

REVIEW

Milestones toward cochlear gene therapy for patients with hereditary hearing loss

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Abstract

A number of genes are reportedly responsible for hereditary hearing loss, which accounts for over 50% of all congenital hearing loss cases. Recent advances in genetic testing have enabled the identification of pathogenic variants in many cases, and systems have been developed to provide personalized treatment based on etiology. Gene therapy is expected to become an unprecedented curative treatment. Several reports have demonstrated the successful use of cochlear gene therapy to restore auditory function in mouse models of genetic deafness; however, many hurdles remain to its clinical application in humans. Herein, we focus on the frequency of deafness genes in patients with congenital and late-onset progressive hearing loss and discuss the following points regarding which genes need to be targeted to efficiently proceed with clinical application: (a) which cells' genes are expressed within the cochlea, (b) whether gene transfer to the targeted cells is possible using vectors such as adeno-associated virus, (c) what phenotype of hearing loss in patients is exhibited, and (d) whether mouse models exist to verify the effectiveness of treatment. Moreover, at the start of clinical application, gene therapy in combination with cochlear implantation may be useful for cases of progressive hearing loss.

KEYWORDS

adeno-associated virus, cochlear implant, electric-acoustic stimulation, gene therapy, genetic deafness, hereditary hearing loss

1 | INTRODUCTION

Congenital hearing loss is a relatively common disorder, occurring in 1 to 2 per 1000 newborns, among which 60%–70% of cases are hereditary.¹ Due to widespread newborn hearing screening, hearing loss has been increasingly detected in the early stages after birth.¹ Identifying the etiology of hearing loss has also improved due to genetic testing, imaging with CT and MRI, and testing for congenital cytomegalovirus infection following auditory testing.² Meanwhile,

hearing aids (HAs) and cochlear implants (CIs) are indicated depending on the severity of hearing loss, with CIs currently being the standard treatment for severe-to-profound hearing loss patients. However, these devices are not biological treatments. Gene therapy is expected to be developed as a treatment targeting causal genes for genetic deafness.³ Here, we report a summary of the current state of genetic testing in humans, gene therapy research in mouse models, and the areas requiring further study, as well as milestones toward the clinical application of gene therapy.

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2 | USEFULNESS OF GENETIC TESTING IN PATIENTS WITH HEREDITARY HEARING LOSS

Approximately 120 deafness genes have been identified as responsible for non-syndromic hearing loss (NSHL) (<https://hereditaryhearingloss.org/>), which refers to hearing loss without other signs or symptoms. Among a number of deafness genes, assumptions regarding the causative gene of NSHL from the severity of hearing loss and mode of inheritance are difficult due to genetic heterogeneity⁴; thus, an accurate diagnosis of NSHL requires genetic testing. Over 400 different syndromes have been reported for syndromic hearing loss (SHL), which is accompanied by various symptoms besides hearing loss.⁵ Although most syndromes can be clinically diagnosed from the accompanying symptoms, many are associated with multiple genes, making accurate diagnosis difficult without genetic testing, similar to NSHL. For example, patients with Usher syndrome types 1 and 2, in which congenital hearing loss is accompanied by late-onset retinitis pigmentosa, exhibit hearing loss alone in childhood, thus appearing similar to NSHL.^{6,7} Patients later show vision symptoms such as night blindness, and Usher syndrome can be clinically diagnosed. Diagnosis before the appearance of vision symptoms can only be made by genetic testing; thus, in cases of SHL, genetic testing may allow early diagnosis and intervention. Identification of causal genes may enable not only accurate diagnosis, but also prediction of the accompanying symptoms, effectiveness of existing therapy, and rate of recurrence.⁸ If gene therapy based on the genetic diagnosis of deafness becomes available in the future, genetic testing will become even more clinically important.

3 | FREQUENCY OF GENES RESPONSIBLE FOR HEREDITARY HEARING LOSS

In recent years, due to the emergence of targeted genomic enrichment and massively parallel DNA sequencing, genetic testing has enabled comprehensive analysis of a number of genes responsible for hearing loss, which has led to a dramatic improvement in the diagnostic rate and turnaround time for diagnosis.^{4,9,10} Sloan-Heggen et al and our research team previously reported that pathogenic variants in deafness genes are identified in approximately 40% of patients with hearing loss.^{9,10} As stated above, over 120 deafness genes have been identified, while their frequency differs and is known to vary by ethnicity. In Japan, *GJB2*, *SLC26A4*, and *CDH23* are reported to be the prevalent deafness genes (Figure 1).⁹ Sloan-Heggen et al reported that *GJB2* is the most frequently identified deafness gene (21.6%), followed by *STRC* (16.1%), *SLC26A4* (6.6%), *TECTA* (5.2%), *MYO15A* (4.8%), *MYO7A* (4.5%), *USH2A* (4.3%), *CDH23* (4.1%), *ADGRV1* (2.7%), *TMC1* (2.3%), *PCDH15* (2.0%), *OTOA* (2.4% in autosomal recessive NHSL patients), and *TMPRSS3* (2.4% in autosomal recessive NHSL patients) in 1119 deafness patients of Caucasian, Hispanic, African American, Asian, Middle Eastern, Ashkenazi Jewish, and mixed ethnicity.¹⁰ When considering which gene should be targeted for gene therapy, estimating the number of patients is important while keeping in mind the frequency of the deafness gene in patients with hearing loss.

