CHAPTER

9 Application of CRISPR/Cas system in iPSC-based disease model of hereditary deafness

Subhajit Giri, Brigitte Malgrange

Developmental Neurobiology Unit, GIGA-Stem Cells, Interdisciplinary Cluster for Applied Genoproteomics (GIGA-R), CHU Sart Tilman, University of Liège, Liège, Belgium

Chapter outline

Abstract

Sensorineural hearing loss (SNHL) is the most prevalent sensory disorders affecting ~ 6% of the world population with partial or complete hearing impairment. The molecular etiologies of SNHL could be inherited genetic or acquired environmental factors. The broadening spectrum of genetic causes of SNHL requires a better understanding of the disease pathogenesis and the development of therapeutic targets. To achieve this, we need an experimental humanoid disease model that is fast and easy to perform genetic manipulation and downstream. In this chapter, we discuss how two revolutionary techniques CRISPR/Cas-based gene editing and human-induced pluripotent cells (hiPSCs) could be implemented and reshape the experimental disease model of hearing loss with a vision toward the development of reliable and sustainable therapeutic approaches.

iPSCs - State of the Science. https://doi.org/10.1016/B978-0-323-85767-3.00009-8

Keywords: CRISPR/Cas9; Deafness; Disease modeling; Gene editing; Gene therapy; Hearing loss; Human embryonic stem cell; Induced pluripotent stem cell; Inner ear hair cell; Organoid; Otic progenitors; Sensorineural.

Introduction

Hearing loss is the most common sensorineural disorder affecting around 466 million (6.1% of the world population) people worldwide (https://www.who.int/health-topics/hearing-loss) and could be accounted for 900 million people by 2050. It imposes an elevated public health concern as well as global financial burden estimated around $750 billion annually. Hearing loss can be categorized based on severity as mild, moderate, severe, and profound deafness. Based on the region(s) of the auditory system affected, the mode of hearing loss can be categorized as conductive, sensorineural, or mixed. Conductive hearing loss occurs when the transmission of sound waves toward the inner ear is disrupted through structural problems of the outer ear, tympanic membrane, or middle ear. Sensorineural hearing loss (SNHL) results from functional and structural impairments and/or damage of cochlear hair cells, spiral ganglion neurons, or cells located in the stria vascularis. The causes of hearing loss can be congenital or acquired that leads to either syndromic or nonsyndromic hearing loss. The congenital causes may be present at birth or acquired postnatally and include pregnancy and birth complications, such as asphyxia, maternal infections, ototoxic drugs consumption during pregnancy, neonatal jaundice, and inheritable genetic factors. Acquired causes affect hearing at any period of life. They include infectious diseases, exposure to ototoxic chemicals, otitis media, ear or head injury, exposure to excessive noise (occupational, recreational, or accidental), and age-related degeneration of cochlear cells. Nonsyndromic hearing loss (NSHL) is characterized by partial or complete loss of hearing ability that is not associated with any other phenotypic symptoms or physiological complications, and it accounts for about 70% cases of hearing loss. The major contributor for NSHL is genetic etiology and based on inheritance pattern. It can be classified in autosomal dominant (20%−25%), autosomal recessive (70%−75%), and X-linked and mitochondrial (1%−2%) NSHL (Venkatesh et al., 2015; Kokotas et al., 2007). Syndromic hearing loss (SHL) occurs with the association with other phenotypic spectrum and/or syndrome affecting multiple body parts and can cause 30% cases of HL. The four major SHL are the well-known Pendred, Waardenburg, Usher, and CHARGE syndromes. The genetic etiology of these syndromes sometimes be multigenic is complex and could associate retinal, renal, musculoskeletal, nervous system, pigmentary, and cardiac disorders and many other anomalies (Koffler et al., 2015). As a whole, genetic factors play the most significant role in the pathogenesis of hearing impairment, and more than 180 loci and 100 genes have been implicated for "deafness." As the detailed discussion about genetics of hearing loss is beyond the scope of this chapter, the list of these genes can be accessed from the "Hereditary Hearing Loss Homepage"

(https://hereditaryhearingloss.org/), and for identified genetic variants for individual "deafness genes," please refer to the "Deafness Variation Database" (http://deafnessvariationdatabase.org/). There are several review papers available in literature where the functional role of these genes for auditory system development is meticulously articulated (Michalski and Petit, 2019; Wu and Kelley, 2012).

Despite the enormous preponderance of hearing loss as a public health issue, treatment and therapeutic options are very limited. As none of the pharmacotherapies is clinically approved to treat SNHL, current management options are relying on amplification of sound waves with the help of hearing aid or cochlear implant. However, none of them can repair or help to regenerate the degenerated cochlear epithelium and inner ear hair cells. As an alternative treatment strategy, gene therapy, and stem cell-based, cell replacement strategies are getting attention as emerging therapeutic approaches (Mittal et al., 2017). In this chapter, we discuss (1) differentiation of human embryonic stem cells (hESCs) and human-induced pluripotent stem cells (hiPSCs) (in a combination called hPSCs) toward inner ear hair cells in 2D and 3D culture system, (2) hiPSC-based disease modeling to study the molecular mechanism of hearing loss, and (3) application of CRISPR (clustered regularly interspaced short palindromic repeats)/Cas (CRISPR-associated protein)-based genome editing in hiPSC model of hearing loss.

