrical transduction machinery are represented. g. The stria vascularis (SV), a three-cell layer epithelium, generates the endocochlear potential (+80 to 100 mV) driving mechano-electrical currents into the hair cells. It also secretes  $K^+$  into the endolymph. Ion channels and transporters, all encoded by deafness genes, are represented.

**Fig 2. Inner ear gene therapy methods.** A- *Gene replacement*: (a) a recombinant AAV (rAAV) with a cargo consisting of a promoter, a cDNA encoded by the therapeutic gene and a polyadenylation signal (pA); (b) a dual AAV approach based on trans-splicing strategy. One rAAV contains a promoter, the 5'-half of the cDNA and a splicing donor (SD) element; the other one includes a splicing acceptor (SA) element, the 3'-half of the cDNA and a pA. ITR-mediated DNA concatemerization of the 2 rAAVs allowing transplicing between SD and SA elements, to remove unwanted sequences from the transcript. A dual approach based on trans-splicing strategy can be associated with recombination *via* recombinogenic sequences (RS, in gray) shared by the two rAAVs as illustrated for the *Otof* gene<sup>112</sup>. B- *Gene editing*: (a) CRISPR-Cas9 editing: Cas9 with the sgRNA unwind the target genomic DNA double helix with the sgRNA pairing the non-PAM strand. The NUC lobe of Cas9 is shown with its HNH (green) and RuvC (yellow) nuclease and PAM-interacting (pink) domains. The HNH and RuvC domains cleave the non-PAM and PAM DNA strands, respectively, creating a blunt DSB, repaired by NHEJ introducing small indels or possibly corrected by HDR using a donor DNA. SpCas9 cleaves (scissor) DNA at a fixed distance of 3 bp upstream from the PAM motif. Adapted from Jian and Doudna<sup>184</sup>. (b) Base editing: cytosine base-editing (CBE) and adenine base-editing (ABE) catalyse the transition of cytosine to uridine (U) and adenosine to inosine (I), respectively, using a sgRNA complementary to the non-PAM strand of the target genomic DNA and a Cas9 derivative, here a Cas9 nickase (Cas9n (D10A)) fused with a single-stranded DNA-specific cytidine deaminase domain (CBE) (orange) or an adenosine deaminase domain (ABE) (purple) binding to the PAM strand, the edit strand. U and I pair as T and G respectively, converting C•G into a T•A and A•T into a

G•C. (c) Prime editing. It uses a Cas9 nickase (Cas9n (H480A)) fused with a reverse transcriptase (RT) (blue) and a prime-editing guide RNA (pegRNA). The 5' end sequence of the pegRNA complementary to the non-PAM DNA strand of the target genomic DNA, directs the Cas9 nickase to cleave the PAM DNA strand at a specific site in the target genomic DNA and its 3' end sequence hybridizes with the PAM strand forming a RT primer-binding site and a template sequence for the RT editing. It results in transitions and transversions, small insertions and deletions.

**Fig. 3: Routes for the delivery of inner ear gene therapy.** Schematic representation of the five main routes for inner ear gene therapy in the mouse using direct injection (indicated by syringes): (1) Round window membrane (RWM) delivery route. After middle ear surgery, the injected therapeutic agent directly diffuses into the scala tympani perilymph and then the vestibular perilymphatic spaces (see FIG 1) (2) Cochleostomy, with delivery of the agent to the scala tympani and/or the scala media endolymph with diffusion to vestibular endolymph. (3) Canalostomy, which involves surgical drilling into a vestibular semicircular canal (usually the posterior canal), with the therapeutic agent injected into the perilymphatic vestibular spaces then diffusing to perilymphatic cochlear spaces and/or injected into the vestibular endolymphatic compartment with diffusion in the scala media. (4) Injection into the utricle protuberance directly targeting the endolymphatic compartment. (5) Local intravenous injection. Injections via canalostomy or cochleostomy have been reported to perform slightly better than RWM injection<sup>109,115,116</sup>. The combination of RWM injection and canal fenestration, creating inner ear flow, has been shown to outperform canalostomy or RWM injection alone<sup>104</sup>. Utricle injection was found to be more efficient than RWM, cochleostomy or canalostomy<sup>135</sup>. Of note, systemic approach was only efficient in early postnatal days<sup>185</sup>. In humans, the endolymphatic sac (6) delivery route to the endolymph is the option primarily explored<sup>94,186,187</sup>; the oval window (OW) (7) delivery route to the endolymph and the RWM (1) perilymphatic route used for cochlear implantation are also considered. Black arrows within the cochlear aqueduct indicate transfer from the scala tympani perilymph to the cerebrospinal fluid, with the possibility of therapeutic agents spreading to the cerebral parenchyma. Adapted from Margarete Anna Überfuh DOI: 10.5282/edoc.24957.

