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The Application of Genome Editing in Studying Hearing Loss

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Abstract

Targeted genome editing mediated by clustered, regularly interspaced, short palindromic repeat (CRISPR)/CRISPR-associated nuclease 9 (Cas9) technology has emerged as one of the most powerful tools to study gene functions, and with potential to treat genetic disorders. Hearing loss is one of the most common sensory disorders, affecting approximately 1 in 500 newborns with no treatment. Mutations of inner ear genes contribute to the largest portion of genetic deafness. The simplicity and robustness of CRISPR/Cas9-directed genome editing in human cells and model organisms such as zebrafish, mice and primates make it a promising technology in hearing research. With CRISPR/Cas9 technology, functions of inner ear genes can be studied efficiently by the disruption of normal gene alleles through non-homologous-end-joining (NHEJ)

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mechanism. For genetic hearing loss, CRISPR/Cas9 has potential to repair gene mutations by homology-directed-repair (HDR) or to disrupt dominant mutations by NHEJ, which could restore hearing. Our recent work has shown CRISPR/Cas9-mediated genome editing can be efficiently performed in the mammalian inner ear *in vivo*. Thus, application of CRISPR/Cas9 in hearing research will open up new avenues for understanding the pathology of genetic hearing loss and provide new routes in the development of treatment to restore hearing. In this review, we describe major methodologies currently used for genome editing. We will highlight applications of these technologies in studies of genetic disorders and discuss issues pertaining to applications of CRISPR/Cas9 in auditory systems implicated in genetic hearing loss.

Keywords

Genome editing; CRISPR/Cas9; Hearing loss; Genetics

1. Introduction

Targeted genome editing defined as modification of the genome at a targeted locus, has long been used as a powerful tool to perform genome function studies in biomedical research. Previous methods such as gene knockdown with small interfering RNA (siRNA) and morpholinos have the potential drawbacks of off-targeting (Jackson et al., 2003; Fedorov et al., 2006; Robu et al., 2007) and incomplete editing (Holen et al., 2002; Elbashir et al., 2001; Bill et al., 2009). Conventional gene editing by homologous recombination (HR) can be used to modify genomes in various organisms (Adachi et al., 2006; Meyer et al., 2010; Rong et al., 2000). However, the extremely low efficiency of HR in mammalian cells (ranging from 1 in 10^8 to 1 in 10^5) limits its routine use (Reh et al., 2014). To overcome the shortcomings of these earlier methods, genome editing using programmable nucleases has become a promising alternative.

Three major programmable nucleases have been adapted as genome engineering techniques: Zinc finger nucleases (ZFNs), transcriptional activator-like effector nucleases (TALENs), and CRISPR/Cas9. All three nucleases can be guided to induce site-specific DNA double-strand breaks (DSBs), which are repaired by both homologous and non-homologous mechanisms. Homologous repair enhances homologous recombination by at least two orders of magnitude (Rouet et al., 1994), while non-homologous repair leads to targeted frame shift mutations (Bibikova et al., 2002). Applications based on the two DSB repair pathways lead to introduction of different types of mutations at target-specific sites of the genome including gene knock-out, knock-in, and point mutations.

Hearing loss (HL) is the most prevalent sensorineural disorder, affecting approximately 1 in 500 newborns (Mehl et al., 1998). It is estimated that more than two-thirds of prelingual HL cases are found to be inherited, most of which are caused by mutations of a single gene that functions in the inner ear (Liu et al, 2001; Hilgert et al., 2009; Morton et al., 2006). More than 150 chromosomal loci and over 80 genes have been identified to cause non-syndromic as well as syndromic forms of deafness (Yan and Liu 2008; Hilgert et al., 2009; Angel et al, 2012). The strong genetic basis of HL and spectacular advancements in CRISPR/Cas9-based genome editing technologies will surely usher in a new era using genome editing techniques

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to study HL, as can be glanced from our recent proof-of-principle study in the auditory system (Zuris et al 2015). This review focuses on what we can learn from applications and challenges of CRISPR/Cas9-mediated genome editing and clinical therapeutic potential of CRISPR/Cas9 in the future, with implications in human genome editing in genetic HL. The current state of genome editing technologies will also be presented.

