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HUMAN GENETICS • REVIEW

CRISPR/Cas9: targeted genome editing for the treatment of hereditary hearing loss

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Abstract

Hereditary hearing loss (HHL) is a neurosensory disorder that affects every 1/500 newborns worldwide and nearly 1/3 people over the age of 65. Congenital deafness is inherited as monogenetic or polygenic disorder. The delicacy, tissue heterogeneity, deep location of the inner ear down the brainstem, and minute quantity of cells present in cochlea are the major challenges for current therapeutic approaches to cure deafness. Targeted genome editing is considered a suitable approach to treat HHL since it can target defective molecular components of auditory transduction to restore normal cochlear function. With the advent of CRISPR/Cas9 technique, targeted genome editing and biomedical research have been revolutionized. The robustness and simplicity of this technology lie in its design and delivery methods. It can directly deliver a complex of Cas9 endonuclease and single guide RNA (sgRNA) into zygote using either vector-mediated stable transfection or transient delivery of ribonucleoproteins complexes. This strategy induces DNA double strand breaks (DSBs) at target site followed by endogenous DNA repairing mechanisms of the cell. CRISPR/Cas9 has been successfully used in model animals to edit hearing genes like calcium and integrin-binding protein 2, myosin VIIA, Xin-actin binding repeat containing 2, leucine-zipper and sterile-alpha motif kinase Zak, epiphycan, transmembrane channel–like protein 1, and cadherin 23. This review discusses the utility of lipid-mediated transient delivery of Cas9/sgRNA complexes, an efficient way to restore hearing in humans, suffering from HHL. Notwithstanding, challenges like PAM requirement, HDR efficiency, off-target activity, and optimized delivery systems need to be addressed.

Keywords CRISPR/Cas9 · Genome editing · Hereditary hearing loss · Genetic deafness

Introduction

Sensorineural hearing loss is a type of deafness wherein hair-cells of inner ear or nerve pathways that lead from ear to brain are damaged. Deafness is categorized on the basis of severity of disease (mild, moderate, severe, profound deafness) as well as location of impairment. Conductive deafness, for instance, is the external or ossicle (middle ear) impairment whereas sensorineural deafness results from cochlear damage; mixed deafness refers to cases where both of these types are involved. Another classification involves age of onset (the disease is either congenital or late-onset), whether the phenotype is progressive (in progressive disease severity increases with age) and whether the disease is syndromic or non-syndromic (Gorlin et al. 1995). It affects 1/500 newborns worldwide and about every third person above 65 years of age. This incidence makes it the most prevalent congenital sensory impairment (WHO 2019; https://www.who.int/news-room/fact-sheets/detail/deafness-and-hearing-loss). Almost 60% of the inherited monogenic or multigenic deafness is triggered by mutations in genetic elements (Balciuniene et al. 1998; Friedman and Griffith 2003; Morton and Nance 2006). Around 70% of inherited cases are non-syndromic (deafness which is not associated with other signs/symptoms) which can be pre-lingual (deafness occurred by birth) or post-lingual

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(deafness arises after the acquisition of speech); the remaining 30% are syndromic (deafness associated with other abnormalities) (Gorlin et al. 1995; Kalatzis and Petit 1998). The inheritance pattern of HHL is mostly autosomal recessive (75 to 80%), then autosomal dominant (12 to 24%) and X-linked (1 to 3%); however, Y-linked and mitochondrial (> 1%) hearing loss is also found (Snoeckx et al. 2005). Due to small and enclosed structure of the inner ear, the cochlea, which is an essential sensory organ, can be separated from the rest of the body via blood-labyrinth barrier where perilymph and endolymph allow liquids to rapidly supply the entire cochlea (Ahituv and Avraham 2002). Because of the delicacy, tissue heterogeneity and critical location of the inner ear down to the brainstem are the major challenges that physically intervene cochlea to be treated through earlier approaches including cochlear implantation, inhibition of hair cell apoptosis, gene manipulation, and stem-cell therapy. Targeted genome editing sounds as the only option left for treating this neurosensory disorder (Kawamoto et al. 2003; Staecker et al. 1998; Suzuki et al. 2000). Targeted genome engineering is a manipulation of a gene into various cell types and organisms. Traditionally, genetic modification is done using homologous recombination (HR) which involves exchange of genetic information between genomic and exogenous DNA during crossing over. However, this event has very low efficiency of about 1 in 108 mammalian cells that restricts its utility for genome editing (Adachi et al. 2006; Rong and Golic 2000). HR is the targeted gene inactivation, basically used for determining gene function (Capecchi 2005). HR approach suffers from low efficacy for designed constructs to be correctly introduced at targeted gene site, is labor-insensitive, and involves laborious selection/screening methods (McManus and Sharp 2002). While targeted gene inactivation using RNAi technology is an economical and high-throughput substitute of HR, it too has some drawbacks such as incomplete gene deletion, off-target activity, and temporary blockage of gene expression; these flaws restrict its practical applications (Carroll 2011; Urnov et al. 2010). To overcome such flaws, precise genetic modifications have been done by using engineered nucleases such as zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), in which sequence-specific DNA-binding modules are joined with non-specific cleavage modules. These chimeric nucleases used to create double-strand DNA breaks (DSBs) at target site followed by DNA repairing mechanisms of the cell include an error-prone non-homologous end joining (NHEJ) and homology-directed repair (HDR) pathways (Chandrasegaran and Carroll 2016). Several factors limit the efficiency of their usage, e.g., specific design expertise, laborious selection methods, and extensive assembly of specific DNA-binding proteins for each targeted gene (Beerli et al. 2000). The emergence of CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas9 (CRISPR-associated protein 9), derived from bacterial type II immune system, has revolutionized precise genome editing by using Cas9 protein guided by 20-nt sequence of single-guide RNA (sgRNA) (Barrangou et al. 2007; Deltcheva et al. 2011; Wiedenheft et al. 2012). The precise genome engineering using CRISPR/Cas9 utilizes a single complex of sgRNA and Cas9 endonuclease (Chandrasegaran and Carroll 2016) where target specificity is entirely dependent upon sgRNA and protospacer adjacent motif (PAM). PAM is usually 3 nucleotide sequence (NGG) that is immediately followed by DNA sequence targeted by Cas9. Upon cutting, cellular endogenous pathways are activated to repair DSBs (Jinek et al. 2012).