Induced pluripotent stem cells model of hereditary deafness

The pioneering effort of Takahashi and Yamanaka discovered the pluripotency factors (i.e., Klf4, Sox2, Oct4, and c-Myc) and derived the iPSCs from mouse and human embryonic fibroblasts for the first time (Takahashi and Yamanaka, 2006; Takahashi et al., 2007). This discovery has an immediate impact on stem cell—based disease modeling and research on regenerative medicine. Previous efforts on cell replacement therapy using hESCs majorly impeded from ethical concerns, restricted access of human embryos, and immune rejection of heterologous hESC-derived specified cells in the host system, which has been obsolete by the advent of iPSC technology. Most importantly, patients' cell-derived iPSCs preserve the patient-specific genomic components, which are responsible for causing that particular disease and associated phenotypic spectrum. With this significant advantage, iPSC technology has been adapted as a method of choice for disease modeling or so-called "disease in a dish." Recent advancement of targeted differentiation protocol with the help of defined factors (e.g., growth factors, signaling pathway inhibitors, etc.) made it possible to derive almost any kind of human cells from hiPSCs nowadays. Throughout the past decade, scientists from all over the world were able to derive the patient-specific iPSCs and study numerous diseases that include the hematological (Georgomanoli and Papapetrou, 2019), cardiovascular (Sharma et al., 2013), neuronal (Ardhanareeswaran et al., 2017; Mertens et al., 2018), gastrointestinal (Dedhia et al., 2016), and hearing (Roccio and Edge, 2019) systems. The "disease in a dish" has a significant advantage to study the pathogenic effect(s) of a

particular gene mutation or a combination of different genetic variants. Indeed, studying the disease mechanism, manipulating gene(s), and drug screening can be done at any differentiation time point of the developmental paradigm. In this chapter, we discuss how iPSC technology has been implemented to study human inner ear hair cell development and disease modeling of hearing impairment.

Otic differentiation from human-induced pluripotent stem cells and human embryonic stem cells

Soon after the discovery of iPSC technology, substantial efforts have been put forward to develop specific differentiation protocols to generate otic progenitors (OPs) and inner ear hair cells from iPSCs and hESCs with synchronization of crucial stages of human inner ear development (Fig. 9.1) (Chen et al., 2012; Ealy et al., 2016; Koehler et al., 2017; Ronaghi et al., 2014; Sakagami et al., 2019; Lahlou et al., 2018). In one of such very first efforts, direct differentiation of OPs from hESCs in monolayer has been achieved through activation of FGF signaling cascade using FGF3 and FGF10 (Chen et al., 2012). The derived OPs were composed of two morphologically distinct colonies identified as otic epithelial progenitors (OEPs) and otic neural progenitors (ONPs). Sequential enrichment of OEPs and culturing with maturation media containing *all-trans* retinoic acid (RA) and epidermal growth factor (EGF) for 2−4 weeks produced "hair cell−like cells" successfully as defined by simultaneous expression of inner ear hair cell markers *ATHO1/BRN3C* or *BRN3C/MYO7A* (please refer to Chen et al., 2012, Supplementary Fig. 7A). Though a small subset of these cells was differentiated as espin (*ESPN*) positive immature apical bundles, this differentiation protocol was not sufficient to produce stereociliary hair bundles, indicating that additional factors are required for complete maturation of inner ear hair cells. However, more recently, the generation of electrophysiologically mature "hair cell−like cells" has been achieved by coculturing OEPs with chicken utricle stromal cells with or without RA and EGF supplementation (Ding et al., 2016; Tang et al., 2016). In another study, monolayer differentiation of iPSCs toward inner ear hair cells has been done in a two-stage manner where induction of preplacodal ectoderm (PPE) has been carried out for 8 days followed by otic placode induction for another 2 weeks (Ohnishi et al., 2015). Finally, hair cell differentiation has been continued for 10 weeks, albeit the efficiency of hair cell formation remained very low. A more refined protocol has been developed where nonneural ectoderm (NNE) induction was introduced before PPE induction to synchronize the differentiation protocol with human embryonic otic developmental paradigm (Ealy et al., 2016; Ronaghi et al., 2014). The NNE induction has been done through inhibition of TGFβ and WNT signaling pathways with or without the treatment with IGF to promote cranial ectoderm. Then PPE induction has been achieved through either combined treatment of RA and FGF2 (Ealy et al., 2016) or sequential FGF activation, WNT activation/BMP inhibition for 3 days, and then BMP activation for 3 days (Ronaghi et al., 2014). Despite these rationally designed protocols, both studies were unable to derive mature

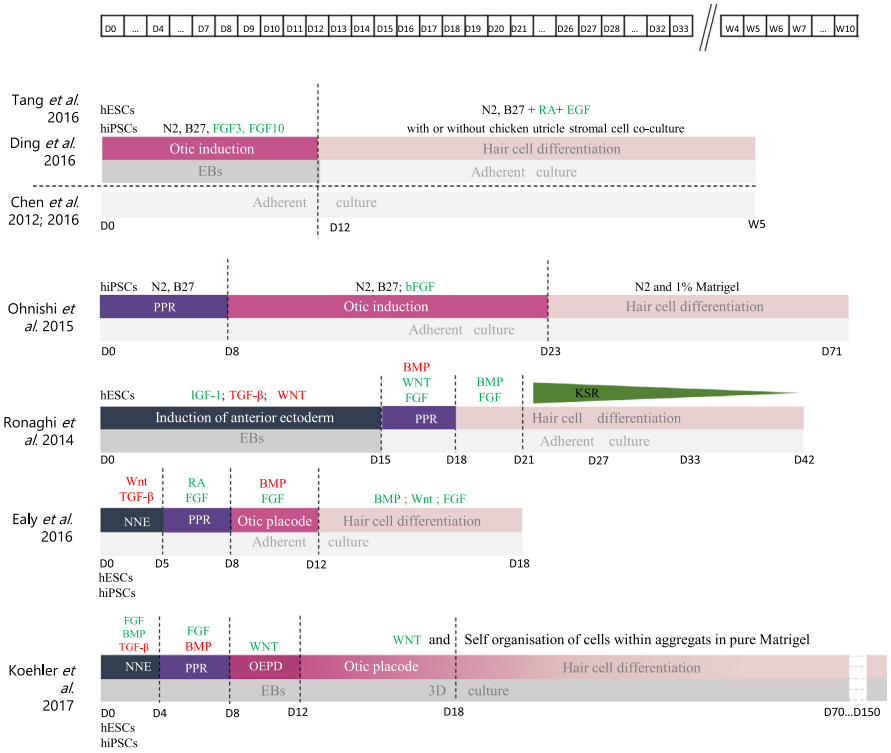

FIGURE 9.1 Comparison of established differentiation protocols of hESCs and hiPSCs into inner ear hair cell–like cells in 2D- and 3D culture system.

