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Review Article

Recent advances in CRISPR-Cas system for the treatment of genetic hearing loss

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Abstract: Genetic hearing loss has emerged as a significant public health concern that demands attention. Among the various treatment strategies, gene therapy based on gene editing technology is considered the most promising approach for addressing genetic hearing loss by repairing or eliminating mutated genes. The advent of the clustered regularly interspaced short palindromic repeats (CRISPR)-Cas system has revolutionized gene therapy through its remarkable gene editing capabilities. This system has been extensively employed in mammalian gene editing and is currently being evaluated through clinical trials. Against this backdrop, this review aims to provide an overview of recent advances in utilizing the CRISPR-Cas system to treat genetic hearing loss. Additionally, we delve into the primary challenges and prospects associated with the current application of this system in addressing genetic hearing loss.

Keywords: Genetic hearing loss, gene editing, gene therapy, clustered regularly interspaced short palindromic repeats (CRISPR), Cas9, Cas13

Introduction

Hearing loss is prevalent across all human life cycle stages, with environmental and genetic factors playing crucial roles in its onset. As of 2019, the global population affected by hearing loss reached a staggering 1.57 billion people, accounting for one-fifth of the total population. Alarmingly, it is projected that by 2050, this number will soar to 2.45 billion, representing a significant increase of 56.1% from 2019 [1]. Given that approximately 1 in 500 births are affected by genetic hearing loss and genetic factors contribute directly or indirectly to about 50-60% of cases of hearing loss [2], hearing loss caused by genetic factors has emerged as an urgent public health concern that demands attention. Presently, the primary clinical treatments for hearing loss involve hearing aids and cochlear implants. However, their effectiveness is contingent upon the number of residual spiral ganglion cells in the patient and does not fully restore natural hearing [3]. Gene therapy, conversely, holds the potential to address these limitations, as mentioned above, at a fundamental level [4]. Gene therapy encompasses three prominent approaches: gene replacement, gene suppression, and gene editing. Gene replacement involves the introduction of normal genes to synthesize functional proteins, while gene suppression tackles gain-of-function mutations by dampening the expression of mutated genes. Gene editing, on the other hand, addresses diseases through base addition, deletion, and replacement [5]. Of particular interest is gene editing, which can be applied to both dominant and recessive genetic hearing loss and has the potential to yield long-lasting effects by permanently modifying cellular genes. Encouraging breakthroughs have already been achieved in gene editing for other genetically related diseases, as exemplified by ongoing clinical trials of CRISPR therapies targeting conditions like β-thalassemia and sickle cell disease [6]. These success stories pave the way for the potential application of CRISPR-based gene editing therapies to treat genetic hearing loss.

CRISPR-Cas systems

The CRISPR locus was initially discovered in Escherichia coli and serves as a crucial component of bacterial and archaeal immune systems, acting as an adaptive defense mechanism [7]. The transcription and translation products of this locus exhibit specific recognition and binding capabilities towards exogenous nucleic acids. In the presence of Cas proteins, they efficiently degrade these nucleic acids, thereby preventing viral attacks on bacteria and archaea. In vitro studies conducted in 2012 demonstrated that CRISPR-Cas9 can cleave target DNA under the guidance of CRISPR RNA (crRNA) and trans-activating CRISPR RNA (tracrRNA) [8]. Subsequently, in 2013, CRISPR-Cas9 showcased its potential for gene editing in mammalian cells [9]. Since then, the CRISPR-Cas system has undergone significant advancements, with various CRISPR/Cas variants emerging. Consequently, this system has found application in gene therapy for diverse genetic disorders [10-13]. Nucleases employed in gene editing can be classified into two groups based on their DNA recognition patterns. The first group includes zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), which achieve DNA binding through protein-DNA interactions. The second group comprises Cas proteins, which target specific nucleic acid sequences via guide RNA that forms base pairs directly with the target nucleic acid [14]. Compared to the other three classes of nucleases, the CRISPR-Cas system offers greater simplicity and versatility. By simply modifying the base sequence of the guide RNA, it can effectively target different nucleic acid sequences. The CRISPR-Cas system is extensive and can be classified into two classes, six types and thirty-three subtypes [15]. Within this large family, this review will primarily focus on type II CRISPR-Cas9 and type VI CRISPR-Cas13, belonging to class 2. These two types treat hearing loss and represent the most prominent CRISPR-Cas systems.