Normal hearing physiology and auditory transduction

Mammalian ear is composed of three distinct structures that function as a unit: outer, middle, and inner ear (Fig. 1). The outer ear includes pinna and external ear canal which collects airborne sound waves and directs them towards the eardrum that vibrates and increases loudness (glands and tiny hairs are present in this canal for protection). The middle ear consists of a chain of three ossicles: malleus, incus, and stapes. It does not only collect vibrations established by the ear drum and convey them into oval window of inner ear but also regulates equilibrium. The inner ear consists of a sensory organ called cochlea, and the vestibular system. Vestibular system is comprised of three semicircular canals stimulated by eighth cranial nerve, transmits sounds to the brain, and upholds balance. A spiral-shaped fluid-filled hearing organ called cochlea is composed of three fluid-containing cavities that go to the entire length. Herein, outer two cavities are of perilymph, which are linked with cerebrospinal fluid, and the third fluid-containing cavity is called cochlear duct which secretes endolymph. The organ of Corti is an auditory transduction sensory apparatus that is located at the sensory epithelia of the inner ear and comprised of well-organized finger-like projections called hair cells. Once sound waves reach inside the inner ear, they cause vibrations within fluid of thousands of the hair cells, and these oscillations are further converted into electrical impulses. Hair cells are associated with nerve fibers, transferring impulses into cochlear branch of eighth cranial nerve/auditory nerve towards brainstem. From brainstem, various nerves along auditory pathways lead towards cerebral cortex in temporal lobes of the brain which interprets neural impulses as a sound (Kalatzis and Petit 1998).

CRISPR/Cas9: a bacterial adaptive immune system

CRISPR locus was first identified in *Escherichia coli* (Ishino et al. 1987). Its occurrence in archaea is approximately 84%, while in bacteria, it is about 45% (Grissa et al. 2007). Bacteria

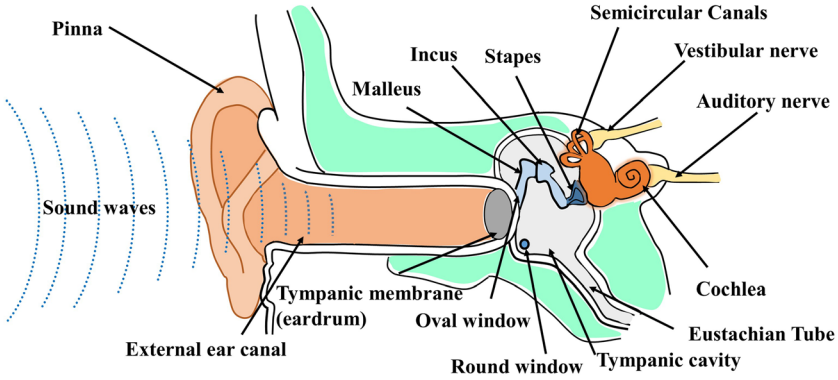

Fig. 1 Mammalian ear consists of 3 main divisions: outer, middle, and inner ear. The outer ear constitutes pinna and external canal, and it collects sound waves and directs them into the middle ear. The middle ear starts with tympanic membrane (eardrum) and 3 bones collectively called ossicles named as malleus, incus, and stapes—the round window, oval window, and eustachian tube. The inner ear, also named as labyrinth of ear, possesses organs of senses of hearing and equilibrium. Temporal bone cavity called bony labyrinth is formed of vestibule, the semicircular canals and cochlea. Inside the bony labyrinth, membranous labyrinth is present which is divided into two sac-like structures named saccule and utricle, and a cochlear duct (hearing part). Hence, from the inner ear, nerve impulses are sent to the brain which interprets them

and archaea possess modified RNA-mediated immune system called CRISPR/Cas9, which defends them against invading viruses and plasmids. Their immune system utilizes short RNAs for sequence-specific recognition, cutting the foreign nucleic acids. The bacterial CRISPR-loci is formed of Cas operon and repeat-spacer array containing genome-targeting sequences (spacers) with integrated identical CRISPR repeats (Bhaya et al. 2011; Terns and Terns 2011; Wiedenheft et al. 2012). In case of viral invasion, bacteria integrate new spacer sequences derived from the attacking viral genome. Both CRISPR and related Cas genes provide resistance against phages, and this resistance specificity is entirely depended upon spacer-phage sequence similarity (Barrangou et al. 2007). Each spacer is an acquired sequence of about 23–44 bp in length, derived from a viral nucleic acid/plasmid DNA which previously invaded the bacteria (Bolotin et al. 2005). It acts as recognition element that finds identical viral genomes and kills them. CRISPR works in association with Cas genes encoding proteins and are essential for immune response (Barrangou et al. 2007; Brouns et al. 2008). To evade autoimmune response, CRISPR/Cas9 has to differentiate between self and non-self (Marraffini and Sontheimer 2010). Foreign DNA having PAM sequence is destined to be cleaved, and potential CRISPR loci targets in the host do not possess PAM; hence, they are not degraded (Garneau et al. 2010; Gasiunas et al. 2012; Sashital et al. 2012).

An effective bacterial CRISPR/Cas defensive response involves 3 major stages (Fig. 2). Adaptation, the first stage, is the attainment of new spacers into CRISPR locus using either naive or primed mechanism. Both of these strategies require protospacer adjacent motif (PAM) and cas1-cas2 complex. Naive spacer integration occurs when there is no previous information regarding target present at CRISPR locus whereas primed spacer acquisition necessitates already present spacer in CRISPR locus that matches the target DNA in the presence of cas3 along cascade complex (Bolotin et al. 2005; Grissa et al. 2007; Jansen et al. 2002). Expression is the second stage where system activation occurs with the expression of Cas9 genes, and transcription of CRISPR leads to the formation of precursor RNA (pre-crRNA) which in turn is converted into mature crRNA via Cas protein and accessory factors. Interference is the last stage in which foreign nucleic acid is recognized by crRNA. crRNA destroys invading DNA presented by Cas protein that works in association with crRNA (Brouns et al. 2008; Hale et al. 2012; Lintner et al. 2011; Semenova et al. 2011; Westra et al. 2012; Wiedenheft et al. 2011; Zhang et al. 2012).