2. Comparisons among three genome editing techniques

2.1 Shared features of three genome editing techniques

All three programmable nucleases contain two functional domains: one is responsible for targeting and binding specific genomic sequence whereas the other is involved in inducing DNA DSBs (Figure 1). For ZFNs, Cys2-His2 zinc fingers are amino termini of ZFNs, each zinc finger can be designed to recognize a three base-pair (bp) DNA sequence, and all zinc fingers (usually 3–6) are joined together to generate a single ZFN unit to target a DNA sequence that is 9-18 bp long (Miller et al., 1985; Nekludova et al., 2000; Urnov et al., 2010; Gaj et al., 2013). For TALENs, transcription activator-like effectors (TALEs) can also be modified to target a predetermined DNA sequence (Figure 2). TALEs are naturally occurring proteins derived from plant pathogenic bacteria *Xanthomonas* spp., most of which contain 13–28 repeats and 33–35 amino acids per repeat (Boch et al., 2010). Each repeat can recognize a single nucleotide in the target sequence, and the nucleotide specificity is determined by a hypervariable region of two adjacent amino acids at positions 12 and 13 within each amino acid repeat, termed repeat-variable di-residue (RVD) (Moscou et al., 2009; Boch et al., 2009; Morbitzer et al., 2010; Streubel et al., 2012; Cong et al., 2012). Multiple TALEs repeats are joined in tandem to target a specific DNA sequence (Bogdanove et al., 2011). For CRISPR, the repeats are derived from prokaryotic RNA-guided adaptive immune system, which are transcribed as target-specific CRISPR RNA (crRNA) and *trans*-acting crRNA (tracrRNA). TracrRNA is essential for the maturation of crRNA and can team up with crRNA guiding Cas9 nuclease to cleave invading phages or plasmids by binding to complementary DNA sequence in viruses or plasmids and inducing DSBs (Jinek et al., 2012; Cong et al., 2013; Mali et al., 2013; Cho et al., 2013).

All three systems can induce DNA DSBs: ZFNs have non-specific DNA nuclease – FokI, FokI nucleases must dimerize to be active to induce DNA DSBs (Vanamee et al., 2001). For CRISPR, type II CRISPR-Cas system is adapted as a genome editing tool, in which Cas9 protein acts as an endonuclease, and is guided by dual RNA complex (crRNA and tracrRNA) to cleave a 23-bp target DNA sequence that contains a 20-bp sequence matching the protospacer of crRNA plus a downstream NGG nucleotide motif (protospacer-adjacent motif [PAM]) (Figure 3) (Jinek et al., 2012; Cong et al., 2013; Mali et al., 2013; Cho et al., 2013). However, the problem with ZFNs is that there is no well-established way to determine how multiple zinc finger modules can be combined to bind longer sequences without empirical testing. There are still some advantages to the TALEN system in the sense that they can be designed for more sites with potentially more specificity to CRISPR.

Finally, the DNA DSBs induced by three genome programming nucleases can be repaired by the same two endogenous cellular repair processes: non-homologous end-joining (NHEJ) and homology-directed repair (HDR) (Rouet et al., 1994; Rouet et al., 1994; Jeggo 1998;

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Van Gent et al., 2001; Bibikova et al., 2002). NHEJ is an error-prone process that can generate small insertions and deletions (indels) of short sequences by quickly ligating DNA DSBs at the target sites (Jeggo 1998). Indels usually cause frameshift mutations in coding regions and eventually lead to gene disruption by knocking out the gene. HDR is a precise process that requires a homologous donor DNA as a template (Rouet et al., 1994). Because of the precision of HDR, point mutations can be corrected or induced to the genome (Bibikova et al., 2001; Bibikova et al., 2003; Porteus et al., 2003; Urnov et al., 2005); additionally, HDR also makes it possible to insert a gene of interest into the genome (Lombardo et al., 2011; Li et al., 2011; Hockemeyer et al., 2011; Doyon et al., 2011; Irion et al. 2014). Induction of two DNA DSBs at the same time can result in chromosomal deletion, duplication or inversion (Lee et al., 2010; Lee et al., 2012; Carlson et al., 2012; Gupta et al., 2013; Cong et al., 2013). Chromosomal translocations can also occur if two DNA DSBs are generated on two different chromosomes (Brunet et al., 2009; Cho et al., 2014).