CRISPR-Cas9, which belongs to type II, consists of three components: the CRISPR RNA (crRNA), the trans-activating crRNA (tracrRNA), and the Cas protein [16], which together form the ribonucleoprotein (RNP) complex to cleave DNA efficiently. The crRNA contains a sequence that can bind to the target DNA base pairing sequence that directs Cas9 RNP to a specific location to form an R-loop. The formation of the R-loop activates the DNA cleavage structural domain of Cas9, RuvC, and His-Asn-His (HNH) to cleave the DNA to form DSBs [17]. The gRNA in CRISPR-Cas9 is formed as a chimeric molecule consisting of tracrRNA and crRNA anteceded by an 18-20-nt spacer sequence complementary to the target DNA adjacent to the protospacer adjacent motif (PAM). The PAM is a 3-nt (NGG) sequence located immediately downstream of the single-guide RNA (sgRNA) target site and together with the first 20-nt of the chimeric sgRNA, which determines the target specificity of the Cas9 endonuclease [18]. The process of gene editing by CRISPR-Cas9 can be summarized as a two-step process: the first step is the expression of nuclear-localized Cas9 protein and gRNA in the target cell and the formation of an RNP complex. The second step is that the RNP complex recognizes the PAM site located near the 3' end of the target site and, under the guidance of the sgRNA Cas9, generates DSBs approximately three bp upstream of the PAM site (**Figure 1A**).

CRISPR-Cas13, which belongs to type VI, differs from type II Cas9 in that it does not require a tracrRNA and consists mainly of crRNA and Cas proteins. The DR region of the crRNA forms a single hairpin flanked on one side by a roughly 20-30-nt target RNA specific to a particular target RNA and mediates its recognition of spacer sequence. In this review, crRNA is referred to as guide RNA or gRNA. After the RNP complex forms a ternary complex with the target RNA, the conformation is rearranged so that the crRNA: target RNA hybrid is surrounded by the nuclease core, and the catalytic nuclease is activated [19, 20]. Unlike CRISPR-Cas9, which cleaves explicitly double-stranded DNA sequences complementary to the crRNA spacer sequence (i.e., target DNA), the Cas13-crRNA complex nonspecifically cleaves its bound activator RNA, as well as any surrounding single-stranded RNA encountered from both phage and host (also called collateral cleavage, collateral cleavage) [21]. The Cas13 protein does not require Protospacer Adjacent Motif (PAM) sequences to recognize its targets and distinguish between autologous and non-autologous nucleic acids. Still, some preference for nucleotides flanking the 3' regions of the protospacer