Mouse model creation using CRISPR/Cas9

Based on how it processes pre-crRNA, the CRISPR-Cas system is classified into three types (I, II, and III). Type II system is modified to be frequently used for eukaryotic genome editing because only Cas9 protein is sufficient for tracing and sniping the targeted sequence. Transgenic mouse can be created by direct delivery of CRISPR-Cas type II components into mouse zygote (Nishimasu et al. 2014). Development of mouse-model requires three components: sgRNA of about 125 nt that recognizes target sequence, Cas9 endonuclease enzyme is responsible for DSBs creation, and a donor oligonucleotide/plasmid that contains repairing material, if required. These components are either directly injected into pronucleus or the cytoplasm of fertilized eggs or delivered through electroporation (Qin et al. 2015). In zygote, sgRNA, which is a complex of tracrRNA and crRNA, finds its target in the mouse genome and triggers its nuclease function against DNA complementary to crRNA (Nishimasu et al. 2014). Base pairing between crRNA and target protospacer DNA

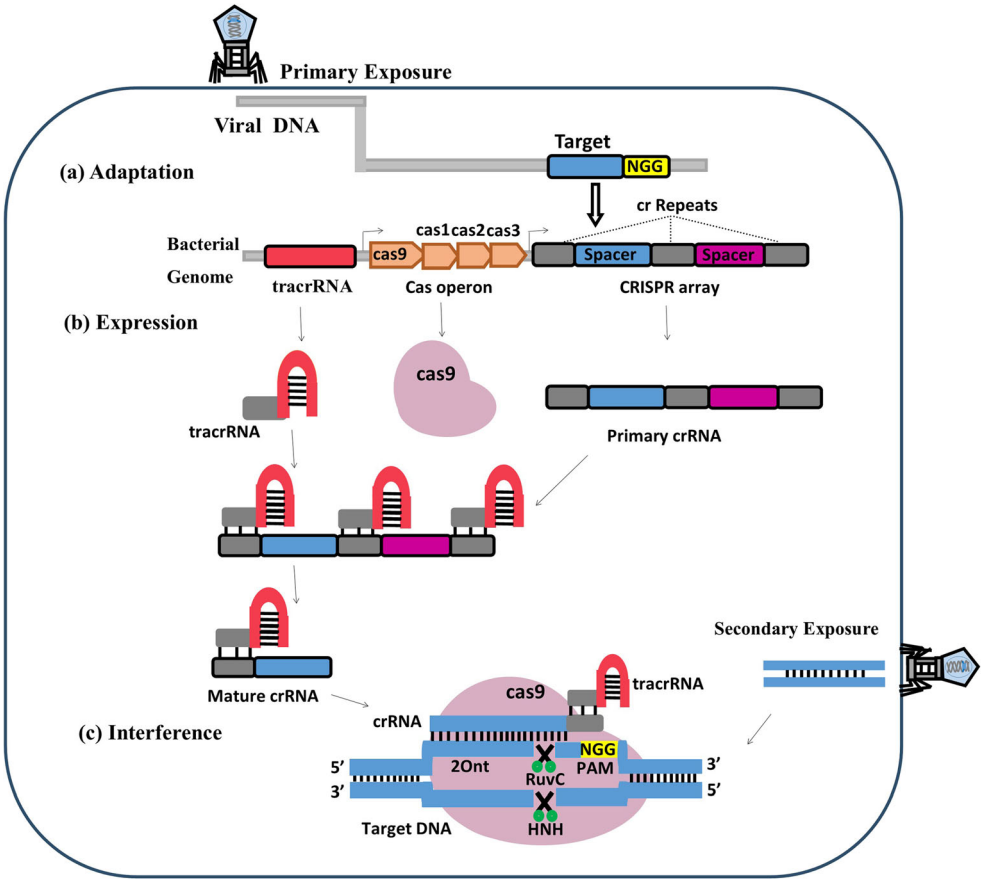

Fig. 2 The bacterial adaptive immune system based on 3 main steps: (**a**) adaptation is a spacer acquisition into CRISPR locus from foreign/viral DNA; (**b**) expression involves transcription of CRISPR locus and processing of crRNA; and (c) interference is the last step which involves target selection having PAM, identification, and breakdown of foreign nucleic acid by gRNA/Cas9 complex

having adjacent PAM determines site-specific DSB generation (Deltcheva et al. 2011). Cas9 endonuclease has a total of six domains (RECI, RECII, Bridge Helix, PAM Interacting, HNH, and RuvC) (Jinek et al. 2012; Nishimasu et al. 2014). The HNH and RuvC are cutting domains. HNH cuts complementary DNA strand while RuvC domain cuts non-complementary strand, and their combined action generates DSB at target site (Chen et al. 2014a; Ran et al. 2013). DNA damage response (DDR) is a coordinated signaling response, which is activated by DSB generation. It promotes DNA repair at DSB and also restricts the expansion of damage in cell using apoptosis or cytostatic mechanisms (Harper and Elledge 2007). DSBs are repaired by two endogenous repairing pathways: If broken ends are joined by error-prone NHEJ, it will leave a scar where frameshift insertions, deletions, and stop-codons are commonly introduced to repair DNA damage. HDR, on the other hand, is the entire DNA renovation that is only possible if donor sequence having homology with target site is inserted along with the nuclease (Low et al. 2014). HDR is a precise repairing method but less efficient. It repairs DSB using sister chromatids as template and is restricted to S and G_2 phases of cell (Richardson and Jasin 2000); however, NHEJ is a simpler and faster method frequently utilized by cells and occurs at all cell stages (Mao et al. 2008; Richardson et al. 2016).

Modes of CRISPR/Cas9 delivery

There are two ways to deliver CRISPR/Cas9 into eukaryotic cells either vector-based delivery for stable expression or Cas9 ribonucleoprotein delivery for transient expression (Fig. 3 and Table 1).

Vector-based stable transfection

Permanent expression requires transgene-mediated vector delivery of CRISPR/Cas9. An all-in-one vector is used for stable delivery of CRISPR/Cas9 into mammalian cells, and for multiplexing or the simultaneous editing of multiple loci (Kim et al. 2014; Sakuma et al. 2014) the multiple gRNAs and a Cas9 are expressed in a single vector to construct all-in-one vector via golden gate cloning. The Cas9 and sgRNA are expressed permanently in case of lentiviral-based transfection (Yin et al. 2016). While in the case of plasmid or adeno-associated virus (AAV)–