2.2 Advantages of CRISPR/Cas9 over the other two techniques

The simplicity of design makes CRISPR/Cas9 more promising than the other two genome editing techniques. First of all, as an RNA-guided nuclease, Cas9 can be guided to target any genomic sequence by easily programming the 20-bp targeting sequence of guide RNA (gRNA), which can then be cloned from the gRNA plasmid backbone. However, for both ZFNs and TALENs, large DNA fragments (500–1500 bp) need to be designed for each new target in order to synthesize proteins (zinc fingers or TALEs) that are responsible for targeting specific genomic sequence. In addition, since FokI is an obligate dimer, both ZFNs and TALENs need two novel proteins to function properly, a process that is labor intensive. The successful binding of Cas9 to the genomic sequences requires the PAM sequence immediately adjacent to the target sequence, with the frequency of a PAM motif every 8 bp (NGG) or 4 bp (NGG and NAG) on average (Cong et al., 2013). In contrast, PAM is not a requirement for ZFNs and TALENs. The target density is one in every 100 bp for ZFNs and one per bp for TALENs, and TALENs appears to be advantageous to both ZFNs and CRISPR/Cas9 in terms of high target density (Sander et al., 2011; Gupta et al., 2012; Reyon et al., 2012).

Another advantage of CRISPR/Cas9 is its amenability for multiplexing by delivering multiple gRNAs to target multiple genes in the same cell simultaneously. This approach has been successfully applied to mammalian cells, mice, zebrafish, and monkeys (Mali et al., 2013; Chang et al., 2013; Jao et al., 2013; Li et al., 2013; Niu et al., 2014). Although using multiple ZFNs or TALEs could achieve the similar goal, formation of mismatched dimers, which will increase off-target effects, limits their routine use for multiplexing (Sollu et al., 2010).

The avoidable off-target effects and acceptable cellular toxicity have largely broadened the application of CRISPR/Cas9 in genome editing. Off-target effects of CRISPR/Cas9 reflect the tolerance of multiple mismatches within the protospacer sequence (Fu et al., 2013; Hsu et al., 2013; Pattanayak et al., 2013). However, these off-target effects are all gRNA specific, which can be reduced through multiple strategies (Fu et al., 2013; Hsu et al., 2013; Pattanayak et al., 2013; Cradick et al., 2013; Cho et al., 2014). Well-designed gRNA could

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significantly reduce off-target effects in comparison with ZFNs and TALENs, as shown by whole-genome analysis of human stem cells (Duan et al., 2014; Kiskinis et al., 2014; Smith et al., 2014; Veres et al., 2014). In addition, modification of CRISPR/Cas9 nuclease specificity by a 5′ truncated gRNA decreases the off-target mutagenesis by over 5000-fold (Fu et al., 2014). An additional strategy is to use a pair of Cas9 nickases, which induce a single-stranded break on each DNA strand, an equivalent to the DSBs generated by normal Cas9. Since the single-stranded break is repaired by base excision pathway with a very low mutation rate, Cas9 nickase can reduce off-target effects (McConnell et al., 2009; Davis et al., 2011). Off-target effects have also been successfully minimized by using a pair of defective Cas9 (dCas9) protein, which lacks the nuclease activity, the dCas9 can then be fused to FokI to induce DNA DSBs (Tsai et al., 2014; Guilinger et al., 2014). As a proof of this concept, we have shown that direct delivery of protein Cas9 with gRNA significantly reduced the off-target effect of genome editing (Zuris et al., 2015).

3. Genetic causes of HL and contribution of CRISPR/Cas9 to hearing research

Most genetic HL cases are caused by monogenic mutations whereas a small portion is resulted from mutations involving more than one gene (Morton et al., 2006; Angeli et al, 2012). The majority of inherited HL is non-syndromic that is often neuroepithelial in origin arising from defects in the function of the organ of Corti – the site of auditory transduction in the inner ear (Yan et al., 2008). Since the discovery of the first nonsyndromic deafness gene in 1993, more than 150 loci for deafness genes have been mapped and more than 80 genes have been implicated in nonsyndromic HL (http://hereditaryhearingloss.org/). These genes belong to very different gene families with various functions, including transcription factors, extracellular matrix molecules, cytoskeletal components, ion channels and transporters (Yan et al., 2008; Hilgert et al, 2010). Various types of mutations have been identified in deaf patients, such as single nucleotide substitution, gene deletion, and gene insertion. These mutations result in missense or/and nonsense mutations of the deafness genes and thus cause HL (http://hereditaryhearingloss.org/). Understanding of functions of human deafness genes has been fueled by the use of vertebrate models for studying hereditary HL.