BMP, bone morphogenetic protein; *EB*, embryoid bodies; *EGF*, epidermal growth factor; *FGF*, fibroblast growth factor; *IGF-1*, insulin-like growth factor 2; *KSR*, knockout serum; *LIF*, leukemia inhibitory factor; *NNE*, nonneural ectoderm; *OEPD*, otic epibranchial placode domain; *PPR*, preplacodal region; *RA*, retinoic acid; *TGF-β*, transforming growth factor-β. Days and weeks in culture are designated as D_n and W_n.

Reprinted by permission from Czajkowski, et al., 2018. Pluripotent stem cell–derived cochlear cells: a challenge in constant progress. CMLS, Springer [COPYRIGHT].

"hair cell–like cells" with organized stereocilia bundles and concluded that additional factors are required for enhanced stabilization of otic progenitors and further maturation of hair cells.

The low efficiency of inner ear hair cell derivation from hPSCs in the 2D-culture system was indicative of requirement of certain undefined factors and tissue microenvironment to facilitate the successful derivation of hair cells, which can be attained by differentiation of hPSCs in aggregates or as organoids (Koehler et al., 2017; Jeong et al., 2018). With this vision, Koehler et al. (2017) designed a differentiation system in 3D, setup to derive "inner ear organoids" complemented with factors allowing fine-tuning in TGFβ, BMP, FGF, and WNT signaling pathways

(Koehler et al., 2017). In this protocol, both hESCs (WA25 line) and hiPSCs are allowed to aggregate as embryoid bodies (EBs), and NNE is induced by culturing in chemically defined media (CDM) supplemented with FGF2, TGFβ inhibitor (SB431542), and optional BMP4 from day 0 to day 4 (d0−d4). Then induction of preplacodal region (PPR) has been carried out (d4−d8) by inhibiting BMP signaling, and further activation of FGF pathway as FGF has shown to be responsible for otic fate specification in embryo (Litsiou et al., 2005; Martin and Groves, 2006). Successive otic epibranchial placode domain (OEPD) induction was carried out (d8−d12) through inhibiting GSK3β, thus activating WNT signaling pathway, and continued until d18 for otic placode formation. Inner ear otic vesicles were enriched with $PAX2^{+}PAX8^{+}$ otic progenitors (refer to Koehler et al., 2017, Fig. 1M and N). After this stage, "inner ear organoids" were allowed to be self-organized until d70 in 100% Matrigel as stationary phase culture (Fig. 9.1). This protocol successfully generated spatially organized, electrophysiologically mature inner ear hair cells expressing specific hair cell markers *MYO7A*, F-actin, *ESPN*, and *TUBA4A* (refer to Koehler et al., 2017, Fig. 2M−R). This culture condition also gave rise to neurofilament+ sensory neurons (unipolar and bipolar), which forms synaptic contacts with the sensory hair cells (please refer to Koehler et al., 2017, Fig. 4). Though this differentiation protocol is comprehensively efficient for inner ear hair cell formation (∼20%), functional characterization of cellular electrophysiological response and bundle morphology was indicative of vestibular hair cell identity rather than cochlear hair cells (Koehler et al., 2017). Improvement of cochlear hair cell derivation could be attained through further optimization of culture condition and/or additional factors to target sonic hedgehog (SHH) signaling pathway, as it has been suggested that SHH could induce ventral identity of otic vesicles (Bok et al., 2007).

Prospects of induced pluripotent stem cell model for hearing loss research

The ability of human inner ear hair cell generation from hPSCs in vitro can be explored in every aspect of stem cell−based hearing loss research and expedite the process of innovation in therapeutic approaches. The potentially unlimited access to the hPSC-derived otic progenitors and inner ear hair cell would be favorable for the large-scale ex vivo drug and small molecule screening and/or genetic screening. These experimental strategies would be beneficial to understand the proper development and regenerative capacity of inner ear hair cells or transdifferentiation of supporting cells into hair cells. The outcome of these experiments would assist the prospective cell replacement therapy for hearing loss (Géléoc and Holt, 2014). To identify the toxic, protective, and regenerative compounds from an extensive library of drugs, iPSC-based inner ear model provides a fantastic opportunity for high-throughput drug screening in terms of feasibility and scalability. This can also be combined with advanced imaging-based techniques such as multiphoton, light-sheet, and automated high-content microscopy for parallel phenotypic screening (Rios and Clevers, 2018). The hPSC-derived inner ear model is also

suitable for studying the functional implications of genetic mutations toward developmental defects and premature degeneration of inner ear hair cells and other sensory components (Tang et al., 2019; Fukunaga et al., 2016). With the help of CRISPR/Cas-based genome editing technique, creating a mutant hPSC line or correcting a mutation already present to generate isogenic control line is the best way to make a genetic model of hearing loss (discussed in the next section). Also, the hPSC model allows scientist to test out different viral vectors for its efficiency and specificity to deliver the CRISPR components or any other substances for gene therapy (Yeh et al., 2018; Gao et al., 2018). Nonetheless, cell replacement and tissue engraftment strategies as a therapeutic option could now be considered through transplantation of hiPSC-derived otic progenitors and sensory cells in the damaged cochlea. In a couple of studies, otic progenitor cells or outer sulcus cell-like cells obtained in vitro from hiPSCs have been transplanted in rodent inner ear and reported different degrees of success in terms of survival, differentiation to sensory cell fate, and synaptic connection to spiral ganglion neurons of transplanted cells (Lopez-Juarez et al., 2019; Chen et al., 2018; Takeda et al., 2018). These preliminary findings would encourage further development of cell replacement strategy, which can be amalgamated with the application of CRISPR/Cas-based genetic correction in iPSCs of inherited hearing loss patients and derivation of otic progenitors for autologous cell transplantation.