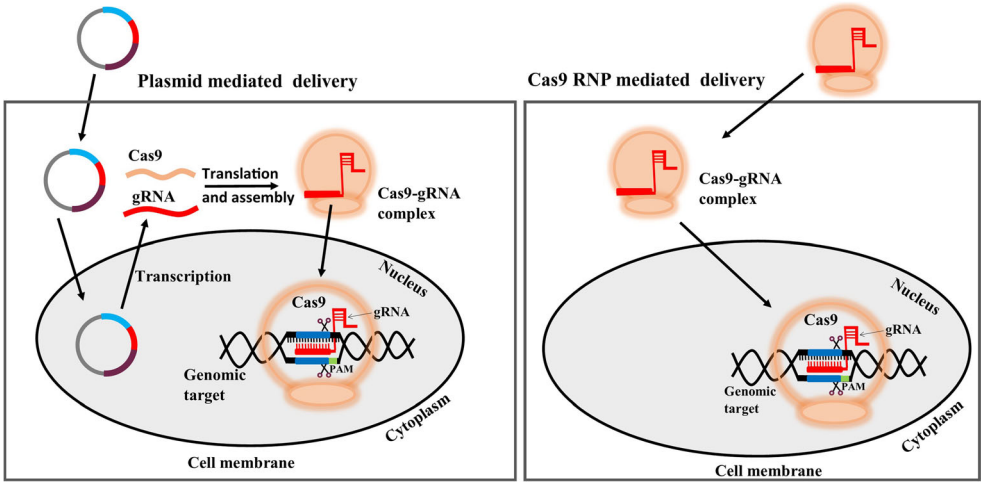

Fig. 3 Modes of CRISPR/Cas9 delivery: Plasmid-mediated delivery requires transcription/translation machinery to be expressed first and assemble as Cas9-ribonucleoprotein complex which then produce permanent mutations, while direct Cas9 RNP-mediated delivery as preassembled complexes into cell (nucleus) provides transient expression of DNA targeting because these are degraded via protein degeneration pathways after performing their function (modified from McDade 2016)

based transfection, expression lasts until the DNA vanishes during cell division (Ran et al. 2015).

Cas9 RNP transient transfection

Ribonucleoproteins (RNPs) are intact complexes of purified Cas9 proteins and in vitro–transcribed gRNA. These are delivered as functional complexes into cells where they induce site-specific mutation after which they are immediately degraded via protein degradation pathways. This immediate clearance of RNPs enhances CRISPR/Cas9 specificity because Cas9 is not available for off-target activity. RNPs are typically delivered into cells by lipid-mediated transfection or electroporation (Liang et al. 2015). Transgene-free RNP delivery drives high rate for HDR, is applied to embryos for quick animal model generation, and is highly implemented on recent genetic engineering applications such as single or multigene deletions of various cell types, genetic modifications using HDR, and long genomic deletions (DeWitt et al. 2017).

Genetics of hearing loss

Genes related to deafness play an important role in ear anatomy such as cochlear fluid homeostasis, stereocilia structure

Table 1 CRISPR expression systems and delivery methods

Expression methods	CRISPR/Cas9 components required	Delivery method	References
Mammalian expression vector	Stable cell lines are generated by expression of (U6) promoter driving CRISPR cassette. GFP-reporter gene is used as selection marker/ to recognize positive cells.	Pronuclear injection is used to attain transient/stable expression of CRISPR/Cas9 in mice cell line	(Mashiko et al. 2013; Mashiko et al. 2014)
Lentiviral transduction	Cas9 and gRNA are expressed into single or separate lentiviral transfer vectors, while packaging and envelope plasmids are co-injected with transfer vectors that encode for lentiviral capsid and envelope.	In vivo delivery via microinjection leads to stable expression of CRISPR/Cas9 into mice cell line.	(Yin et al. 2016)
Adeno-associated virus (AAV) transduction	Compatible with *Staphylococcus aureus* Cas9 (SaCas9) with a packaging limit of 4.5 kb. SaCas9 and gRNA expression cassette are packaged into a single AAV transfer vector and delivered into mice for targeted mutation.	AAV is least toxic method for in vivo delivery and microinjected into dividing or non-dividing cells for stable or transient expression of CRISPR/Cas9.	(Ran et al. 2015)
Cas9 mRNA and gRNA	CRISPR components containing plasmids are in vitro–transcribed to make mature complexes and then transiently delivered into mice cell.	Pronuclear injection leads to transient delivery of CRISPR/Cas9	(Mashiko et al. 2013)
RNPs (ribonucleoprotein complex)	In vitro–transcribed gRNA and Cas9 protein are joined to form Cas9-gRNA complex called RNPs which are transiently expressed into cells and are then degraded via protein degradation pathways.	Transiently delivered into cells via lipid-mediated transfection or electroporation	(Liang et al. 2015)

and functionality, synaptic communication, and gene expression. Mouse models are extensively used to study the pathogenesis associated with these genes. Deafness is frequently caused by the mutation in a single gene or can be a multigenic disorder. Such mutations are mostly single-nucleotide substitutions, insertions/deletions (indels) that lead to missense/non-sense mutations in deafness genes, thereby causing hearing loss (HL) (Angeli et al. 2012; Morton and Nance 2006). Congenital deafness is, in most of the cases, non-syndromic and has a neuroepithelial origin resulted from malfunctioning of the Corti (Yan and Liu 2008). Since the discovery of the first non-syndromic gene in 1993 (Angeli et al. 2012), around 145 chromosomal loci associated with non-syndromic and many other with syndromic deafness have been reported. In addition, X-linked and mitochondrial DNA disorder are known to contribute in deafness (http://hereditaryhearingloss.org) (Shang et al. 2018). These loci are related to diverse gene families and have distinct functionality such as transporters, ion channels, and transcription factors (Hilgert et al. 2009; Yan and Liu 2008). Progressive deafness or age-related hearing loss (ARHL) occurs mainly due to autosomal dominantly inherited genes. Genetic alterations that lead to gradual changes in protein expression are directly related with aging and disease progression. The reason for this progressive nature depends on the extent of malfunction in proteins seeded by genetic mutations over the period of time. In order to have normal transcription and translation levels and to avoid disease onset, mutations/changes in disease-related genes need to be repaired (Liu and Yan 2007).

Potential therapeutic approaches to prevent HL

Genetic mutations cause malfunctioning of auditory hair cells which leads to HL. No hearing aid can work for sensorineural/hereditary deafness albeit available treatments for conductive hearing loss are hearing amplification (for patients with mild to severe deafness) and cochlear implantation (for patients with severe to profound deafness). Cochlear implantation is a good surgical remedy for profound deaf children, but it has certain limitations in efficiency of implants, and outcomes cannot be compared with the native hearing. These cochlear implant amplifies the sense of sound rather than restoring hearing loss in the patients (Kral and O'donoghue 2010). Some gene-specific approaches such as RNA interference; gene therapy; CRISPR/Cas9; and gene non-specific approach, i.e., stem cell therapy, are the available therapeutic options to reestablish the normal organ of Corti and restore hearing in deaf patients.