Progressive HL can initiate at any age, including in the first few years of life. Genes that have been associated with autosomal dominant progressive HL are excellent candidates for age-related hearing loss (ARHL) (Liu et al., 2007). The progressive nature of these diseases could be explained by a gradual increase in the ratio of damaged to normal protein or changes in protein expression. This could result from alterations that influence the transcription, translation and/or degradation of the altered proteins. Developing alternative biological treatments to stop or reverse the progression of HL by repairing these alterations is highly desirable.

The strong genetic basis of HL provides a significant platform to combine CRISPR/Cas9 genome editing technology with model organisms to mimic causal mutations of genetic HL and understand the pathology of the disease. Traditional methods of making transgenic animals to model HL are time-consuming, costly and labor intensive. With CRISPR/Cas9

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technology, embryonic stem cells can be efficiently generated through the NHEJ mechanism to create the deletion models or by the HDR mechanism that combines a mutant donor template with Cas9 nickase activity to produce single nucleotide mutation and gene insertion models. We envision the utilization of CRISPR/Cas9 would greatly accelerate the process.

The majority of gene mutations causing HL are associated with dysfunction in auditory hair cells. Hair cell targeted rescue of deleterious gene mutations via expressing or delivering corresponding wild type proteins, or the knock-down of a mutant allele via delivering or expressing silencing RNAs could help protect against the occurring and/or the progression of certain forms of deafness. This makes the cochlear hair cell a preferential target for potential intervention. Adeno-associated virus (AAV) has been the choice in the development of gene therapy for inhereditary HL (Akil et al., 2012). However the size limit of inserts (less than 4.7 kb) severely constrains its use. Advances in methods for delivering *in vitro*-transcribed mRNA offered a new promising alternative, but still face challenges related to immunogenicity and RNA stability. Thus, CRISPR/Cas9-mediated genome editing could be explored as a new approach to achieve disruption of a dominant mutation or to achieve the repair of recessive mutations for functional recovery of hearing. It is possible that in situations such as the use of CRISPR/Cas9 to edit mRNA where continuous availability of Cas9:gRNA that can be provided by viral delivery will be needed (O'Connell et al., 2014). Researchers have demonstrated that Cas9 can be guided to bind specifically to target RNAs without interfering corresponding DNA sequences by the use of specially designed PAM-presenting oligonucleotides (PAMmers) (O'Connell et al., 2014). This strategy has been proved to isolate GAPDH mRNA from HeLa cells (O'Connell et al., 2014).

CRISPR/Cas9 has been shown to correct genetic disorders including Duchenne muscular dystrophy and liver disorder (Long et al., 2014; Yin et al., 2014). In those studies the corrections were achieved by viral-delivered Cas9/gRNA, resulting in permanent production of the Cas9/gRNA complex. Given the potency of CRISPR/Cas9 in genome editing, transient delivery of Cas9/gRNA complex would be preferred, with permanent editing results while reducing the risk associated with continuous genome editing activities. One emerging route in the development of therapy for genetic HL is the delivery of Cas9/gRNA in protein/nucleic acid complex to achieve CRISPR/Cas9-mediated genome editing for inner ear application.

We have shown that a complex formed between protein Cas9 and nucleic acid gRNA by commercially available cationic lipids can be directly delivered to mouse inner ear hair cells *in vivo* (Zuris et al, 2015) (Figure 4). Further such complexes were shown to induce efficient genome editing by knockdown of GFP signal in the Atoh1-GFP transgenic mice and by detection of indels by the HST sequencing. There was minimum toxicity associated with the delivery of the complex or genome editing. As Cas9 and gRNAs are rapidly degraded upon entering cells, the duration of their activities is short-lived, yet with the permanent genome editing results. However, so far our strategy is only able to target outer hair cells efficiently. We are in the process of developing different methods such as the use of super-charged proteins to carry Cas9 with gRNAs, which is likely to target more inner ear cell types. The immediate implication in the treatment for genetic HL with the approach is to design gRNAs