4 | SELECTION OF TARGET GENES FOR GENE THERAPY

As mentioned above, a preclinical study of the prevalent deafness genes in humans is needed prior to clinical application. Not only the

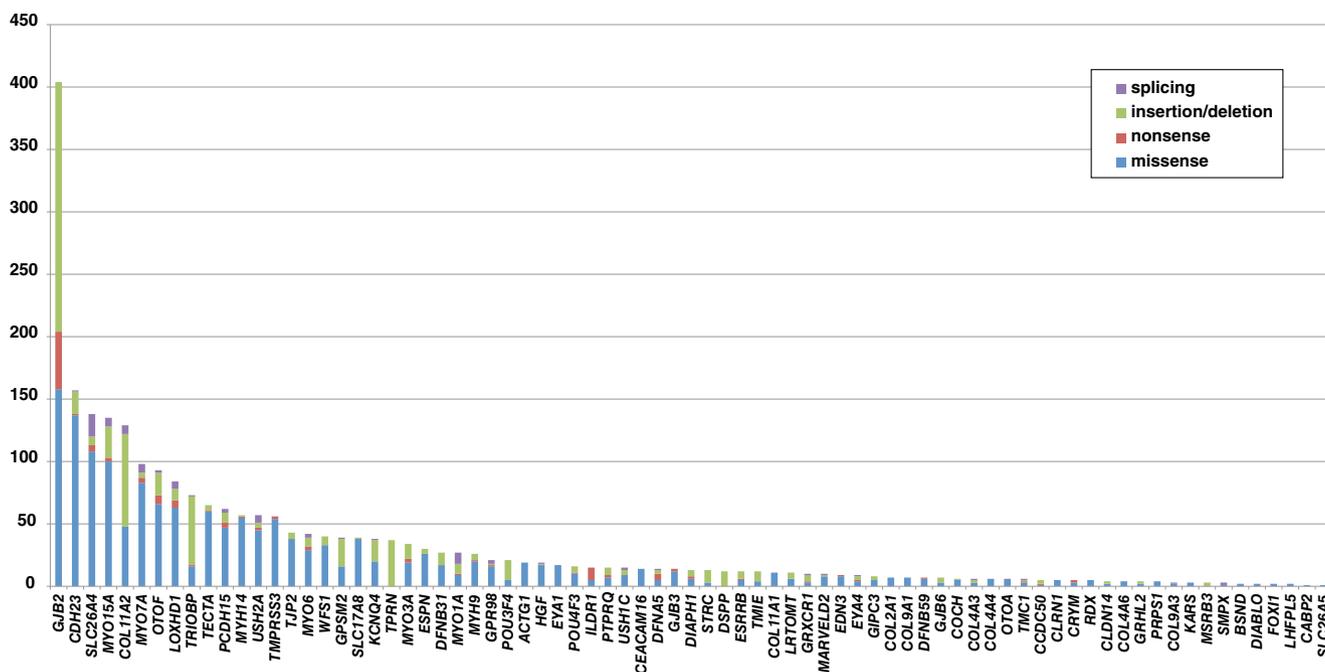


FIGURE 1 The frequency of genetic mutations found in 1120 Japanese patients with hearing loss (Nishio et al., 2015).⁹ Relatively prevalent deafness genes, such as *GJB2*, *CDH23*, and *SLC26A4*, are described