Application of CRISPR/Cas9 in induced pluripotent stem cells for hearing loss disease modeling

The discovery and advancement of iPSC technology in the past decade has been serendipitously complemented with a breakthrough discovery in gene editing through "CRISPR−Cas" system. In the past decade, the CRISPR−Cas technique transformed the thought and approaches in biomedical research and revolutionized the field of long-sought "gene editing" technique. In 2007, CRISPR−Cas system was demonstrated as a prokaryotic immune mechanism against phage invasion (Barrangou et al., 2007). In 2012, collaborative work of Jennifer Doudna and Emmanuelle Charpentier established that CRISPR−Cas9 system as an RNA programmable "genome editing" technique can be used to create DNA double-strand break (DSB) at a targeted locus in genome (Jinek et al., 2012). This observation is further supported by two independent studies in the following year (Cong et al., 2013; Mali et al., 2013). Following these discoveries, a race to find different kinds of naturally occurring CRISPR system and their mechanism of "gene editing" has been cataloged 6 CRISPR−Cas types and 29 subtypes so far (Shmakov et al., 2017; Koonin et al., 2017). Among them, the widely used *Streptococcus pyogenes* Cas9 (SpCas9) belongs to class 2 type II CRISPR system. Cas9 is a dual-RNA-guided DNA endonuclease enzyme, and upon DNA recognition, it generates a DNA DSB. DNA recognition of SpCas9 relies on 20-nucleotide spacer sequence adjacent to 5′-NGG

(N stands for any nucleotide) PAM (protospacer adjacent motif) sequence (Jinek et al., 2012). DNA targeting CRISPR RNA (crRNA) hybridizes with *trans*-activating crRNA (tracrRNA) to form a complex with Cas9 protein at the targeted genomic locus. The crRNA and tracrRNA can be engineered as a single guide RNA (sgRNA) in the experimental setup. Apart from SpCas9 PAM (5′-NGG) specificity, directed evolution-based approaches generated a plethora of Cas9 variants with diverse PAM specificities (Makarova et al., 2018). Several mutant variants of Cas9 have been engineered for alternative PAM recognition (e.g., VQR SpCas9, VRER SpCas9, xCas9-3.7) (Kleinstiver et al., 2015; Hu et al., 2018) and enhanced target specificity (e.g., eSpCas9 1.1, evoCas9, HiFi Cas9) (Table 9.1) (Slaymaker et al., 2016; Kleinstiver et al., 2016; Chen et al., 2017; Casini et al., 2018; Vakulskas et al., 2018). In addition to SpCas9 and its variants, another class 2 type V CRISPR system namely Cas12a (previously known as Cpf1) and class 1 type 1 CRISPR system called "cascade" with Cas3 significantly contribute toward the expansion of the CRISPR toolbox (Table 9.1) (Zetsche et al., 2015; Halpin-Healy et al., 2020).

Gene editing by CRISPR/Cas9

The genome editing through CRISPR−Cas9 depends on cellular intrinsic DNA break repair pathways at the site of Cas9-induced DSBs. Eukaryotic cells predominantly rely on error-prone nonhomologous end joining (NHEJ) pathways after which repair small insertion and/or deletion could be incorporated in single or both DNA strands. Harnessing the characteristics of the NHEJ pathway, experimental gene knockout strategies have been designed. Generally, when a coding exon at or near the start codon had been targeted, Cas9 induced DSB would be repaired and introduce indel-mediated frameshift mutation and/or premature stop codon to disrupt the expression of the gene of interest. Large genomic deletion and chromosomal translocation can be induced by targeting two sites in a genomic locus or two nonhomologous chromosomes, respectively (Essletzbichler et al., 2014; Torres et al., 2014). An error-free precise genome editing can be taken place through homology-directed repair (HDR) pathway, albeit in a low efficiency compared with the NHEJ pathway. Careful genome editing can be achieved through the delivery of repair template containing desired nucleotide(s) flanked by two homology sequences of the target site along with CRISPR components. The efficiency of HDR-mediated editing can be stimulated by repressing the activity of repair components of NHEJ pathway either by chemical and genetic modulators, the timing of CRISPR component delivery, and usage of modified Cas9, thus favoring the HDR pathway (Liu et al., 2018a,b). The desired nucleotide(s) in the repair template can be a single base for point mutation alteration to create a mutant protein or restoration to native one or a specific sequence encoding an epitope tag or a fluorescent protein to be fused with the endogenous protein of interest to monitor its activity in cellular context (Paquet et al., 2016). Recently, the highest level of precise genome editing efficiency has been achieved with the development of "prime editing" (Anzalone et al., 2019). Single base editing can also be done without DSB using Cas9 nickase

Table 9.1 CRISPR/Cas system variants with respective protospacer adjacent motif and target specificities.

CRISPR class	Name	Description	PAM sequence (5′–3′)	Target	Notes
Class 2 type II	SpCas9	Native *Streptococcus pyogenes* Cas9	NGG	DNA	1368 amino acids, two nuclease domains RuvC and HNH, blunt end cut (Jinek et al., 2012; Cong et al., 2013; Mali et al., 2013)
	VRER SpCas9	D1135V, G1218R, R1335E, T1337R	NGCG	DNA	Altered PAM variant (Kleinstiver et al., 2015)
	VQR SpCas9	D1135V, R1335Q, T1337R	NGAN/NGNG	DNA	Altered PAM variant (Kleinstiver et al., 2015)
	EQR SpCas9	D1135E, R1335Q, T1337R	NGAG	DNA	Altered PAM variant (Kleinstiver et al., 2015)
	xCas9-3.7	A262T, R324L, S409I, E480K, E543D, M694I, E1219V	NG/GAA/GAT	DNA	Altered PAM variant (Hu et al., 2018)
	eSpCas9 (1.1)	K810A, K1003A, R1060A	NGG	DNA	Improved target specificity (Slaymaker et al., 2016)
	Cas9-HF1	N497A, R661A, Q695A, Q926A	NGG	DNA	Improved target specificity (Kleinstiver et al., 2016)
	HypaCas9	N692A, M694A, Q695A, H698A	NGG	DNA	Improved target specificity (Chen et al., 2017)
	evoCas9	M495V, Y515N, K526E, R661Q	NGG	DNA	Improved target specificity (Casini et al., 2018)
	Sniper-Cas9	F539S, M763I, K890N	NGG	DNA	Improved target specificity (Lee et al., 2018)
	HiFi Cas9	R691A	NGG	DNA	Improved target specificity (Vakulskas et al., 2018)
	ScCas9	Native *Streptococcus canis* Cas9	NNG	DNA	1375 amino acids, different PAM compared with SpCas9 (Chatterjee et al., 2018)
	StCas9	Native *Streptococcus thermophilus* Cas9	NNAGAAW	DNA	1121 amino acids, different PAM compared with SpCas9 (Cong et al., 2013)