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that target dominant hair cell gene mutations such as dominant Myo7a mutations in Usher 1B for disruption by NHEJ. By delivery of Cas9/gRNAs to the mutant inner ear, it is likely that efficient disruption of mutations could be achieved, leading to potential hearing recovery. The easy constitution of Cas9/gRNA complex makes it feasible to study functions of virtually all hair cell genes by guiding RNAs to target the gene of interest. However, we did not test HDR in the inner ear *in vivo*, primarily due to low efficiency (less than 1%) even for *in vitro* assay compared to NHEJ where over 85% efficiency can be achieved. It is an area that needs substantial improvement in efficiency. Further studies are warranted to demonstrate that HDR is feasible in the inner ear.

4. Potential challenge and prospect

One major challenge of broad application of CRISPR/Cas9 in hearing research appears to be its requirement of a PAM motif, which lowers its design density and may be a potential problem for small-range precise mutations like single nucleotide substitution because there could not be a PAM motif nearby. The most commonly used Cas9 from *S. pyogenes* requires a 5′-NGG PAM motif, which limits the design density to one per eight base pairs on average in genome (Jinek et al., 2012; Cong et al., 2013; Mali et al., 2013; Cho et al., 2013). One potential strategy is to combine Cas9 proteins from other bacteria that recognize different PAM motifs, and therefore the target scope of CRISPR/Cas9 can be extended. Some of these Cas9 proteins have already been adapted as genome editing techniques, including Cas9 from *Neisseria meningitides* (5′-NNNNGATT), *Streptococcus thermophilus* (5′-NNAGAAW), and *Treponema denticola* (5′-NAAAAC) (Cong et al., 2013; Esvelt et al., 2013; Hou et al., 2013).

Since its emergence just a few years ago, CRISPR/Cas9 technology has shown great promise to transform biomedical research and to be developed as a new type of treatment-based genome editing for a wide range of genetic disorders. The easy use and high efficiency distinguish it from other existing genome editing technologies, and we fully anticipate its productive applications in hearing research. For the study of inner ear genes, the utilization of reagents including Cre-dependent Cas9 knock-in transgenic mice (Platt et al., 2014) in combination with targeted delivery of gRNAs by AAV *in vivo* and *in vitro* could rapidly reveal gene functions by NHEJ-mediated disruptions without the process of germline transmission. The same route can be used to test intervention of mutations to restore hearing in genetic hearing models. However, to develop potential treatment for genetic HL in humans, we consider transient protein/nucleic acid delivery would be a better choice especially in reducing the risk associated with off-target effect. Further development for transient Cas9/gRNA delivery in the inner ear includes the lipid formulations that can target different cell types at different ages. In addition to the disruption of dominant mutations by NHEJ, one major challenge is to improve the efficiency of HDR that can be applied to recessive mutations. Given the rapid progress in the field, it is likely that these obstacles will be overcome in the near future, thus potentiating novel hearing treatments for genetic deafness.

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Abbreviations

AAV	Adeno-associated virus
ARHL	age-related hearing loss
Cas9	CRISPR-associated nuclease 9
CRISPR	clustered, regularly interspaced, short palindromic repeat
crRNA	CRISPR RNA
dCas9	defective Cas9
DSBs	double-strand breaks
gRNA	guide RNA
HDR	homology-directed-repair
HL	hearing loss
HR	homologous recombination
NHEJ	non-homologous-end-joining
PAM	protospacer-adjacent motif
RVD	repeat-variable di-residue
siRNA	small interfering RNA
TALENs	transcriptional activator-like effector nucleases
TALEs	transcription activator-like effectors
tracrRNA	*trans-acting* crRNA
ZFNs	Zinc finger nucleases

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Highlights

- Targeted genome editing using CRISPR/Cas9 is a powerful tool to perform genome function studies.
- Applications and challenges of CRISPR/Cas9-mediated genome editing are discussed.
- CRISPR/Cas9-mediated technology has implications in human genome editing in genetic hearing loss.
- Zebrafish, mice, and primates can serve as model organisms to harness the potential of CRISPR/Cas9-mediated genome editing.
- CRISPR/Cas9 genome editing holds great potential to modulate the function of genes involved in hearing loss.