### **Box 1. Viral and non-viral therapeutic gene delivery**

Several recombinant viruses have been tested as possible vectors for therapeutic gene transfer into the inner ear. AAVs — linear single-stranded DNA viruses — are currently the preferred option, due to their non-pathogenicity and limited immunogenicity in humans<sup>99</sup>. Natural capsid serotypes (AAV1-12) and pseudo-serotypes of these viruses have been studied, and strategies have been developed for modifying AAV capsids to extend or restrict their cellular tropism, increase their transduction efficiency- and reduce their immunogenicity by genetic engineering<sup>188,189</sup>. AAVs transducing the IHCs, or both IHCs and OHCs very efficiently in mice are now available<sup>78</sup> but their transduction is rarely restricted to hair cells. AAVs targeting the other cochlear cell types — epithelial cells (various SC and SV cell types) and fibrocytes — have yet to reach this standard. AAVs transducing several cell types, required for the treatment of genetic forms of deafness affecting several cochlear cell types<sup>190,191</sup>, remain few in number. Finally, before their use in humans can be envisaged, explorations of the cell tropism of AAVs in the cochlea of non-human primates (NHP) at various ages are required; such validation has been reported for only a few hair cell-transducing AAVs to date<sup>100,183,192,193</sup>.

The major drawback of AAVs is their small packaging capacity (~4.7 kb), limiting the size of the therapeutic sequence. Many deafness genes are much longer (up to 15 kb, for *USH2A*). Cotransduction with two AAVs can double the length of the therapeutic sequence to be delivered, through the generation of concatemers of rAAVs via their inverted terminal repeats (ITRs)<sup>194</sup>; triple-AAV strategies make it possible to use even longer therapeutic sequences, of up to ~14 kb<sup>195</sup>. In dual-AAV strategies, one AAV contains the 5' cDNA fragment and a tail-tagged splicing donor sequence, and the other contains the 3' cDNA fragment preceded by a splicing acceptor sequence. Splicing across the ITR junction leads to efficient mRNA production. This approach has been successfully used to restore hearing postnatally<sup>112,113</sup> and at adult<sup>112</sup> stages in a mouse model of DFNB9, with a defective *Otof* gene, which encodes otoferlin via a 6.9 kb mRNA. An alternative dual-AAV approach based on protein-trans-splicing mediated by split-inteins<sup>196</sup> has been developed and was recently used to prevent HI in mouse models of DFNB16 and DFNA2A<sup>180,135</sup> (Table 2).

Liposomes, lipid nanoparticle (LNP), dendrimers and carbon nanotubes, the principal non-viral vehicles for gene therapy, have the major advantage of being able to accommodate large therapeutic nucleic-acid molecules. Liposomes are highly

versatile<sup>197</sup> for clinical applications, but their unnatural lipid content can render them toxic. Considerable efforts have therefore been made to modify the composition of these phospholipid vesicles to limit their toxicity. However, the low transfection rates achieved with liposomes may limit their use. Exosomes have recently been developed as alternative non-viral vehicles<sup>198,199</sup>. Surface modifications can increase exome functionality, by increasing transfection rates and cell-specific targeting. Exosomes can also encapsulate AAVs to form exosome-associated AAVs (Exo-AAVs) carrying rAAV genomes. These vectors have a higher transduction efficiency than conventional AAVs<sup>200</sup>, are more resistant to neutralizing anti-AAV antibodies, and elicit no anti-AAV antibody response<sup>201,202</sup>. When tested in the cochlea, Exo-AAV1 outperformed AAV1 in terms of OHC transduction rate<sup>101</sup>. Moreover, an Exo-AAV1 approach partially rescued the hearing phenotype in the *Lhfp15<sup>-/-</sup>/Tmhs<sup>-/-</sup>* mouse model of DFNB67<sup>101</sup>. However, exosomes have a smaller oligonucleotide molecule packaging capacity (~6 kb) than liposomes. Efforts have, therefore, been made to develop hybrid exosome-liposome nanoparticles capable of accommodating large nucleic acid molecules, including the CRISPR-Cas9 system, and in which the natural lipids of the exosome mitigate the cytotoxicity of the unnatural lipids of the liposome<sup>203–205</sup>.

## **GLOSSARY**

### **AAV**

Adeno-associated virus is a single-stranded DNA virus from which recombinant AAVs (rAAVs) are engineered for DNA delivery to target cells. rAAVs being non-pathogenic are widely used as vectors for gene therapy.

### **Antisense oligonucleotides**

Antisense oligonucleotides (ASO) are short synthetic single-stranded strings of nucleotides (15 to 30 nucleotides) designed to target other nucleotide sequences, usually coding or non-coding RNAs. They can mediate target RNA degradation by recruiting RNase H for cleavage of the ASO-RNA duplexes or act as steric blockers, masking complementary sequences on RNAs and potentially interfering with several RNA processing steps. They can modulate exon splicing through steric blocker activity, including the masking of cryptic splice sites in mutated pre-mRNA.

### **Canalostomy**

Surgical approach to the inner ear involving fenestration of a vestibule semicircular canal, generally the posterior semicircular canal, used in inner ear gene therapy interventions in mice.