Continued

Table 9.1 CRISPR/Cas system variants with respective protospacer adjacent motif and target specificities.—*cont'd*

CRISPR class	Name	Description	PAM sequence (5′–3′)	Target	Notes
	NmCas9	Native *Neisseria meningitidis* Cas9	NNNNGATT	DNA	1082 amino acids, different PAM compared with SpCas9 (Hou et al., 2013)
	SaCas9	Native *Staphylococcus aureus* Cas9	NNGRRT	DNA	1053 amino acids, different PAM compared with SpCas9 (Ran et al., 2015)
	CjCas9	Native *Campylobacter jejuni* Cas9	NNNVRYM	DNA	984 amino acids, different PAM compared with SpCas9 (Yamada et al., 2017)
	CasX	Phyla Deltaproteobacteria and Planctomycetes	TTCN	DNA	980 amino acids, different PAM compared with SpCas9 (Burstein et al., 2017)
Class 2 type V	Cas12a (Cpf1)	CRISPR-associated endonuclease in Privotella and Francisella 1	TTTV	DNA	1300 amino acids, contains RuvC but lacks HNH domain, staggered end cut (Zetsche et al., 2015)
Class 2 type VI	Cas13a	RNA-guided RNAase	Does not require PAM sequence	RNA	1120 amino acids (Cox and Gootenberg, 2017)
Class 1 type I	Cas3	*Streptococcus thermophilus* Cas3	TTC	DNA	926 amino acids, single-stranded DNA nuclease and ATP-dependent helicase (Sinkunas et al., 2011)

M *stands for amino (A or C);* N *stands for any nucleotide;* R *stands for purines (A or G);* V *stands for A, C, or G;* W *stands for weaker interactions (A or T);* Y *stands for pyrimidines (C or T).*

(nCas9), or catalytically dead Cas9 (dCas9) fused with cytidine deaminases like rat APOBEC1 or lamprey cytidine deaminase 1 for site-specific direct conversion of C → T (or G → A) (Komor et al., 2016). Continued improvement of base editing technique developed a third-generation base editor (BE3) where APOBEC1 fused with XTEN linker; nCas9 (A840H) and uracil glycosylase inhibitor significantly improved the editing efficiency in vitro and in vivo (Komor et al., 2016; Kim et al., 2017; Rees et al., 2017; Chadwick et al., 2017). Recently, adenine base editors (ABEs) have been developed by protein engineering of a tRNA adenosine deaminase for direct conversion of A → G (or T → C) (Gaudelli et al., 2017). These recently developed techniques are continuously enriching the arsenal of "molecular scissors" and broadening the scope of gene editing for a diverse range of purposes.

Epigenome editing and gene regulation by CRISPR/Cas

In addition to gene editing activity, Cas9 can be modified to catalytically inactive Cas9 (so-called dead Cas9 or dCas9) as DNA recognition complex, and by tethering various effector proteins to it, site-specific gene regulation and epigenome editing can be achieved (Qi et al., 2013). Through the fusion of "transcription modulators" to dCas9, gene-specific CRISPR-based transcription inhibition (CRISPRi) or activation (CRISPRa) can be performed. In CRISPRi, dCas9 is tethered with a strong transcription repressor domain of Krüppel-associated box (KRAB) and targeted to either promoter, 5′ UTR, or proximal and distal enhancer regions of a specific gene for robust transcription repression (Fig. 9.2A) (Gilbert et al., 2013). It has been shown that KRAB-mediated gene repression is mediated by a local decrease in histone H3 acetylation and an increase in H3K9me3 repressive mark on gene regulatory regions (Thakore et al., 2015). The robust heritable long-term gene silencing can be achieved by the simultaneous use of dCas9-KRAB in combination with the catalytic domain of eukaryotic DNA methyltransferase (DNMT3A) fused to dCas9 (dCas9-DNMT3A), which periodically precipitates methylation at CpG islands near gene promoter sequence (Fig. 9.2C) (Amabile et al., 2016; Liu et al., 2016). In another study, dCas9 was fused to a histone demethylase LSD1, which removes H3K4me2 and H3K27ac marks from the active enhancer element and reduced the specific gene expression (Fig. 9.2C) (Kearns et al., 2015).