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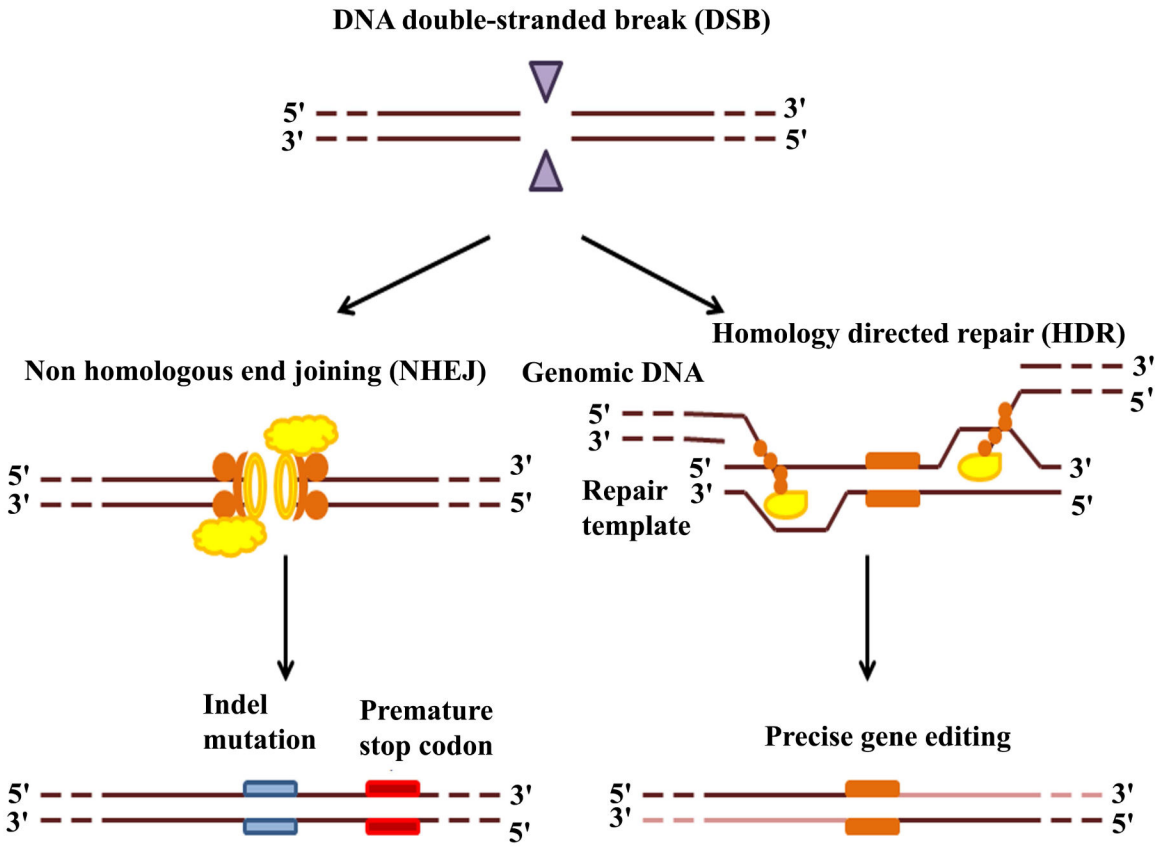

Figure 1.
A schematic representation of programmable nucleases demonstrating two functional domains.

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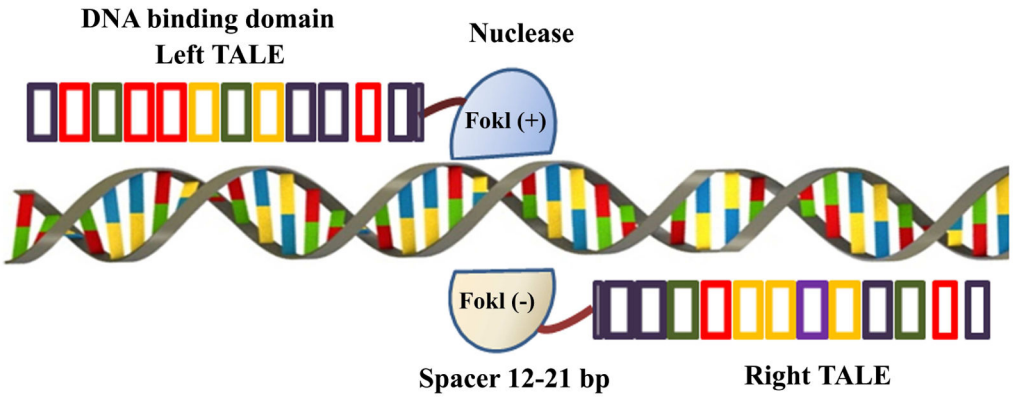