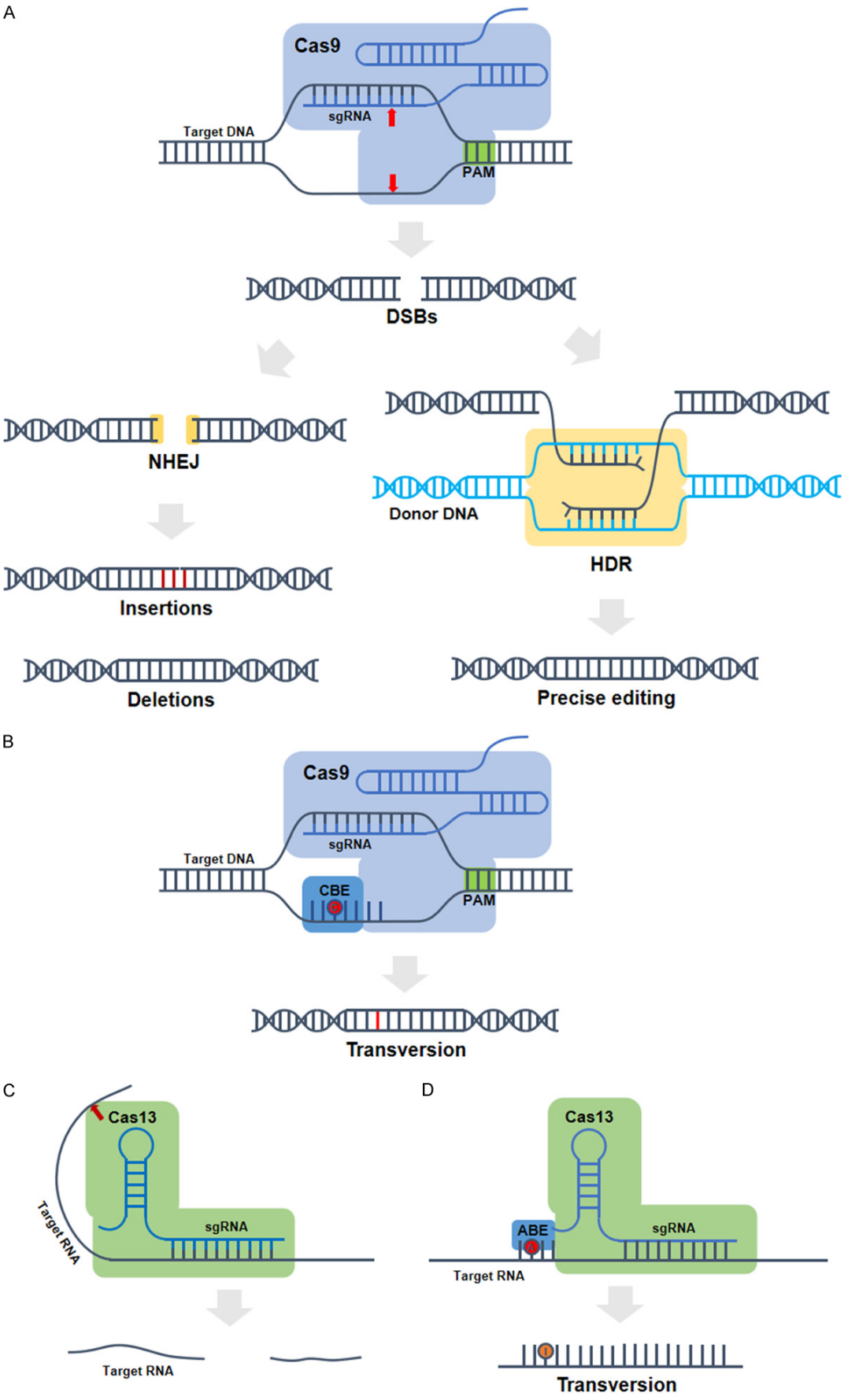
A
Cas9
sgRNA
Target DNA
PAM
DSBs
NHEJ
Donor DNA
HDR
Insertions
Deletions
Precise editing
B
Cas9
sgRNA
Target DNA
CBE
PAM
Transversion
C
Cas13
sgRNA
Target RNA
Target RNA
D
Cas13
ABE
sgRNA
Target RNA
Transversion

Figure 1. CRISPR-Cas-mediated gene editing. A. CRISPR-Cas9-mediated gene editing. Cas9 produces DSBs by shearing the target DNA in the presence of sgRNA. Repair of DSBs through the NHEJ pathway is likely to result in indel mutations, whereas through the HDR pathway, in the presence of donor DNA template, can perform precise repair. B. Combining CRISPR-Cas9 with CBE for single base editing of DNA. C. CRISPR-Cas13-mediated RNA editing. D. Combining CRISPR-Cas13 with ABE for single-base editing of RNA. CRISPR, clustered regularly interspaced short palindromic repeat; sgRNA, single-guide RNA; HDR, homology-directed repair; NHEJ, non-homologous end-joining; DSBs, double-strand breaks; indel, insertions or deletions; ABE, adenine base editor; CBE, cytosine base editors.

has been observed and has been termed the protospacer flanking site (PFS) [22, 23]. We can also summarize the process of gene editing by CRISPR-Cas13 as a two-step process: the first step is to express Cas13 proteins and gRNAs in target cells and form RNP complexes. The second step is for the RNP complexes to form a ternary complex with the target RNAs under the guidance of the sgRNAs, and to cleave the target RNAs (**Figure 1C**).

CRISPR-Cas delivery forms, vectors, and route

The delivery forms of the CRISPR-Cas system can be categorized into DNA, RNA, and RNP complexes. The DNA form refers to the delivery of DNA into the target cell's nucleus, where it is transcribed into mRNA and then translated into Cas proteins, which permits sustained Cas expression [24]. Still, the prolonged-expression also increases the likelihood of off-target effects [25], and the molecular weight of this type of DNA exceeds 6400 kDa to hinder its delivery efficiency [26]. RNA form refers to the delivery of Cas mRNA and gRNA into the cell; compared to the DNA form, it does not require transcription to express Cas protein faster. The stability of mRNA/gRNA is not as good as DNA, so it is difficult to ensure the continuous expression of the Cas protein. RNP is a complex formed by Cas protein and gRNA that enters the nucleus and performs genome editing.