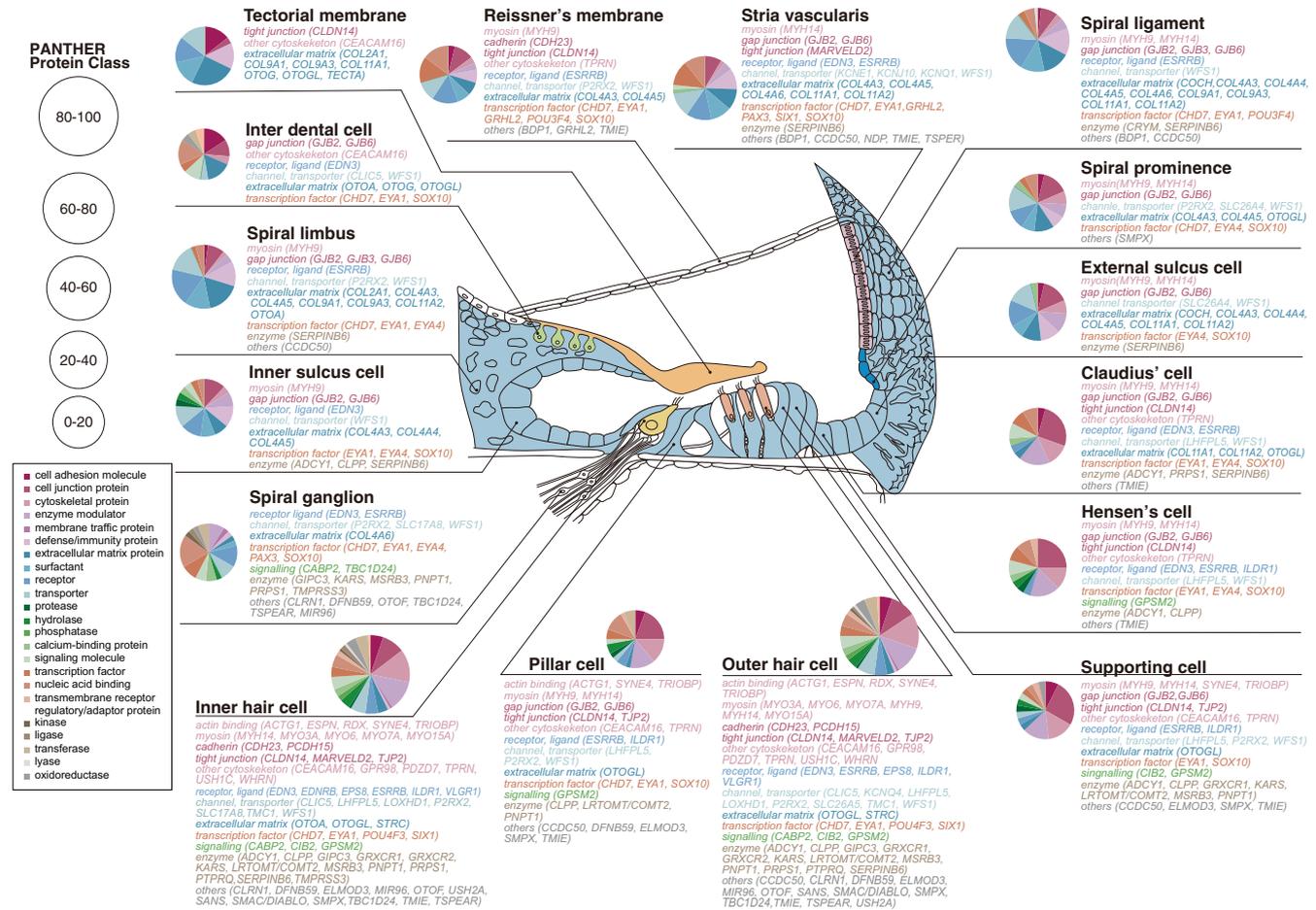


FIGURE 2 The expression sites of causal genes of hearing loss in the cochlea (Nishio et al., 2015).¹¹ The cochlea contains various types of cells that have distinct functions. The genes responsible for genetic deafness expressed in each cell type are shown

genetic frequency needs to be determined, but also the type of cells in which each gene is expressed and the role it plays in cellular function. The cochlea, which has a highly complex structure, consists of various types of cells that possess functionally distinct roles owing to their distinct gene expression patterns (Figure 2).¹¹ For example, of the prevalent genes responsible for hearing loss shown in Table 1, those that are mainly expressed in the inner/outer hair cells include *MYO15A*,²⁶ *MYO7A*,²⁹ *USH2A*,³⁵ *CDH23*,³⁸ *ADGRV1*,⁴⁴ *TMC1*,⁴⁶ and *PCDH15*,³⁸ while *STRC*¹⁷ is mainly expressed in the outer hair cells and *OTOF*⁵² is mainly expressed in the inner hair cells. Moreover, *GJB2*¹² is reportedly expressed in the supporting cells, spiral limbus, and lateral wall, while *SLC26A4*¹⁹ is expressed in the outer sulcus and the epithelial cells of the spiral prominence, *TECTA*²³ is expressed from the inter dental cells and encoding protein is localized in tectorial membrane, and *TMPRSS3*⁵⁴ is expressed in the hair cells and spiral ganglion. In the development of gene therapy, the target cells of the gene transfer will depend on where the target gene is expressed in the inner ear.