Similar to CRISPRi, in CRISPRa method dCas9 can be tethered to transcriptional activators such as herpes simplex viral protein 16 (VP16) alone or multiple copies of it VP64 (4 copies), VP160 (10 copies), or SunTag array, which recruits several copies of VP64 (Fig. 9.2B) (Tanenbaum et al., 2014; Gilbert et al., 2014; Cheng et al., 2013). To increase the activation efficiency, dCas9 has been tagged with tripartite transcriptional activators VP64, transactivation domain of NF-κB p65 subunit (p65AD), and Epstein−Barr virus R transactivator (Rta) in tandem as dCas9-VPR, which showed significantly higher gene activation efficiency compared with VP64 for neuronal differentiation from iPSCs (Chavez et al., 2015). In another approach, synergistic activation mediator (SAM) system has been developed, where single-guided RNAs (sgRNAs) contain two copies of MS2 RNA hairpins, each of

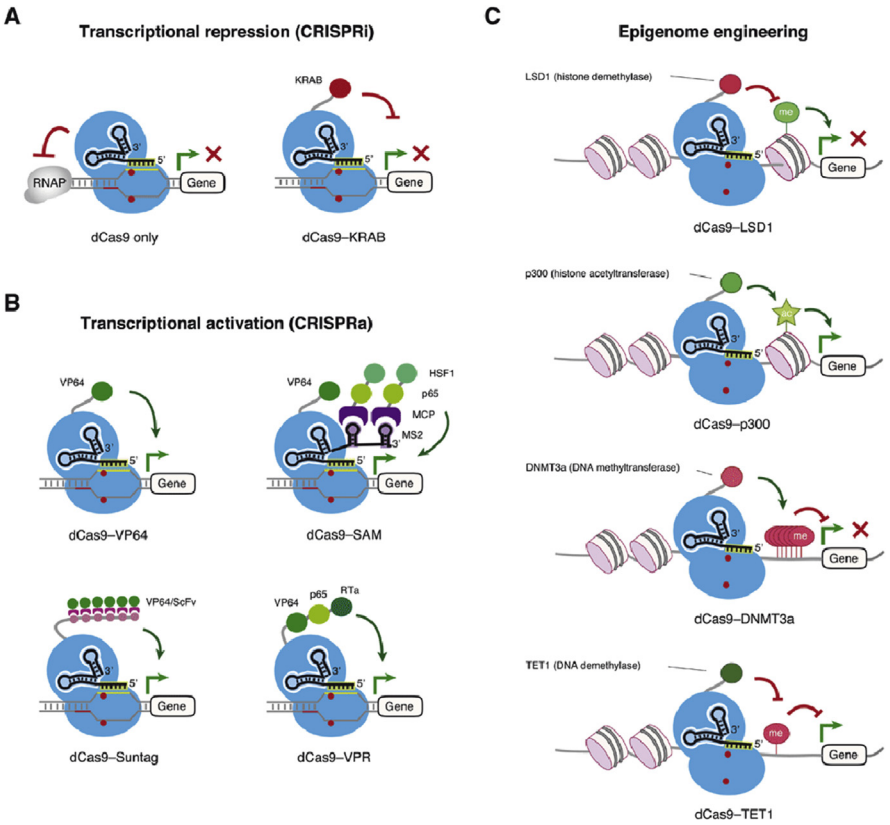

FIGURE 9.2 Epigenomic regulation of specific gene expression by catalytically inactive CRISPR/Cas system (dCas9 system).

(A) CRISPR interference (CRISPRi) system where dCas9-KRAB complex can effectively inhibit the activity and assembly of RNA polymerase (RNAP) complex to disrupt the gene expression, (B) CRISPR activation (CRISPRa) system where dCas9 is coupled with different transcriptional activators such as four copies of herpes simplex viral protein 16 (VP16) to form VP64, synergistic activation system (SAM), SunTag system, and VPR (VP64, p65, and RTa). (C) CRISPR-dCas9-based epigenomic modifications where dCas9-LSD1 complex demethylase H3K4me2 near enhancer to disrupt gene expression, dCas9-p300 complex acetylate the H3K27 histone mark at enhancer and promoter regions to promote gene expression, and finally DNA methylation and demethylation at the promoter regions of specific gene can be modulated by dCas9-DNMT3a and dCas9-TET1 complex, respectively, to manipulate gene expression.

which recruits cognate MS2 phage coat protein (MCP) fused with p65AD and heat shock factor (HSF1) (Fig. 9.2B). The incorporation of three specific activation domains—VP64, p65, and HSF1—into the complex facilitates robust transcriptional activation through synergy (Konermann et al., 2015). Gene activation has also been induced by CRISPR-based epigenome manipulation, either introduction of active histone marks H3K27ac at enhancer and H3K4me3 at the promoter, or by erasing the DNA methylation at gene promoters. The introduction of the catalytic domain of histone acetyltransferase p300 fused with dCas9 (dCas9-p300) resulted in a significant increase in local H3K27ac level and subsequent activation in gene expression (Fig. 9.2C) (Hilton et al., 2015). Other dCas9 tools to modify histone marks have been developed for CRISPRa, including methyltransferases (DOT1L, SMYD3, and PRDM9) or DNA demethylases (TET1). Indeed, the introduction of dCas9-PRDM9 and dCas9-DOT1L significantly enriched H3K4me3 in promoter and H3K79me in the transcribed region of genes and followed by remarkable activation of targeted gene expression (Cano-Rodriguez et al., 2016). Gene expression activation has also been achieved through targeted demethylation of CpG island at gene promoter and 5′ UTR regions by catalytic domain of DNA demethylase ten−eleven translocation 1 (TET1) fused to dCas9 (dCas9-TET1) and subsequent reactivation of silenced gene (Fig. 9.2C) (Liu et al., 2016). For example, reactivation of *FMR1* expression and rescue of fragile X-syndrome (FXS) patient-specific iPSC-derived neurons have been done by targeted demethylation of disease-causing CGG repeats hypermethylation using dCas9-TET1 system (Liu et al., 2018a,b).