The delivery vectors of the CRISPR-Cas system can be categorized into biological, chemical, and physical methods according to the delivery mechanism into the cells. Biological methods use viruses or cellular components; viral vectors include adeno-associated virus (AAV), AdV, LV, VLP, etc. The main advantage of viral vectors is that they can target a specific type of cell, and their structure can primarily protect the CRISPR-Cas system from degradation by intracellular host enzymes. Still, viruses have disadvantages such as mutation, high immunogenicity [27, 28], and hepatotoxicity [29]. To avoid the risk of immunogenicity of protein components targeting the delivery system, a chemical approach using artificially synthesized materials also seems feasible, with commonly used chemical components including polymers, lipids, or metal. Still, they are incompetent for in vivo use due to the high toxicity of cationic lipids and low transfection efficiency of in vivo tissues because of massive interaction with anionic cellular membranes [26]. There are also physical methods that rely on the physical energy of electricity or ultrasound to deliver the genes into cells, such as electroporation, sonoporation, and microinjection. However, physical methods are mainly limited to in vitro and surface delivery [30-32], and it is difficult to ensure the safety and effectiveness of in vivo delivery to deep organs such as the cochlea, so it may be possible to achieve better delivery results if physical methods are combined with chemical or biological methods.

The primary delivery routes for the CRISPR-Cas system include the round window membrane (RWM), canalostomy, cochleostomy, and RWM combined with canal fenestration (CF) [33]. The RWM is currently the most established and preferred method for inner ear drug delivery, and cochlear implantation is also performed through the round window. However, studies have demonstrated that virus delivered by RWM tends to occur in a base-to-apex gradient in adult mice [34]. Canalostomy is an alternative to RWM, allowing the drug to enter the cochlea and vestibule by injecting it through a window in the posterior semicircular canals. Nevertheless, it remains challenging to determine whether the drug enters the exolymph or endolymph after injection. In contrast, cochleostomy involves directly injecting the drug into the endolymph by creating a hole between the basal turn of the cochlea and the round window. This technique offers better delivery efficiency by allowing the drug to be injected into the scala media. However, it is more traumatic to the ear compared to the previous two options. As previously mentioned, transduction following injection via the RWM route tends to

occur in a base-to-apex gradient in adult mice. Increasing the injection volume only leads to additional hearing loss. Therefore, combining RWM with CF, which enables the drug to flow longitudinally in the inner ear while ensuring uniform distribution, presents a promising approach if transient vestibular dysfunction associated with canal fenestration in the posterior semicircular canals [34] can be effectively avoided. Additionally, Wang et al. demonstrated good safety and delivery efficiency by introducing the drug into the scala media of the cochlea through injection in the lateral wall [35].

CRISPR-mediated hearing loss therapy

As mentioned above, the CRISPR-Cas system is a well-suited technology for application in gene therapy for genetic hearing loss. This section will discuss the CRISPR-Cas systems used for in vivo gene therapy for genetic hearing loss according to CRISPR-Cas targeting DNA and RNA, respectively.

DNA-targeted gene editing

In 2015, CRISPR-Cas9 showed the first in vitro and in vivo gene editing ability for inner ear hair cells [36], laying the foundation for gene editing therapy for genetic hearing loss. Subsequently, CRISPR-Cas9 was applied to treat hearing loss associated with genetic factors such as Cdh23, Klhl18, Tmc1, Kcnq4, and Myo6 (**Table 1**) [37-41].