In addition, effective gene therapy for hearing loss requires an understanding of the audioprofile of deafness genes. In hereditary hearing loss, the time of onset, severity, and presence/absence of progression vary depending on the gene responsible. For example, *GJB2*-related hearing loss, which is inherited in an autosomal recessive manner, is congenital with mild-to-profound hearing loss that rarely

progresses.¹³ Similarly, *STRC*,¹⁸ *TECTA*,^{24,25} *USH2A*,³⁶ and *ADGRV1*-related⁶ hearing loss are congenital with no progression, but the severity of hearing loss is relatively mild. *SLC26A4* causes congenital and fluctuating progressive hearing loss.²⁰ In contrast, *MYO15A*,²⁷ *MYO7A*,³⁰⁻³² *CDH23*,^{39,40} and *TMPRSS3*⁵⁵ are responsible for congenital profound or progressive hearing loss. *CDH23* and *PCDH15* are responsible for both NSHL and Usher syndrome. For these two genes, a genotype-phenotype correlation has been reported, and Usher syndrome is known to result from a truncating mutation.^{50,57} In *CDH23*-related NSHL, hearing loss is progressive, starting with high-frequency hearing loss that gradually affects the low frequencies and, finally, hearing loss occurs across all frequencies.³⁹ Moreover, for several deafness genes, the course of disease reportedly varies according to ethnicity.⁵⁸ Understanding the audioprofile for each deafness gene enables prediction of the appropriate therapeutic time window for gene therapy intervention.

5 | LIMITATIONS OF MOUSE MODELS FOR HEREDITARY HEARING LOSS FOR PRECLINICAL STUDY

When considering the application of gene therapy to humans, preclinical studies using mouse models of hereditary hearing loss are essential

TABLE 1 Summary of hearing loss phenotype and gene expression profiles of the prevalent deafness genes

Gene (OMIM)	Length (bp)	Locus (OMIM)	Hearing loss phenotype in human	Expression profiles in the cochlea	Mouse model	Hearing loss phenotype in mice	Reference
<i>GJB2</i>	2290	DFNB1A	Congenital mild to profound	SCs	Conditional <i>Gjb2</i> KO mouse	Moderate	12-16
	[NM_004004.6]	DFNA3A	Late onset mild to moderate		Tg Cx26 R75W	Profound	
<i>STRC</i>	5515	DFNB16	Congenital/childhood onset mild to moderate, nonprogressive	Stereocilia of the OHC	<i>Strc</i> ^{-/-}	Progressive	17,18
	[NM_153700.2]						
<i>SLC26A4</i>	4737	DFNB4	Congenital, high-frequency, fluctuating	Outer sulcus	<i>Pds</i> ^{-/-}	Profound	19-22
	[NM_000441.2]			Spiral prominence epithelial cells	<i>Slc26a4</i> insufficient	Fluctuating	
<i>TECTA</i>	7353	DFNA12	Mild to moderate, mid- or high-frequency	Tectorial membrane	(None)		23-25
	[NM_005422.4]						
<i>MYO15A</i>	11 811	DFNB3	Congenital profound or late onset moderate to severe, progressive	HCs	Shaker-2	Profound	26-28
	[NM_016239.4]						
<i>MYO7A</i>	7483	DFNA11	Late onset mild to severe, progressive	HCs, cuticular plate, synaptic region	Headbanger	Low frequency	29-34
	[NM_000260.4]	DFNB2, USH1B	Congenital profound		Shaker-1	Progressive	
<i>USH2A</i>	6372	USH2A	Congenital moderate to severe	Ankle link of the HCs	<i>Ush2a</i> ^{-/-}	Moderate, nonprogressive	35-37
	[NM_007123.6]						
<i>CDH23</i>	11 138	DFNB12	Moderate to profound, high-frequency progressive	Tip link of the HCs	<i>salsa</i> , C57BL/6J	Progressive	38-43
	[NM_022124.6]	USH1D	Congenital profound		Waltzer	Early-onset profound	
<i>ADGRV1</i>	19 557	USH2C	High-frequency	Ankle link of the HCs	CBA-2 ^{ush/ush}	Severe	6,44,45
	[NM_032119.4]						
<i>TMC1</i>	5340	DFNA36	Postlingual progressive	HCs	Beethoven	Progressive	46-49
	[NM_138691.3]	DFNB7/11	Prelingual, severe to profound		Baringo	Early-onset profound	
<i>PCDH15</i>	6962	DFNB23	Prelingual profound	Tip link of the HCs	Ames waltzer	Profound	38,50,51
	[NM_033056.4]	USH1F	Prelingual profound				
<i>OTOF</i>	7214	DFNB9	Congenital or prelingual severe to profound	Synaptic vesicles of the IHC	<i>Otof</i> ^{-/-}	Profound	52,53
	[NM_194248.3]						
<i>TMPRSS3</i>	2399	DFNB8/10	Congenital severe or postlingual progressive, high frequency	HCs, SGNs	<i>Tmprss3</i> ^{Y260X/Y260X}	Profound	54-56
	[NM_024022.4]						

Abbreviations: HC, hair cell; IHC, inner hair cell; KO, knockout; OHC, outer hair cell; SC, supporting cell; SGN, spiral ganglion neuron.