Hearing loss disease modeling by CRISPR/Cas9

As the genetic factors contribute extensively for SNHL pathogenesis, genome editing through CRISPR/Cas technique would undoubtedly pave the way of gene therapy as a coherent therapeutic approach. For this purpose, it is essential to understand the molecular nature of the disease progression for a specific gene mutation in a relatively faster and inexpensive, humanoid in vitro disease model, which can specifically be achievable using a combination of hiPSC and CRISPR/Cas technique. With this aim, several studies have generated iPSC-based inherited hearing loss disease models with specific gene mutation to detect the cellular phenotypic abnormalities and tried to rescue the phenotype by correcting the mutation through CRISPR/Cas-based gene editing. In one such experiment, Chen et al. (2016) generated iPSCs from a Chinese family carrying a compound heterozygous mutation in *MYO15A* gene (c.4642G>A; p. A1548T and c.8374G>A; p. V2792M) and differentiated into inner ear hair cell−like cells in monolayer culture following the published protocol of Chen et al. (2012, 2016). Mutation in *MYO15A* causes deafness autosomal recessive 3 (DFNB3), a profound congenital SNHL (Wang et al., 1998). In this study, authors found that precise genetic correction of

MYO15A mutations by CRISPR/Cas9 rescued the abnormal morphological and structural abnormalities in terms of F-actin disorganization, short stereocilia bundle formation, and functional response in terms of electrophysiological properties in iPSC-derived hair cell−like cells (Chen et al., 2016). The same research group investigated the effect of a compound heterozygous mutation in the *MYO7A* gene (c.1184G>A and c.4118C>T) in patient-specific iPSC-derived hair cell−like cells in similar experimental setup (Tang et al., 2016). The patient having this mutation was suffering from severe congenital hearing loss and inherited each of the heterozygous mutations from asymptomatic parents in an autosomal recessive fashion. Upon CRISPR/Cas9-based gene correction of the only c. 4118C>T mutation, investigators have successfully restored the stereociliary bundle formation and electrophysiological response of gene-corrected hair cell−like cells compared with the mutant form (Tang et al., 2016). In a recent study on iPSC-based disease modeling of Pendred syndrome (DFNB4), investigators differentiated cochlear epithelial cells (induced outer sulcus cells) from patient-derived iPSCs harboring different mutations in *SLC26A4* gene (Hosoya et al., 2017). The extensive biochemical and imaging-based analysis found that mutant pendrin protein remains in a diffused cytoplasmic distribution and forms aggregate like puncta. The pendrin mutant-induced outer sulcus cells (OSCs) showed increased cellular stress-induced cell death and degenerative phenotypes. Gene editing−based generation of an isogenic line of a mutant iPSC and subsequent production of induced OSCs rescued the degenerative phenotypes in terms of reduced puncta formation and cellular stress and increased cell viability (Hosoya et al., 2017). In another study, CRISPR-based genetic correction of *USH2A* gene mutation (causing Usher syndrome) restored the *USH2A* mRNA expression to normal level in an iPSC-based cellular model (Sanjurjo-Soriano et al., 2020). Collectively, these studies reflect the fact that CRISPR/Cas9-based gene editing in iPSC-based modeling would have an essential advantage to delineate the disease mechanism in experimental mutant-isogenic control cell line pairs.

Moreover, in the era of single-cell analysis, lineage tracing for cell-fate commitment for otic progenitors (a.k.a. otic stem cells) or monitoring the development of inner ear hair cells would be much more informative to identify the molecular signatures. Here, CRISPR/Cas9 comes into play as the marker genes of each specified cell type could be tagged with fluorescent reporters (e.g., GFP, mCherry, DsRed2, etc.) by CRISPR/Cas-based genome editing. Indeed, Koehler et al. (2017) tagged *ATOH1* gene (an inner ear hair cell marker) with eGFP (enhanced green fluorescent protein) to visualize the inner ear hair cell formation in hPSC-derived organoid model (Fig. 9.1) (Koehler et al., 2017). In another report, early otic lineage marker gene *Pax2* has been tagged with eGFP using CRISPR/Cas9 technique to generate a $Pax2^{EGFP}$ transgenic mice line. The mESCs were isolated from the transgenic mice embryos and differentiated for inner ear organoid development, and otic vesicles were visualized in terms of EGFP expression (i.e., *Pax2* expression) from day 12 of differentiation onward, which helped to delineate the critical stages of inner ear hair cell development (Schaefer et al., 2018). It is evident that

fluorescence-activated cell sorting (FACS) of these reporter expressing cells under different experimental conditions (i.e., the influence of mutation, signaling pathway manipulation etc.) and single-cell analysis in terms of the transcriptome, the proteome, and so on would provide outstanding sets of experimental data. On this basis, planning of novel therapeutic strategies could be drafted.

Future perspectives

The advancement in the field of inner ear hair cell generation from hPSCs, gene editing and manipulation techniques, and single-cell analysis provides an unprecedented opportunity to create hearing loss disease-specific models and study the molecular mechanism of disease onset and progression in developmental paradigm. Though the hPSC model of hearing loss provides an alternate inexpensive and faster experimental model compared with transgenic animal models, it has several hurdles, which need to be solved to adopt it as a reliable ex vivo model. Until now, the best method to derive inner ear hair cells is through a 3D-organoid model, though the hair cells are more vestibular rather than cochlear. The embedding of 3D organoids in extracellular matrix component (e.g., Matrigel) and the substantially large dimension of organoid could hinder the access of extracellular source of growth factors, signaling pathway modulators for differentiation and cellular maturation purposes, and also drugs or small molecules for drug screening purposes. To overcome these issues, the application of bioengineering techniques to provide optimal extracellular matrix stiffness (Brassard and Lutolf, 2019; Yin et al., 2016), cellular contacts and tissue architecture by microfluidics chamber (Yu and Hunziker, 2019), and organ-on-chip technology(Park and Georgescu, 2019) would be transformative for improvement of targeted cellular differentiation and maturation as well as tissue accessibility. In addition to that, the versatile applicability of CRISPR/Cas-based gene editing and genome manipulation is yet to be explored rigorously.

In comparison with gene editing–based disease modeling, epigenetic manipulation through CRISPRi/CRISPRa remained as an unexplored niche. CRISPRi/a, an ex vivo manipulation of gene expression regulation could potentially explain the functional importance of spatiotemporal expression of specific genes and molecular consequences of any perturbation in it during developmental time frame. This CRISPRi/a technique also provides an ample opportunity to test whether inhibition and/or activation of specific genes could facilitate transdifferentiation of supporting cells into functional inner ear hair cells in the cochlear compartment. Furthermore, to study the molecular mechanism of disease development, CRISPR-based genome imaging (Ma et al., 2016), chromatin–protein interaction (Fujita and Fujii, 2013; Myers et al., 2018), and single RNA tracking and imaging (Yang et al., 2019) techniques can also be exploited. In the era of the technological revolution in the field of stem cell biology and molecular genetics, innovation in therapeutic approaches is imminent to tackle one of the most common public health problems, hearing loss.