Double-stranded breaks (DSBs) of DNA generated by CRISPR-Cas9 cleavage of mutant genes rely primarily on the homology-directed repair (HDR) or non-homologous DNA end joining (NHEJ) pathways for rehabilitation [14, 42] (**Figure 1A**). The Cdh23ahl allele is a synonymous single nucleotide polymorphism (SNP) affecting the last nucleotide of the seventh coding exon of the Cdh23 gene (c.753). The presence of an adenine (A) but not guanine (G) at this position results in an increased frequency of exon 7 skipping, predisposing inbred mice carrying the A allele to age-related hearing loss [43]. The researchers injected offset-nicking Cas9 nickase with paired RNA guides and a single-stranded oligonucleotide donor template in C57BL/6NTac zygotes to repair a point mutation in the Cdh23 gene via the HDR pathway. Encouragingly, mice that underwent successful mutation repair exhibited normal hearing thresholds and maintained hair cells and hair bundles at 36 weeks. However, the survival rate of the mice in this study was meager, with only 104 out of the 456 fertilized eggs injected surviving, and only four mice were successfully repaired with mutations [37]. Treatment of Cdh23 mutant mice achieved salvage of hearing loss in mice, but the HDR pathway hardly occurs in nondividing, terminally differentiated cells/organs [14]. It is only carried out in dividing cells, so if it is desired to achieve efficient gene repair in nondividing mammalian cells (including hair cells), efficient gene repair needs to be completed by the HMEJ-based system. The researchers devised a new HMEJ-based strategy using CRISPR/Cas9-mediated cleavage of the transgene donor vector, which contains guide RNA target sites and a homology arm, and division of the targeted site in the genome. The CRISPR/Cas9 knock-in system delivered into the inner ear using AAV successfully corrected the hair cells mutation in Klhl18lowf mice, restored the inner hair cell static cilia morphology, and significantly improved the hearing loss of the mice for up to 6 months after treatment [38].

The NHEJ pathway is the primary DSB repair mechanism but tends to introduce random insertions or deletions without the donor vector, so it is widely used in gene disruption [44-48]. Tmc1 (transmembrane channel-like 1) protein is essential to mechanotransduction channels in mammalian hair cells. Dominant-negative mutation in TMC1 (p.M418K, c.T1253A) cause progressive post-lingual sensorineural hearing loss in humans [49-51]. Beethoven mouse model carrying a homozygous mutation (p.M412K, c.T1235A) in the human Tmc1 gene exhibits progressively elevated auditory response thresholds and progressive loss of hair cells starting at one month [52]. In 2017, researchers used Lipofectamine 2000 to deliver Cas9-sgRNA RNP complexes into the inner ear, disrupting dominant deafness-associated alleles in a Bth mouse model. They succeeded in partially alleviating hearing loss in $Tmc1^{Bth/+}$ mice [39]. The study provides evidence of the efficacy of in vivo disruption of target genes using the CRISPR-Cas system to treat specific autosomal dominant hearing loss disorders.

To achieve more precise targeting and improve the therapeutic efficiency of CRISPR-Cas9, researchers have also tried to use AAV as a delivery vector. Myosin VI (MYO6) is an uncon-

Table 1. Summary of CRISPR-Cas gene therapy for the treatment of genetic hearing loss

Gene	Mutation	Expression site	Forms	Vector	Route	Injection age	CRISPR	Reference
TMC1	p.M412K, c.T1253A	HCs	Plasmid	Lipofectamine 2000	PSCC	P0-2	Cas9	[39]
		HCs	Plasmid	AAV-Anc80	Inner ear	P1-P2	SaCas9-KKH	[67]
		HCs	Plasmid	AAV9-PHP.B	Utricle	P1	SpCas9	[68]
	p.Y182C, c.A545G	HCs	Plasmid	AAV2/Anc80 L65	Inner ear	P1	SpCas9-based AID-BE4max	[62]
MYO6	p.C442Y, c.1325G > A	HCs	Plasmid	AAV-PHP.eB	Scala media	P0-P2	SaCas9-KKH	[41]
KCNQ4	p.W276S, c.830G > C	OHCs	Plasmid	AAV2/Anc80 L	Utricle, PSCC, RWM, scala media	P1-P3	SpCas9	[40]
	p.G228D, c.683G > A	OHCs	Plasmid	AAV-PHP.eB	Scala media	P1-P2	SaCas9-KKH	[69]
PCDH15	av3j “A” insertion	HCs	Plasmid	AAV2/9	Scala media	P0-P2	SpCas9	[44]
KLHL18	p.V55F, Chr9: 110455454 C > A	IHCs	Plasmid	AAV9, AAV-PHP.eB	Inner ear	P1	SaCas9-KKH	[38]
CDH23	753A > G	HCs	Plasmid	Microinjected		One-cell-stage mouse embryos	Cas9	[37]
MYO6	p.C442Y, c.1325G > A	HCs	Plasmid	AAV-PHP.eB	Scala media	P0-P2	Mini dCas13X-based adenine base editor (mxABE)	[66]
TMC1	p.M412K, c.T1253A	HCs	Plasmid	AAV-PHP.eB	RWM	P1-P2	RfxCas13d	[63]