(Table 1). However, mouse models of deafness that possess similar phenotypes and mutations as in humans with hearing loss are extremely rare. For example, truncating mutations (c.235delC in Asian countries or c.35delG in the non-Asian population) in *GJB2* are commonly reported in humans,⁵⁹ whereas *GJB2* knockout is known to be lethal in embryonic mice.⁶⁰ At present, only conditional *GJB2* knockout has been reported in a mouse model for the most common, autosomal recessive, *GJB2*-related hearing loss.¹⁴ A mouse model for *TMC1*-related hearing loss, named Beethoven mice, demonstrates a similar genetic mutation, hearing loss severity, and presence/absence of progression as in humans.⁴⁷ Beethoven mice, generated by ENU mutagenesis, have a c.1235T>A mutation, which is orthologous to the *TMC1* mutation found in patients with hearing loss.^{61,62} As a phenotype of hearing loss, both are known to exhibit progressive hearing loss; thus, this is one of ideal mouse models for the study of human hereditary hearing loss. Unfortunately, it remains difficult to generate mice with mutations orthologous to those in each gene responsible for human hearing loss, and that exhibit similar hearing loss to that in humans. In preclinical studies, mice with a similar hearing loss phenotype to that in humans are considered useful. However, regenerating the lost hair cells and a denatured cochlear structure is considered extremely difficult; thus, using a mouse model of hereditary hearing loss exhibiting progressive hearing loss instead of congenital profound hearing loss is considered a straightforward strategy.⁶³ In addition, the therapeutic effectiveness of a model with progressive hearing loss is expected to be poor unless intervention is performed before irreversible changes occur.⁶⁴ Mouse models for *CDH23*-related hearing loss, *salsa*⁴¹ and *C57BL/6J*⁴² mice, are attractive as they exhibit similar progressive hearing loss to that in humans. For congenital hearing loss, a mouse model of hearing loss caused by *OTOF*, associated with neurotransmission and not cochlear architecture, reportedly provides a good target to determine therapeutic effectiveness.⁶⁵ Furthermore, attention needs to be paid to the differences in inner ear development between mice and humans. The inner ear matures by 26 weeks' gestation in humans, whereas in mice, the inner ear does not mature until 15 days after birth. Therefore, the results of studies using neonatal mice are difficult to apply directly to humans, and experiments using adult mice are needed for clinical application in humans.⁶⁴

6 | GENE THERAPY STRATEGIES FOR HEREDITARY HEARING LOSS

HAs and CIs are effective therapeutic devices for sensorineural hearing loss. However, gene therapy is expected to be a therapeutic method that can provide curative treatment.³ Moreover, gene therapy may potentially suppress hearing loss progression or restore hearing function, which cannot be achieved with HAs or CIs. Gene therapy primarily includes three approaches. The first approach, "gene replacement," is the most common method by which a functional protein is supplied by delivering a normal gene. The appropriate candidates for this strategy are inherited disorders caused by loss-of-function mutations, such as recessive diseases. The second approach, "gene silencing," is a method to treat diseases with gain-of-function mutations by suppressing the expression of the mutated

gene. The third approach, "gene editing," enables correction of pathogenic variants by genome editing, such as the CRISPR/Cas9 system.

7 | SELECTION OF TARGETED GENES, VECTORS, AND DELIVERY ROUTES

In the selection of any gene therapy strategy, genetic material needs to be delivered to the inner ear by use of a vector. Some papers have already summarized delivery routes and viral vectors for cochlear gene therapy.^{3,66} Established routes for direct local administration have included (a) round window membrane (RWM) injection, (b) RWM injection with semi-circular canal fenestration, (c) cochleostomy, and (d) canalostomy (Figure 3).^{3,66,67} Among them, the two injection routes via the RWM enable the vector to be delivered into the perilymph, which is clinically feasible. While a number of viral or non-viral vectors have been reported, adeno-associated virus vectors (AAVs) have been used in most hereditary hearing loss studies due to their non-pathogenicity or minimal immunogenicity.⁶⁶ There are several AAV serotypes, each of which exhibits distinct tropism. AAV cell tropism, as dictated by virus capsid proteins, is an important factor affecting transduction efficiency and specificity across cell types. The gene transfer efficiency to the murine inner ear by AAV vectors depends on the titer and promoter of the AAV, mouse age, delivery route, and presence/absence of an enhancer, in addition to tropism.⁶⁶ As approximately one-third of deafness genes are expressed in the hair cells,⁶⁸ AAV serotypes that enable efficient gene transfer to the hair cells are important. We previously reported that AAV2/2 exhibited the highest total transduction rate for both the inner and outer hair cells when AAV serotypes 2/1, 2/2, 2/8, 2/9, and 2/Anc80L65 were introduced into the adult murine cochlea under the same titer.⁶⁹ Omichi et al⁶⁶ summarized the tropism of AAV serotypes in the adult mouse cochlea. To date, no AAV reportedly enables robust transduction in the supporting cells, in which the most common deafness gene (*GJB2*) is expressed, and in the outer sulcus and the spiral prominence cells, in which *SLC26A4* is expressed; thus, further investigation of appropriate vectors (eg, synthetic AAV capsid) enabling efficient gene transfer to these types of cells is warranted.