Hair cell regeneration/stem-cell therapy

Supporting cells lie beneath hair cells, and molecular interactions occur between them to produce normal sense of hearing. Any disruption in these molecular interactions causes loss/damage to hair cells. In avian cochlea, damaged hair cells, induced by noise or ototoxic drugs, can be replaced by regenerating new hair cells either via mitosis of supporting cells or trans-differentiation. In the latter strategy, a mature somatic cell transforms into another mature somatic cell without forming intermediate pluripotent state / progenitor cell-type. Both of these approaches depend on the condition of supporting-cells as well as associated intracochlear drug delivery method (Di Domenico et al. 2011). In an experiment, the potential role of epidermal growth factor (*EGF*) and transforming growth factor-α (*TGFα*) was investigated in which aminoglycoside a toxic agent was used to induce free radicals and permanent damage to neonatal rat organ of Corti grown in a culture (Zine and de Ribaupierre 1998). Quantitative analysis of phalloidin staining of ototoxic cultures showed that auditory epithelium has the potential to replace damaged hair cells during neonatal development if *EGF* or *TGFα* supplemented during recovery process. In comparison with untreated control cultures, ~ 36% and ~ 40% of damaged hair cells were replaced by supplementing *TGFα* and *EGF*, respectively (Zine and de Ribaupierre 1998). Various mice studies proved that naïve cells stimulate forming of supporting cells, and then converting them into hair cells. These naïve cells respond preferentially to molecular signals rather than transdifferentiation because they have limited transdifferentiating ability (Gonzalez et al. 2016). Alternatively, stem cell transplantation has been employed to restore lost cochlear cells by using embryonic stem cells (ESCs), adult stem cells (ASCs), or induced pluripotent stem cells (iPSCs), and act as seeds from which hair cells originated. All stem cells carry general characteristics of self-renewal (cell division with maintenance of undifferentiated state) and differentiation (changing a cell type into more specialized cell type). This differentiation ability of stem cells is objectively utilized to regenerate hair cells and auditory neurons (Clarke et al. 2000; Hu and Ulfendahl 2013; Peng et al. 2014). ESCs are derived from inner-cell mass of mammalian blastocyst and have the potential to differentiate into any tissue of 3 primary germ layers and hence characterized as pluripotent cells. ESCs have been differentiated into hair cells (Oshima et al. 2010), otic sensory neurons (Perny et al. 2017), and spiral ganglion neuron–like cells (Matsuoka et al. 2017). Pluripotency is the most striking feature for clinical applications and cellular therapies, but in humans, ESCs raise serious ethical and safety concerns as embryos are destroyed for their separation, and possess high risk for immunological rejection during transplantation (Hu and Ulfendahl 2013; Volarevic et al. 2018). ASCs preclude such ethical issues but they have limited

pluripotency, so iPSCs have been utilized in most of the studies. iPSCs have gained success in developing hair cells in mice that respond to mechanical transduction (Chen et al. 2018). Stem-cell therapy will be a promising technique for restoring hearing in the near future by overcoming drawbacks of this technology given as follows: hair cells regenerated by stem cells are closely related to vestibular instead of cochlear hair cells (Koehler et al. 2013; Koehler et al. 2017). After culture, stems cells are injected into the inner ear, which are self-inserted into a suitable place within membranous labyrinth. It seems very tricky (Fu et al. 2013). Lastly, carcinogenic potential of these cells must be carefully traced (Sugai et al. 2016). After considering all above challenges, mice can still be deaf if regenerated hair cells have disoriented morphology.

Gene-specific: gene therapy

Gene therapy refers to the replacement of defective gene(s) with a normal or wild-type copy in order to restore the normal functioning of the cell. Patients with hereditary amaurosis and hemophilia are successfully being treated by this approach. The first successful inner-ear gene therapy was done on mice lacking vesicular glutamate transporter-3 (*Vglut3*). These mice were born deaf due to absence of glutamate in inner-ear afferent synapse. The corrected gene copy of *Vglut3* was delivered into cochlea via adeno-associated virus 1 (AAV1). The introduction of corrected *Vglut3* resulted in overexpression within inner hair cells (IHCs) which subsequently rehabilitated the ribbon synapse morphology and startle response and rescued the normal hearing ability. Although, transgene *Vglut3* expression within the inner ear was not confined to IHCs; however, some post-transcriptional regulatory mechanisms, acting on *Vglut3* mRNA, resulted in the selective expression of protein only within IHCs (Akil et al. 2012). There are certain restrictions on the implementation of this study because non-syndromic autosomal recessive HL underlying *Vglut3* is yet to be reported in humans. Adeno-associated virus (AAV) delivery vehicle is used for deafness-related gene therapy; however, the size of the inserts is in excess of 4.7 kB which limits its utility. Moreover, results were effective on P1 time-point but varied level of success was achieved on later time-points of P10-12 because maturation of the inner ear and auditory function did not fully develop at initial stages until P15. Translating the same work in humans can only help to restore hearing if gene therapy works for mature murine ear because humans have matured inner ears by birth (Akil et al. 2012).

Gene suppression by RNA interference

RNA interference is a post-transcription gene-silencing mechanism in which RNA molecules hinder gene expression or translation by specifically neutralizing the targeted mRNA. Its therapeutic applications utilize mainly two types of small RNA molecules—small interfering RNA (siRNA) and micro RNA (miRNA), a novel class of therapeutic agents used to treat various infections and cancers (Maeda et al. 2009). RNAi-based therapeutic drug is now marketed for the treatment of congenital transthyretin-mediated amyloidosis in adults (Adams et al. 2018). Although siRNA and miRNA molecules exhibit similar attributes, both are small duplex RNA molecules and are involved in targeted gene silencing at post-transcriptional level, but differ in their modes of action and medical implementations. Artificial miRNAs are synthesized to complementary base pair with targeted mRNA sequence. These artificial miRNAs similar to designer siRNA function as RNAi-mediated gene-specific or even allele-specific mRNA repression (Kim et al. 2009). In a study, siRNA was used to post-transcriptionally silence the expression of *R75W*-allele variant of *GjB2* gene that was previously involved in causing deafness by dominant-negative effect in vivo in mice (Maeda et al. 2005). Based on these consequences, another study utilized a single intracochlear injection of artificial miRNA carried in viral vector, to slow down the progressiveness of HL in Beethoven mouse treated at P1-2 time-point, which selectively silenced mutated deafness-causing allele of transmembrane channel–like protein 1 (*TMC1*) (Shibata et al. 2016). Reconsidering the same prospective in another study showed miRNA-mediated silencing at P15- or P30-treated adult mice could slow down the progressive HL but resulted outcomes of this experiment were not dramatic because no significant effects were observed in mice treated at P60 duration. These results suggested that RNAi-mediated suppression of *TMC1*-related deafness is temporally defined and above certain time-point mutated allele suppression has no impact on HL. This experiment rose the question whether these restrictions are valid for all types of gene therapies targeting *TMC1*-related deafness or other genetic HL exhibit temporal windows for treatment? For addressing this question, further extensive research is required for continual development of gene therapy as an option to restore hearing in humans (Yoshimura et al. 2019). Moreover, in vitro–transcribed mRNA delivery itself provokes challenges of immunogenicity and RNA constancy, i.e., longer half-life and chemical stability of transcript should be ensured, otherwise unmodified RNAs will be degraded by RNases present within cells.