### **Cochlear implant**

This surgically implanted neuroprosthesis is an electrical device that processes sounds and transforms acoustic stimulation into electrical stimulation delivered directly to the primary auditory neurons via electrodes inserted into the cochlea.

### **Conductive**

Conductive hearing impairment results from the defective transmission of sound waves to the cochlea due to external and/or middle-ear defects.

### **Dominant-negative variant**

The product of a mutated gene carrying a variant with a dominant-negative effect has not only lost its function, but it also interferes in a deleterious manner with the

function of the normal, wild-type gene product present within the same cell of heterozygous organisms.

### **Episome**

A genetic determinant (such as the DNA of some bacteriophages) that can replicate autonomously in bacterial cytoplasm or as an integral part of the chromosomes.

### **Gene editing**

Gene editing refers to processes that change genomic DNA by adding, removing or altering sequences. Gene-editing technologies produced site-specific modifications.

### **Gene replacement**

Gene replacement is a procedure for correcting the effects of defective genes by transferring a normal copy of the gene into the targeted diseased cells.

### **Genetic architecture**

The genetic architecture of disease is the underlying genetic basis of its phenotypic features. It includes all the causal variants, the magnitude of their effects, their frequency and their interactions with each other and with environmental factors.

### **Genome-wide association study**

Genome-wide association studies (GWAS) are a genetic approach for identifying genetic risk factors for diseases or particular traits, through the scanning of single-nucleotide polymorphisms (SNPs) across the genome for association with these phenotypes.

### **Haploinsufficient gene**

A haploinsufficient gene must have two normally functioning alleles for leading to a normal phenotype.

### **Hearing aid**

A hearing aid is an auditory prosthesis, an electrical device amplifying sound at selected frequencies.

## **Hearing impairment**

In this Review, the terms ‘hearing impairment’ and ‘deafness’ are used interchangeably (deafness being not restricted to profound hearing impairment). Hearing impairment in humans is classified as mild, moderate, moderately severe, severe and profound, for threshold elevations ranging from 26–40 dB hearing level (HL), 41–55 dB HL, 56–70 dB HL, 71–90 dB HL and >90 dB HL, respectively, relative to normal human hearing thresholds.

## **Hearing threshold**

Auditory thresholds refer to pure-tone audiometry (PTA) testing the audibility threshold, that is the minimal intensity a sound of a given frequency must reach in order to be detected. It is measured in dB SPL (sound pressure level), with 0 dB SPL corresponding to 20 µPa. In clinical context, thresholds are obtained at 4 frequencies of 0.5, 1, 2 and 4 kHz and are expressed in dB on the Hearing Level (dB HL) scale. 0dB HL represents for each frequency the average auditory threshold of individuals with normal hearing.

## **Heterochrony**

Evolutionary change in timing and rates of developmental processes

## **Homozygosity mapping**

Homozygosity mapping identifies genomic regions containing causal mutations for recessively inherited disease through homozygous polymorphic markers coinherited with the disease. Homozygous regions are generally large for recent consanguineous unions and shorter in cases of ancestral population homozygosity.

## **Menière’s disease**

A chronic inner-ear disorder characterized by episodes of vertigo (dizzy spells), tinnitus (perception of sound without an external sound stimulus) and hearing loss.

## **Ribbon pre-synapse**

A synaptic ribbon is an electron-dense structure present in the sensory cells of various sensory systems, to which synaptic vesicles are tethered. In the mature auditory system, presynaptic ribbons are present only in IHCs. Each ribbon



presynapse faces the post-synaptic ending of the single dendrite of the primary afferent neuron.

### **siRNA**

Small-interfering RNAs are the molecular effectors of RNA interference (RNAi). These double-stranded small RNAs, 19–25 base-pairs long, produced by the cleavage of longer double-stranded RNAs, can be used to inhibit transcription, degrade RNA and repress mRNA translation.

### **SNP**

Single nucleotide polymorphisms (SNPs) are single-base genomic variants. A variant is considered to be an SNP if its frequency exceeds 1% in a large population of unrelated individuals.

### **Stria vascularis**

The stria vascularis is a highly specialized three-layered epithelium of the lateral wall of the cochlear duct housing a dense capillary network. The stria vascularis is involved in  $K^+$  secretion into the endolymph and the generation of endocochlear potential.

### **Tonotopic**

Tonotopic organization expresses gradients in the representation (maps) of sound properties, such as their frequency-to-place representation, or frequency maps of pure tone stimuli, from the cochlea to the primary auditory cortex and in all the nuclei of the auditory sensory pathway in between.

### **Usher syndrome**

Usher syndrome (USH) is a multisensory disorder inherited in an autosomal recessive mode, with three clinical subtypes (USH1-3). USH1 is the most severe subtype; it combines congenital severe-to-profound sensorineural deafness, balance defects of vestibular origin and retinitis pigmentosa leading to blindness.

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Related links:

Van Camp, G., Smith, R. J. H. Hereditary Hearing Loss  
Homepage <https://hereditaryhearingloss.org>