Figure 2.

A schematic representation of transcriptional activator-like effector nucleases (TALENs). TALENs are composed of TALE repeats and FokI nuclease. TALENs work in paris, binding two complementary DNA strands across a spacer over which FokI nuclease dimerizes to create a double-stranded break. Each TALE repeat can recognize one single nucleotide, and multiple TALE repeats are joined in tandem to target a specific DNA sequence.

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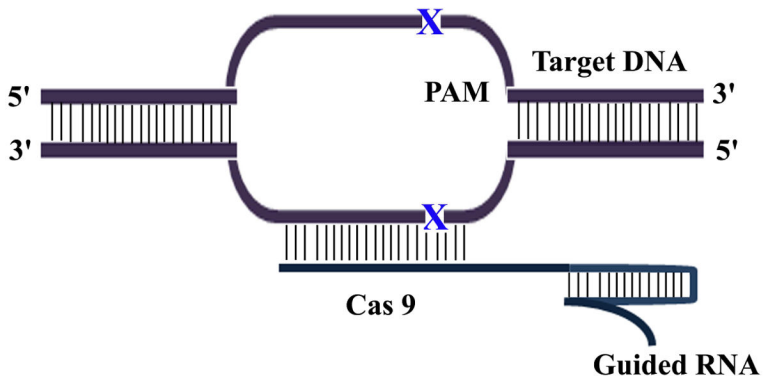

Figure 3.
In CRISPR system, guide RNA directs Cas 9 enedonuclease to cleave the target DNA sequence 3′ bp upstream of PAM.

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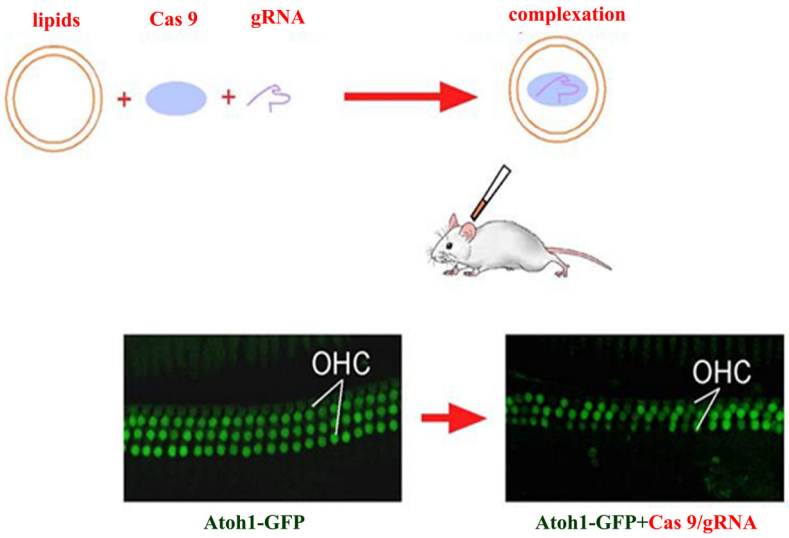

Figure 4.

CRISPR/Cas9-mediated genome editing in hair cells *in vivo.* Cas9 protein and the GFP gRNA were complexed with the commercial lipids (Lipofectamine 2000), which were then injected into postnatal Atoh1-GFP mouse cochlea in which all outer hair cells were GFP positive. Genome editing was achieved in the outer hair cells by the absence of GFP signal in the injected inner ear, without affecting our hair cell survival. High-throughput sequencing confirmed the disruption of the GFP gene by indels. OHC: Outer Hair Cells