8 | TRENDS IN PAST GENE THERAPY RESEARCH

In 2012, Akil et al⁷⁰ first reported the effectiveness of gene therapy in a mouse model of hereditary hearing loss using *Vglut3* knockout mice. These researchers performed gene transfer by administering AAV1 containing *Vglut3* cDNA to the inner ear of mouse pups via the round window and demonstrated improved hearing loss. Similarly, several successful studies in neonatal mice were reported using a gene replacement approach.^{65,71-83} As described above, hereditary hearing loss is largely classified by mode of inheritance, including cases resulting from loss-of-function mutations (autosomal recessive inheritance) and from gain-of-function mutations (autosomal dominant inheritance).³ The therapeutic target of gene replacement is hearing loss due to

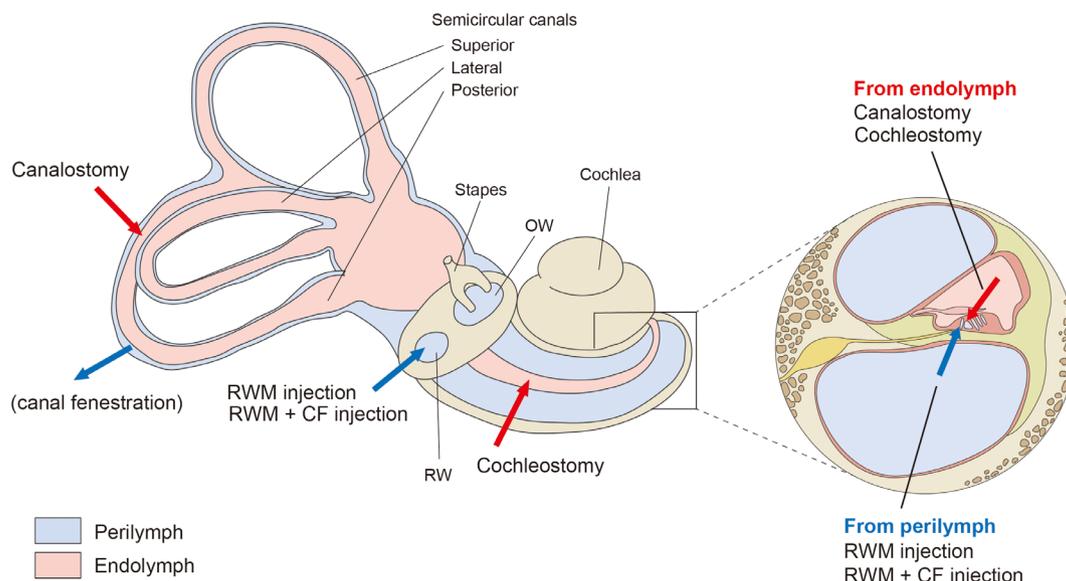


FIGURE 3 Inner ear schematic showing established delivery routes. Vector delivery into the perilymph via a round window membrane (RWM) or a RWM combined with a semicircular canal fenestration (CF); vector delivery into the endolymph via cochleostomy or canalostomy. OW indicates oval window

loss-of-function mutations. Lentz et al⁶⁴ and Shibata et al⁶³ reported the possibility of using gene therapy for hearing loss due to gain-of-function mutations by gene silencing via RNA interference. In addition, Gao et al⁶⁵ reported a study using gene editing in 2018. However, all the above studies were conducted with neonatal mice without mature inner ears (ie, mice younger than 2 weeks of age). Therefore, we performed gene therapy via gene silencing in a mouse model of human hereditary hearing loss in 2- to 8-week-old mice, demonstrating suppression of hearing loss progression, protection of the hair cells, and suppression of their degeneration.⁶⁴ This was the first study reporting the effectiveness of gene therapy in a mouse model of genetic deafness with fully developed inner ears. The targeted allele suppression by miRNA utilized in that study is a mutation-specific therapy that requires miRNAs designed for each targeted variant. Thus, the target patients in any clinical application include only those who have a mutation orthologous to that in mice, which is an extremely limited population. It is impossible to generate a mouse model of genetic deafness to verify the therapeutic effectiveness of manipulating a number of deafness genes as well as genetic mutations. To address this limitation, the effectiveness of gene therapy may be verified in the future using disease-specific induced pluripotent stem cells,⁸⁶ such as the outer sulcus cells in which *SLC26A4* is expressed, as this technique enables induction of differentiation to various cell types in the cochlea.