Hearing loss treatment with CRISPR/Cas9 technology

Harmful genetic alterations causing onset of disease can be avoided by targeting hair cells via expressing/delivering CRISPR/Cas9 components. The CRISPR/Cas9 technology

has successfully been used to interrupt dominant as well as recessive mutations as an efficient treatment of deafness (O'Connell et al. 2014). CRISPR/Cas9 editing is done in altered inner ear wherein knocking out the mutation would lead to regain hearing. The dominant form of deafness is treated in a human genetic mice model of *TMC1*, called Beethoven. The Beethoven has a point of mutation (transversion of 1235T into A) in *TMC1*, leads to progressive decrease in hair cells, and causes deafness in heterozygous mice of 3 days after birth. In vivo injection of Cas9-gRNA-lipid complexes is given into cochlea of neonatal (1–2 days old) Beethoven mouse. These Cas9-gRNA complexes effectively target mutant dominant *TMC1* allele that is different from wild-type allele by a single transversion. A DSB is induced by CRISPR/Cas9, and random indels are introduced by cells' endogenous repairing mechanisms around mutation locus which lead to inactivation of mutant allele, but the wild-type allele remained unharmed to carry normal hair cell mechanotransduction. CRISPR/Cas9 improves hair cells and restores hearing in genetically deaf Beethoven mice (Gao et al. 2018). This *TMC1* mutation is also reported in humans, CRISPR/Cas9 editing can potentially treat deaf patients having same *TMC1*mutation as treated in Beethoven model or other autosomal mutations (Zhao et al. 2014).

Cas9 is a versatile tool for genetic engineering and gene regulation. It was initially supposed to be incompetent for targeting RNA (Gasiunas et al. 2012), but now, by using specially designed PAM-presenting oligonucleotides (PAMers), Cas9 can be directed to bind single-stranded RNA (ssRNA) targets corresponding to Cas9-associasted gRNA sequence when PAM is offered in trans as an isolated DNA oligonucleotide. It cuts ssRNA target while avoiding corresponding DNA sequence. PAM initiates site-specific endonucleolytic cutting of ssRNA similar to PAM-mediated Cas9-catalyzed DNA cleavage (Sternberg et al. 2014). This approach is used to separate GAPDH mRNA from HeLa cells (Chu et al. 2011; Engreitz et al. 2013; Simon et al. 2011).

In some conditions while editing through CRISPR/Cas9, constant supply of Cas9/gRNA is given by viral delivery. CRISPR/Cas9 has been used to correct genetic disorders by constant viral supply of Cas9/gRNA components which results in a permanent nucleoprotein complex establishment and cures the disorder forever. However, transient delivery of CRISPR/Cas9components is generally preferred because it decreases the hazards of continual genome editing. Another emerging treatment for hereditary deafness is the supply of Cas9/gRNA components in protein/nucleic acid complex for the implementations of genetic engineering in the inner ear structure. A complex is generated between protein Cas9 and nucleic acid gRNA due to cationic lipids, and is injected into the inner ear hair cells in vivo (Zuris et al. 2015). These complexes are used to edit the genome by deleting the GFP-signal in Atoh1-GFP mouse and removing indels via HST sequencing. The transient delivery of RNPs is successfully used to edit outer hair cells because it reduces toxicity caused in case of permanent expression, and after creating indels, these RNPs are immediately degraded by protein degradation pathways and do not find enough time to perform off-target activity. Various other approaches using super-charged proteins to transport Cas9/gRNA into inner ear cell types are being used. Instant application of this approach is to engineer gRNA which targets dominant hair cell MYO7 mutations in Usher 1B for deletion via NHEJ repairing. Effortless design of CRISPR/Cas9 makes it viable to research on hair cell genetics by designing gRNA according to gene of choice. For hearing research, in vivo gene correction of the inner ear via HDR-mediated repairing has a little progress due to low efficacy of HDR (< 1%) as compared with NHEJ (> 85%). Improving its efficacy is the first prerequisite for its wide application (Cho et al. 2013; Cong et al. 2012; Jinek et al. 2012; Mali et al. 2013).

Human deafness genes are experimentally studied by using vertebrate models for hearing loss. CRISPR/Cas9 genetic engineering is applied on model organisms to decrease the mutation level and to gain knowledge of the disease pathology. Embryonic stem cells are designed by NHEJ repairing method to form deletion models whereas single-nucleotide mutation/insertion models are created using HDR repairing method (Akil et al. 2012). The following human deafness genes have been successfully edited using the CRISPR/Cas9 technology to regain hearing.

Transmembrane channel–like protein 1

TMC1 gene is destined to encode transmembrane protein and has a specific role in mechanotransduction channels of mammalian hair cells (Pan et al. 2013). A single letter change in the genetic code of *TMC1* can cause recessive and dominant deafness in humans, and this mutation leads to progressive deafness which results from the loss of inner ear's hair cells over time (Van Camp and Smith 2006). In vivo delivery of Cas9-sgRNA complex can lead to allele-specific gene disruption in Beethoven (Bth) mouse model of human genetic disease. A single Cas9-Tmc1-mut3-lipid injection into the inner ear of *Tmc1Bth*/+ mice would cure autosomal dominant progressive hearing loss relating hair cell dysfunction by inactivating mutant allele. After 8 weeks, healthy hair cells appeared in these mice (Zuris et al. 2015).

Leucine-zipper and sterile-alpha motif kinase Zak

Sterile alpha motif (*SAM*) and *leucine-zipper containing kinase* (*ZAK*), a human gene, sometimes also referred to as

ZAK, is expressed in mouse cochlea (Yang 2002; Francis et al. 2013). Alterations in SAM domain of protein kinase *ZAK* would lead to an autosomal recessive disease categorized by split-foot/split-hand malformation (SHFM) and hearing loss (Spielmann et al. 2016). *ZAK* is a member of MARKKK family and regulates the growth, differentiation, and gene expression (Cheng et al. 2009). CRISPR/Cas9-mediated deletion of two *ZAK* isoforms is done via single gRNA in exon 2 and transfection of CRISPR construct into mouse embryonic stem cells. This mutation is fatal for mice embryos (Kraft et al. 2015; Lupiáñez et al. 2015), while SAM domain deletion via CRISPR/Cas9 ultimately resulted in a complex hind limb defect/hearing loss and downregulation of *SHFM* gene such as Trp63 (Ruf et al. 2011).