ventional myosin vital for auditory and vestibular function. The MYO6 p.C442Y mutation causes DFNA22. Carriers of the Myo6 p.C442Y mutation begin to develop progressive hearing loss during childhood and show profound sensorineural hearing loss by middle age [53]. The researchers used AAV-PHP.eB as a vector to deliver Cas9 (SaCas9-KKH)-sgRNA complexes into the $Myo6^{WT/C442Y}$ mouse model that recapitulated the phenotypes of human patients. At week 15 post-treatment, auditory brainstem response (ABR) was restored by approximately 17 dB at 16 kHz in AAV-SaCas9-KKH-Myo6-gRNA-treated ears compared to untreated ears, with lower distortion product otoacoustic emission (DPOAE) thresholds, increased outer hair cell survival, and more regular hair bundle morphology. Recovery of auditory function was observed up to 5 months after treatment [41].

The aforementioned studies provide compelling evidence to support the potential of utilizing CRISPR-Cas9 gene editing technology to treat dominant and semi-dominant inherited hearing loss. Furthermore, recent findings have shed light on the non-random nature of CRISPR-Cas9 cleavage-induced insertions/deletions (indels) introduced through the NHEJ pathway in dividing cells, which appear to be dependent on the specific guide RNA sequence [54-59]. The PCDH15 protein is one of two components that form the tip link to gate the mechanotransduction channel in hair cells [60], a key component for Hair cells to detect mechanical force. DFNB23-Pcdh15 av-3J is a code shift mutation in a single adenine (A) nucleobase insertion. This mutation results in the premature appearance of the stop codon to form a truncated PCDH15 protein lacking transmembrane and intracellular structural domains [61]. The researchers introduced non-random CRISPR-Cas9 cleavage-induced insertions/deletions (indels) via the NHEJ pathway to block the early appearance of the stop codon to achieve repair of the shifted-code mutation, and 30 of 52 injected mice developed ABRs at 4-5 weeks; however, no response was observed in mice that were not injected [44].

For hearing loss due to recessive loss-of-function mutations, in addition to precise gene repair under the guidance of a donor, single-base editing offers a therapeutic option that can efficiently and permanently correct the causative mutation without forming DSBs (**Figure 1B**). Using a dual AAV base editing delivery system, researchers delivered cytosine base editing (CBE) into the inner ear of Baringo ($Tmc1^{Y182C/Y182C}$; $Tmc2^{+/+}$) mice, which have a T-A to C-G mutation in the Tmc1 (c.A545G) gene, and succeeded in restoring sensory transduction and hair cell morphology in inner hair cells, and four weeks after injection at 5-35 kHz temporarily rescued 10-20 dB of hearing [62]. Although the degree of hearing salvage is very limited, the experimental results demonstrate that it is entirely feasible to utilize in vivo base editing to restore recessively inherited hearing loss.

Gene editing that targets DNA will cause irreversible genetic changes if it edits non-targeted normal genes, so the off-target effect of gene editing is an essential aspect of judging the safety of gene editing therapy, and it is encouraging to note that no off-target mutations were detected or reported, or only very low off-target mutations were demonstrated in these research (**Table 2**), suggesting that Cas9-based editing has a very good safety profile.

RNA-targeted gene editing

The exploration of utilizing the CRISPR/Cas13 RNA editing system to treat genetic hearing loss is also underway. To evaluate the therapeutic efficacy of CRISPR/Cas13 researchers evaluated the therapeutic efficacy of CRISPR/Cas13 and compared the editing specificity and efficiency of the PspCas13b and CasRx systems. The CasRx and sgRNA were delivered into the inner ear of postnatal mice using AAV-PHP.eB43 as a vector and successfully reduced the transcript of Tmc1 mutants by 70.2% within two weeks and recovered ABR thresholds by 20 to 2 dB at 4 kHz~32 kHz within eight weeks after treatment, with few off-target effects across the transcriptome [63]. This research suggests that CRISPR-CasRx-based RNA editing is a promising therapeutic approach for treating autosomal dominant hearing loss.