9 | PRECLINICAL STUDY REQUIRED FOR THE FUTURE DEVELOPMENT OF GENE THERAPY

Phase I/II gene therapy trials for patients with severe-to-profound hearing loss were started in the United States in 2014 ([https://](https://clinicaltrials.gov/ct2/show/NCT02132130)

clinicaltrials.gov/ct2/show/NCT02132130); however, at present, gene therapy for hereditary hearing loss in humans has not been approved. The advantage of gene replacement is that it enables treatment regardless of the mutation position. However, the vector may need to be administered multiple times depending on the period of transduction to the target cells, in which immune responses to AAV in the inner ear need to be verified. In addition, the size of the cDNA that can be packaged using an AAV is approximately 4.7 kb, which may require splitting the transgene into two or three parts. Akil et al⁸⁷ summarized the deafness genes that need to be split to avoid exceeding the packaging capacity of AAV. We previously reported the feasibility of a dual AAV vector approach,⁶⁹ but no triple AAV vector approach has been reported and this warrants further study. In contrast, the disadvantage of gene silencing and gene editing is that miRNA and gRNA need to be designed for each targeted mutation. In gene editing using the CRISPR/Cas9 system, off-target mutations have had serious adverse effects, although gene editing using a base editor that can minimize off-target effects, in which double-stranded DNA breaks are not included, has been reported in recent years⁸⁸ and future advancements are expected. Regardless of the method selected, achievements using prevalent deafness genes as targets in patients with hearing loss are desirable.

10 | MILESTONES TOWARD THE CLINICAL APPLICATION OF GENE THERAPY IN HUMANS

As described above, there are many hurdles to overcome for the clinical application of gene therapy for hereditary hearing loss. However, in humans, it is important to consider how gene therapy will be