Xin-actin binding repeat containing 2

Xin-actin binding repeat containing 2 (*XIRP2*) gene was initially reported for cardiac and skeletal muscles (Wang et al. 2012), but its genetic locus intersects two human deafness loci such as DFNB27 and DFNB16; this makes it vital for deafness study (Fukushima et al. 1999; Pulleyn et al. 2000). *Xirp2* is located in stereocilia and pericuticular area of hair cell. Hair bundle maintains a tiny splice isoform which is different from variant expressed in striated muscle. To knockout *XIRP2* gene in vivo and create null mutant mouse-line, the CRISPR/Cas9 technology is used (Fu et al. 2013; Hsu et al. 2013). It results into a deaf mouse and provides evidence that *Xirp2* is crucial for hearing function (Cong et al. 2013). Hair bundle damage and disruption of *Xirp2* function at cellular junction would result in progressive high-frequency deafness (Wang et al. 2013).

Epiphycan

Epiphycan (*EPYC*) is a member of leucine-rich repeat proteoglycan family. Its transcript is mainly expressed in cochlea and present in supporting cells of Corti of neonatal and adult mice. Knockout (KO) models were created via the CRISPR/Cas9 technology. Abnormal cochlear morphology was not observed in *EPYC* KO mice (Mashiko et al. 2013), and their brain stem measurement revealed *EPYC* KO mice have a raised hearing threshold above 16, 24, and 32 kHz which depicts *EPYC* has an important role in normal auditory function (Chen et al. 2014b; Kono et al. 2007).

Calcium- and integrin-binding protein 2

Calcium- and integrin-binding protein 2 (*CIB2*) belongs to calcium- and integrin-binding family which contains numerous Ca-binding EF-hand domains. This family has four members named *CIB1*, *CIB2*, *CIB3*, and *CIB4*. From which *CIB1* and *CIB2* are expressed in mice cochlear hair cells (Gentry et al. 2005), and these genes also related with USH protein family. Any genetic change in *CIB2* gene leads to non-syndromic deafness (DFNB48) and syndromic deafness (Usher syndrome type 1J) (Patel et al. 2015; Riazuddin et al. 2012; Seco et al. 2016). For further confirmation of function of these genes in auditory function, *CIB1* and *CIB2* KO mice are generated via the CRISPR/Cas9 technology. Deleting *CIB1* did not influence auditory function in mice, whereas *CIB2* deletion led to profound deafness and stopped mechanoelectrical transduction currents in auditory hair cells (Wang et al. 2017).

Cadherin 23

Cadherin 23 (*CDH23*) gene encodes a member of cadherin superfamily which consists of calcium-dependent cell to cell adhesion glycoproteins and forms hair tip, present in the organ of Corti (Zhang et al. 2017). Any genetic change in it can cause age-related progressive deafness (Liu et al. 2012). To avoid stain-specific genetic alteration as Cdh23ahl, genome editing is done at zygote level of inbred C57BL/6NTac mice via CRISPR/Cas9-mediated HDR in which Cas9 (D10A) nickase enzyme coupled with gRNA and single-stranded oligonucleotide donor (template) has been used to successfully repair defected allele. It genotypically amends defected gene while phenotypically rescues their auditory function (Mianné et al. 2016).

Myosin VIIA

Myosin VIIA (*MYO7A*) gene encodes unusual myosin named myosin VIIA, expressed in cytoplasm and stereocilia of inner/outer hair cells of cochlea which is involved in joining of stereocilia in stereociliary bundles (Hasson et al. 1997). Mutated *MYO7A* gene provides a basis for mice recessive/dominant deafness mutation and Usher syndrome (USH) type 1B, while Usher syndrome is an autosomal recessive disorder and a dual sensory deaf-blindness characterized by deafness and vision loss. Mutated *MYO7A* is also known to be associated with non-syndromic deafness (DFNB2) in humans. Various types of mutations in *MYO7A* can lead to syndromic or non-syndromic types of deafness (Yan and Liu 2010). Instant application of RNP delivery targets dominant hair cell MYO7A mutations in Usher type 1B and inactivates its function by deleting MYO7A gene which is then repaired via NHEJ repairing (Mali et al. 2013).

A few failure stories of CRISPR-mediated editing in humans

CRISPR/Cas9 has been proved as a promising approach to edit genes in model systems, mammalian zygotes, and human cells but there is a severe information gap in understanding DNA repair mechanisms in early human embryos, efficacy, and off-target activity of CRISPR/Cas9 in human preimplantation embryos. To explore CRISPR-mediated editing in humans, tri-pronuclear (3PN) zygotes were objectively utilized. 3PN egg cells fertilized by 2 sperms are non-viable human zygotes that cannot develop into fetus. Results showed that CRISPR components successfully generated DSB at endogenous HBB gene but owing to low efficiency of HDR, edited embryos showed mosaicism. Another endogenous HBD gene homologous to HBB competed to act as a repairing template and hence generated unwanted mutation. Off-target cleavage was also observed in these 3PNS (Liang et al. 2015). In another study, CRISPR components were co-injected into 3PN embryos to introduce CCR5Δ32 allele into them using HDR repairing (Fig. 4). Due to low HDR efficacy, resulting embryos were mosaics. Edited embryos contained CCR5Δ32 allele, while other alleles at the same locus could not be controlled because these persisted as wild type or possessed indels (Kang et al. 2016). In comparison with 2PN embryos, 3PN embryos possess only one extra sperm nucleus and the aforementioned findings suggested that CRISPR-based approach can be used to correct disease-causing mutations in early human embryos if ethical and scientific concerns are resolved.

Future challenges and prospects

Commonly used *Streptococcus pyogenes* Cas9 (SpCas9) requires that 5′NGG′3 PAM is present after every 8–12 bp in human genome. This prerequisite restricts CRISPR/Cas9 design and limits single base pair editing because there might be no PAM present in the proximity of the target sequence. To overcome this challenge, PAM-independent target editing strategies should be sought (Kleinstiver et al. 2015). Target scope for CRISPR/Cas9 can be diversified either by deriving Cas9 homologue from other bacteria for identifying variant PAM or using SpCas9 variants with altered PAM recognition (Deltcheva et al. 2011; Kleinstiver et al. 2015). To improve low HDR efficiency, a mutant Cas9 nickase (inactivated one of the two nuclease domains) is used to create a nick at specific site, and when used with 2 adjacent gRNAs, it can decrease off-target activity, and toxicity. HDR-mediated repair could potentially treat recessive mutations underlying deafness (Gasiunas et al. 2012; Jinek et al. 2012). CRISPR/Cas9 genome editing is distinguished from other exiting editing techniques due to its easy design and relatively simple methodology. It has reformed biomedical research and developed novel treatments for many genetic disorders. Cationic-mediated CRISPR/Cas9 in vivo delivery has been proved efficient enough to treat autosomal-dominant HL associated with hair cell dysfunctioning (Shibata et al. 2016). Multiplexed genome editing using CRISPR/Cas9 is applied to edit complex and heterologous sequences in mammalian cells in which many defective genes can be edited concurrently. This unique feature of CRISPR/Cas9 may be used for simultaneous targeting as a treatment for multigenic deafness in the near future. In vivo delivery methods