In addition to using Cas13 to cleave target RNAs, RNA single-base editors developed based on Cas13 can realize single-base changes at the RNA level by targeted RNA editing techniques (**Figure 1D**). Minibase editors developed based on compact Cas13 proteins that exhibit high targeting and low off-targeting effi-

Table 2. Hearing and hair cell rescue and off-target analysis

Gene	ABR threshold recovery (compared to the untreated group)	DPOAE threshold recovery (compared to the untreated group)	The hair cell	Off-target analysis	Reference
TMC1	Recovered 20 dB~0 dB at 5.66 kHz to 45.24 kHz at 4 weeks	DPOAE thresholds were slightly elevated in the injected ears at 4 weeks	Preserved part of stereocilia of IHCs at 8 weeks	Modification of only one off-target site (off-T1, 1.2% indels) was detected in primary fibroblasts from $Tmc1^{Bth/+}$ mice	[39]
	The mean threshold increased by 40 dB at 8 kHz at 12 weeks	Recovered 40 dB~0 dB at 5 kHz to 30 kHz at 12 weeks	Preserved normal hair bundle at 24 weeks	Did not detect off-target effects in cultures of primary fibroblasts from $Tmc1^{Bth/WT}$ mice	[67]
	Recovered 40 dB~0 dB at 5 kHz to 35 kHz from no response at 4 weeks	Recovered 40 dB~0 dB at 5 kHz to 35 kHz from no response at 4 weeks	IHCs: Rescued: 3.3 in apex, 5.4 in middle, 4.7 in base; OHCs: 15.8 in apex, 10.8 in middle: 2.5 in base at 24 weeks	-	[68]
	Recovered 20 dB~10 dB at 5 kHz to 35 kHz at 4 weeks	None of the treated mice showed recovery of DPOAE	Promoted preservation of normal hair bundle morphology at 4 weeks	No off-target editing at any protospacer position above that of an untreated control sample (≤ 0.1% mutation frequency above the untreated control) at any of the nine tested off-target sites tested	[62]
MYO6	Recovered 30 dB~5 dB at 4 kHz to 32 kHz at 10 weeks	Recovered 10 dB~5 dB at 4 kHz to 32 kHz at 12 weeks	IHCs: Rescued 5.22 in middle, 4.89 in basal; OHCs: 20.34 in middle at 10 months; Observed more organized hair bundles in OHCs and IHCs	No obvious indel mutations were observed in 15 off-target sites in mESCs	[41]
KCNQ4	Recovered 30 dB~10 dB at 6 kHz to 30 kHz at 7 weeks	Recovered 10 dB~0 dB at 6 kHz to 30 kHz at 7 weeks	OHC viability in treated mice was not significantly different from that in non-treated mice at 7 weeks	No off-target editing at any protospacer position above that of an untreated control sample (≤ 0.1% mutation frequency above the untreated control) at any of the nine tested off-target sites tested	[40]
	Recovered 20 dB~10 dB at 5.6 kHz to 32 kHz at 8 weeks	Recovered 15 dB~5 dB at 5.6 kHz to 30 kHz at 8 weeks	The treatment promoted survival of OHCs at middle and basal turns at 12 weeks	No significant editing occurred at the four sites in treated mice compared with the control	[69]
PCDH15	Recovered 20 dB~10 dB at 4 kHz to 32 kHz at 4-5 weeks from no response	-	Restored PCDH15 expression and mechanotransduction of HCS	Based on inner ears collected from treatment, 2 of 16 selected off-target sites showed detectable editing, with average indel frequencies of 0.7% and 0.3%	[44]
KLHL18	Recovered 20 dB~15 dB at 4 kHz to 32 kHz at 12 weeks	Normal DPOAE thresholds for this Klhl18 mutation	Rescued stereocilia morphology in some inner hair cells at 12 weeks	No obvious indel mutations for the off-target sites were detected in homozygous Klhl18lowf fibroblasts	[38]
CDH23	Normal ABR thresholds at 36 weeks	-	Restored normal hair cells and hair bundles	No induced sequence changes were identified at assessed the 'off-target' sites predicted for the guide RNAs (≤ 4 nucleotide mismatches)	[37]
MYO6	Recovered 25 dB~10 dB at 4 kHz to 32 kHz at 12 weeks	Recovered 30 dB~0 dB at 4 kHz to 32 kHz at 12 weeks	OHCs: Rescued 13.20 in middle turn, 29.6 in basal turn	Inducing A-to-I conversion without substantial off-target edits in vivo	[66]
TMC1	Recovered 20 dB~10 dB at 4 kHz to 32 kHz at 4 weeks	Recovered 20 dB~0 dB at 4 kHz to 32 kHz at 4 weeks	OHCs: 10.8 in apical turn, 17.2 in middle turn; IHCs: 10.2 in middle turn at 10 weeks; Preserved hair bundles in the middle turn	No difference in RNA expression for 9 of the top 10 most likely off-target genes, one gene was not detected in vivo compared with untreated mice	[63]