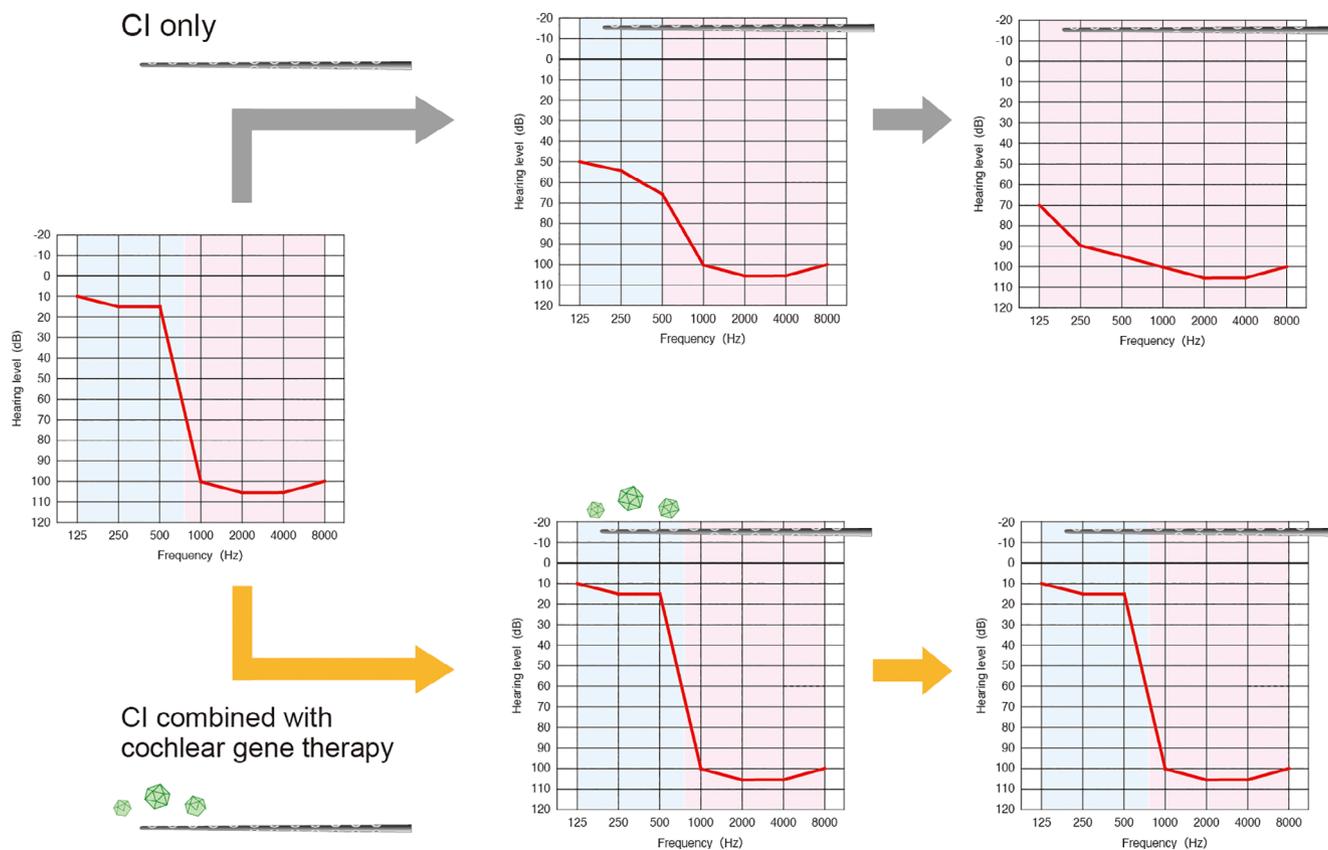


FIGURE 4 The schema of hybrid gene therapy based on a combination with cochlear implants. In patients with high-frequency hearing loss, cochlear implants (CIs) improve hearing ability, while the natural course of low-frequency hearing loss is observed (upper). Residual hearing can be preserved by performing cochlear gene therapy through CIs (lower)

combined with present therapies. At present, the therapeutic options for sensorineural hearing loss are HAs and CIs, which are not biological treatments, but are highly useful. Even in cases of congenital severe-to-profound hearing loss, favorable language development is frequently reported with bilateral CI at an early age. Similarly, the effectiveness of HAs for mild-to-moderate hearing loss has been demonstrated. Thus, even if gene therapy potentially becomes a curative treatment, it is unlikely to immediately become an alternative therapy to CIs and HAs. In addition, even when the gene transfer method to the inner ear is via round window injection, canalostomy, or a combination,^{66,67} the method is invasive and patients with mild-to-moderate hearing loss are expected to prefer non-invasive therapy with HAs. Therefore, starting with a hybrid therapy based on CIs combined with gene therapy for severe-to-profound hearing loss is considered clinically feasible. Drug delivery through CIs has been studied, and administration of neurotrophins and glucocorticosteroids through CIs reportedly reduces insertion trauma, immune reaction, degeneration of spiral ganglion cells, and fibrosis and ossification in cochlear implantation.⁸⁹ Gene transfer to the inner ear through CIs in patients with profound hearing loss is reasonable, and is considered a useful step to confirm the safety of gene therapy delivery. Further, as the achievement successful gene therapy for patients with profound hearing loss may be extremely challenging, patients with genetic

progressive hearing loss, for which CIs are applied, are considered good targets. For example, in most patients with severe-to-profound high-frequency hearing loss with only mild-to-moderate hearing loss at low frequencies, sufficient hearing could not be obtained with HAs. For such ski-slope hearing loss, electric-acoustic stimulation (EAS) was developed to perform acoustic stimulation that amplifies low-frequency residual hearing and deliver electrical stimulation (ES) through a CI so as to improve high-frequency hearing loss with a single device.⁹⁰ Greater improvements in speech recognition in noise, music appreciation, and sound localization by EAS than by ES alone have demonstrated the significance of preserving residual hearing.^{91,92} Hearing at low frequencies is reportedly preserved after cochlear implantation, while most cases of low-frequency hearing loss are progressive; consequently, most patients experience hearing loss across all frequencies as part of the natural course.⁹³⁻⁹⁵ Therefore, preserving low-frequency residual hearing by gene therapy via CIs is clinically significant (Figure 4). This strategy is considered applicable to hereditary hearing loss cases associated with the *CDH23*, *TMPRSS3*, and *ACTG1* genes. In trials of drug delivery through CIs, coating and incorporating drugs into the CI itself is common at present, while a pump will conceivably need to be developed and installed in CIs to deliver multiple drug administration.

11 | CONCLUSION

Herein, we discussed gene therapy research for hereditary hearing loss, focusing on the frequency of deafness genes, hearing loss phenotypes in patients, and mouse models of genetic deafness. If gene therapy can be provided as a precise treatment for hereditary hearing loss in the future, restored hearing function and prevention of hearing loss progression, which cannot be achieved by conventional medicine, can be realized. The number of treatable deafness genes is expected to be expanded by further study, in which research based on data from patients with hearing loss is desirable. In order to actualize gene transfer to the human inner ear, gene therapy in combination with CIs is considered a reasonable strategy for clinical application in humans.

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CONFLICT OF INTEREST

Authors declare no potential conflicts of interest.

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