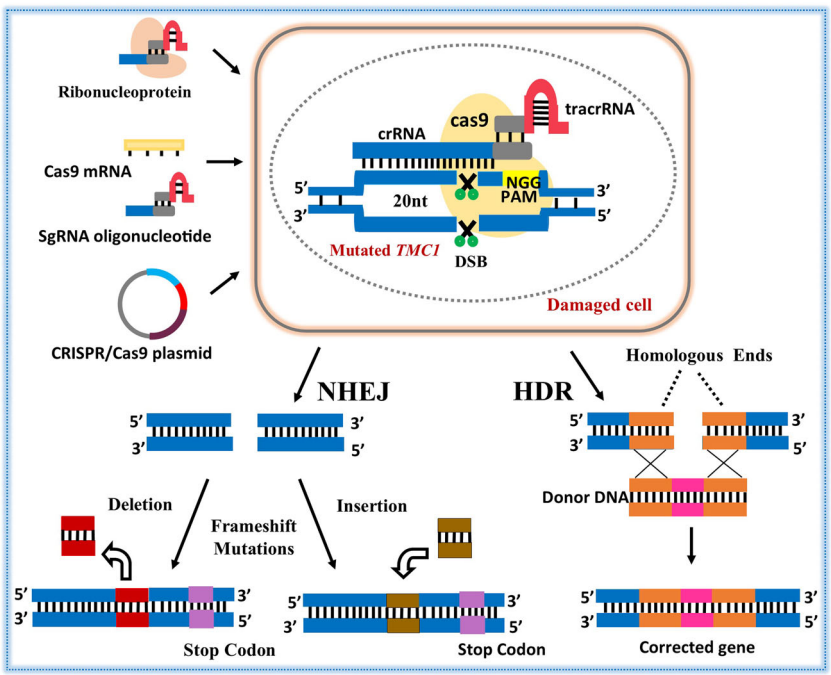

Fig. 4 Different forms of CRISPR/Cas9 components: Plasmid, Cas9 mRNA/SgRNA oligonucleotide, and RNP can be introduced into a damaged cell. gRNA targets mutant *TMC1* allele and recruits Cas9 to create a DSB at target site. Upon DSB generation, cell's endogenous repairing pathways are activated to repair it. NHEJ is an error-prone repairing and creates random indels at target site to inactivate dominant *TMC1* mutant allele while wild-type allele remains intact to carry normal function of hearing. HDR is a precise repairing mechanism which corrects defected gene by replacing it with a corrected *TMC1* sequence (donor/template)

Table 2 Genetically edited deafness genes using CRISPR/Cas9 technology

Gene name	Normal function	Deafness type Genetics	Treatment via CRISPR/Cas9	References
TMC1	Transmembrane channel–like protein 1 has specific role in mechanotransduction channels in hair cells of mammalian inner ear.	Non-syndromic and autosomal recessive/dominant deafness	A single Cas9-Tmc1-mut3-lipid injection into inner ear of *Tmc1Bth*/ + mice cured autosomal dominant progressive hearing loss.	(Gao et al. 2018; Pan et al. 2013)
XIRP2	Xin-actin binding repeat containing 2is located in stereocilia and pericuticular area of hair cell. Hair bundle damage and depletion of Xirp2 function result progressively high-frequency deafness	Non-syndromic and autosomal recessive/dominant pattern	Null mutant mouse line is created via in vivo CRISPR/Cas9 delivery, which resulted in deaf mice showing *XIRP2* is crucial for auditory function.	(Francis et al. 2015)
EPYC	Epiphycan is a member of leucine-rich repeat proteoglycan family, expressed into cochleae and present in supporting cells of Corti of neonatal and adult mice.	Non-syndromic	EPYC knockout (KO) models were created via the CRISPR/Cas9 technology, showing EPYC is essential for normal hearing function.	(Hanada et al. 2017)
ZAK	Leucine-zipper and sterile-alpha motif kinase Zak is a member of MARKKK family of signal transduction molecules. ZAK gene is expressed in mouse cochlea and regulates growth and gene expression.	Syndromic and autosomal recessive	Two *ZAK* isoforms are knocked out via CRISPR/Cas9. SAM domain deletion results in hind limb defect/deafness and downregulation of Trp63 involved in by split--foot/split-hand malformation.	(Gross et al. 2002; Spielmann et al. 2016)
CDH23	Cadherin 23 gene encodes a member of cadherin superfamily and forms hair tips localized in organ of Corti. The mutated CDH23 gene would result profound deafness.	Syndromic USH type 1D/non-syndromic and autosomal recessive/dominant inheritance	Stain-specific mutation asCdh23ahlis cured via CRISPR/Cas9-mediated delivery into zygote of C57BL/6NTac mice	(Mianné et al. 2016)
CIB2	Encodes calcium and integrin-binding protein 2 and a member of family CIB1 to CIB4. While CIB1 and CIB2 genes are present into mice cochlear hair cells.	Non-syndromic/syndromic USH type 1J and inherited as autosomal recessive	*CIB1* and *CIB2* knockout mice were generated via CRISPR/Cas9. But only CIB2 gene depletion influenced the hair function and resulted profound deafness.	(Wang et al. 2017)
MYO7A	Encodes myosin VIIA and is involved in stereocilia maintenance. Any mutation in this gene would result deafness and Usher syndrome type 1B.	Syndromic USH type 1B/non-syndromic and can be inherited as autosomal recessive/dominant.	RNP transient delivery repairs dominant hair cell mutations in Usher 1B, and deleting mutated gene results gene knockdown in mice.	(Yan and Liu 2010; Zou et al. 2015)

should be improved to provide support for treatment of diseases that cannot be targeted ex vivo. Transient protein/nucleic acid delivery would be a potential treatment for congenital deafness in humans because it limits off-target risks. But additional progress in this area is required especially in the case of lipid formulations which can target various cells at different stages. Table 2 provides some examples of mutated genes that have been successfully treated in mouse models to regain hearing and restore hair cell function. A similar approach in humans could potentially restore hearing in deaf patients. This review provides an evidence that precise editing through CRISPR/Cas9 can be used to cure hereditary hearing loss by overcoming the barriers for auditory research.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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