ciency in A to I and C to U substitutions [64, 65] provide for in vivo treatment of genetic disorders using the CRISPR-Cas13 system only via a single AAV vector. Myo6$^{C442Y/+}$ mice have a C-G to T-A change in the mutant gene Myo6 (NM_001039546.1: c.1325G > A), so the researchers used AAV-PHP.eB to deliver the minibase editor, mxABE, into Myo6$^{C442Y/+}$ mice to induce the Myo6C442Y A-to-G conversion of RNA. The treatment rescued auditory function, with ABR at 12 weeks showing 20-10 dB of hearing loss rescued at 4-32 kHz, and DPOAE also showing 20-0 dB of hearing loss rescued at 4-32 kHz [66]. The results demonstrate the potential of RNA base editing therapies to treat autosomal dominant hearing loss associated with hair cell dysfunction and provide complementary strategies to other approaches using RNA interference and DNA alteration.

In conclusion, RNA-targeted gene editing exhibits comparable effectiveness to DNA-targeted gene editing in addressing the manifestations of genetic hearing loss. Throughout the experimental duration, RNA-targeted gene editing demonstrated therapeutic efficacy on par with DNA-targeted gene editing (**Table 2**).

Conclusion

Gene therapy based on the CRISPR-Cas system holds great promise for treating genetic hearing loss as it enables the restoration of regular gene expression at the genetic level. Different Cas proteins in the CRISPR-Cas system are targeted to edit DNA and target RNA, so we discuss recent studies on CRISPR-Cas gene editing technology in inner ear gene therapy in the order of targeting DNA and RNA. The application of the CRISPR-Cas system in the treatment of both dominantly inherited hearing loss and recessive inherited hearing loss has shown surprising results. Still, at the same time, we also found that the delivery forms, vectors, and pathways of the CRISPR-Cas system in the inner ear limit its therapeutic effect, so we also briefly introduce the delivery forms, vectors, and pathways.

The research advancements cited in this review demonstrate the potential of CRISPR-Cas-based gene editing therapies for genetically related hearing loss (**Table 2**). However, several challenges need to be addressed. Currently, most gene therapies based on the CRISPR-Cas system focus on mutations in inner ear hair cells (**Table 1**), and treatments in supporting cells and the stria vascularis remain unexplored. For instance, GJB2, the primary causative mutant gene for genetic hearing loss, is predominantly expressed in non-sensory epithelial cells such as supportive cells. Although attempts at gene therapy for GJB2-associated genetic hearing loss have been made, they mainly involve gene replacement.

In addition, current experiments are modeled on mice. Still, there are significant species-related physiological and anatomical differences between mice and humans. Thus the CRISPR-Cas system will need to be validated for efficacy and safety in animals with genetic mutation patterns and disease phenotypes closer to those of humans, such as non-human primates before it can be applied to humans. Although non-human primates are more comparable to humans regarding genetic mutation patterns and disease phenotypes, they are not the best choice because of their long breeding cycles and high prices. The genetic hearing loss pig model being constructed by our lab and the Army Medical University has a cochlea size, thickness, and strength of the inner ear bone wall, genetic mutation patterns, and disease phenotypes that are highly similar to those of humans, and has a shorter breeding cycle than that of non-human primates. It is reasonably priced and has potential value for evaluating human gene therapy.

CRISPR-Cas system-based gene therapy had a limited rescue effect on hearing function, could not restore the hearing of mutant mice to the level of wild-type, and the rescue effect decreased significantly with the age of the mice, which indicated that the editing efficiency of CRISPR-Cas system-based gene therapy needed to be further improved. Finally, since the ultimate goal of the research is to apply the CRISPR-Cas-based system to humans, its safety is of paramount importance, and there is a need for rational ways to detect off-target effects of gene editing in the human cochlea, as well as the development of new tools to enable the timely termination of off-target gene editing.

Although the current gene therapy based on CRISPR-Cas system has some limitations, with the application of more new and expanded

CRISPR-Cas systems and delivery vectors, the gene transduction efficiency and targeting can be further improved, and it is reasonable to believe that the application scenarios of CRISPR-Cas system in the treatment of genetic hearing loss will be more extensive.

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