References

Amabile, A., et al., 2016. Inheritable silencing of endogenous genes by hit-and-run targeted epigenetic editing. Cell 167, 219−232.e214. https://doi.org/10.1016/j.cell.2016.09.006.

Anzalone, A.V., et al., 2019. Search-and-replace genome editing without double-strand breaks or donor DNA. Nature 576, 149−157. https://doi.org/10.1038/s41586-019-1711-4.

Ardhanareeswaran, K., Mariani, J., Coppola, G., Abyzov, A., Vaccarino, F.M., 2017. Human induced pluripotent stem cells for modelling neurodevelopmental disorders. Nat. Rev. Neurol. 13, 265−278. https://doi.org/10.1038/nrneurol.2017.45.

Barrangou, R., et al., 2007. CRISPR provides acquired resistance against viruses in prokaryotes. Science 315, 1709−1712. https://doi.org/10.1126/science.1138140.

Bok, J., et al., 2007. Opposing gradients of Gli repressor and activators mediate Shh signaling along the dorsoventral axis of the inner ear. Development 134, 1713. https://doi.org/10.1242/dev.000760.

Brassard, J.A., Lutolf, M.P., 2019. Engineering stem cell self-organization to build better organoids. Cell Stem Cell 24, 860−876. https://doi.org/10.1016/j.stem.2019.05.005.

Burstein, D., et al., 2017. New CRISPR-Cas systems from uncultivated microbes. Nature 542, 237−241. https://doi.org/10.1038/nature21059.

Cano-Rodriguez, D., et al., 2016. Writing of H3K4Me3 overcomes epigenetic silencing in a sustained but context-dependent manner. Nat. Commun. 7, 12284. https://doi.org/10.1038/ncomms12284.

Casini, A., et al., 2018. A highly specific SpCas9 variant is identified by in vivo screening in yeast. Nat. Biotechnol. 36, 265−271. https://doi.org/10.1038/nbt.4066.

Chadwick, A.C., Wang, X., Musunuru, K., 2017. In vivo base editing of PCSK9 (proprotein convertase subtilisin/kexin type 9) as a therapeutic alternative to genome editing. Arterioscler. Thromb. Vasc. Biol. 37, 1741−1747. https://doi.org/10.1161/atvbaha.117.309881.

Chatterjee, P., Jakimo, N., Jacobson, J.M., 2018. Minimal PAM specificity of a highly similar SpCas9 ortholog. Sci. Adv. 4, eaau0766. https://doi.org/10.1126/sciadv.aau0766.

Chavez, A., et al., 2015. Highly efficient Cas9-mediated transcriptional programming. Nat. Methods 12, 326−328. https://doi.org/10.1038/nmeth.3312.

Chen, W., et al., 2012. Restoration of auditory evoked responses by human ES-cell-derived otic progenitors. Nature 490, 278−282. https://doi.org/10.1038/nature11415.

Chen, J.R., et al., 2016. Effects of genetic correction on the differentiation of hair cell-like cells from iPSCs with MYO15A mutation. Cell Death Differ. 23, 1347−1357. https://doi.org/10.1038/cdd.2016.16.

Chen, J.S., et al., 2017. Enhanced proofreading governs CRISPR−Cas9 targeting accuracy. Nature 550, 407−410. https://doi.org/10.1038/nature24268.

Chen, J., et al., 2018. Differentiation and transplantation of human induced pluripotent stem cell-derived otic epithelial progenitors in mouse cochlea. Stem Cell Res. Ther. 9, 230. https://doi.org/10.1186/s13287-018-0967-1.

Cheng, A.W., et al., 2013. Multiplexed activation of endogenous genes by CRISPR-on, an RNA-guided transcriptional activator system. Cell Res. 23, 1163−1171. https://doi.org/10.1038/cr.2013.122.

Cong, L., et al., 2013. Multiplex genome engineering using CRISPR/Cas systems. Science 339, 819. https://doi.org/10.1126/science.1231143.

Cox, D.B.T., Gootenberg, J.S., 2017. RNA editing with CRISPR-Cas13. Science 358, 1019−1027. https://doi.org/10.1126/science.aaq0180.

Dedhia, P.H., Bertaux-Skeirik, N., Zavros, Y., Spence, J.R., 2016. Organoid models of human gastrointestinal development and disease. Gastroenterology 150, 1098−1112. https://doi.org/10.1053/j.gastro.2015.12.042.

Ding, J., et al., 2016. Induction of differentiation of human embryonic stem cells into functional hair-cell-like cells in the absence of stromal cells. Int. J. Biochem. Cell Biol. 81, 208−222. https://doi.org/10.1016/j.biocel.2015.11.012.

Ealy, M., Ellwanger, D.C., Kosaric, N., Stapper, A.P., Heller, S., 2016. Single-cell analysis delineates a trajectory toward the human early otic lineage. Proc. Natl. Acad. Sci. USA 113, 8508−8513. https://doi.org/10.1073/pnas.1605537113.

Essletzbichler, P., et al., 2014. Megabase-scale deletion using CRISPR/Cas9 to generate a fully haploid human cell line. Genome Res. 24, 2059−2065. https://doi.org/10.1101/gr.177220.114.

Fujita, T., Fujii, H., 2013. Efficient isolation of specific genomic regions and identification of associated proteins by engineered DNA-binding molecule-mediated chromatin immunoprecipitation (enChIP) using CRISPR. Biochem. Biophys. Res. Commun. 439, 132−136. https://doi.org/10.1016/j.bbrc.2013.08.013.

Fukunaga, I., et al., 2016. In vitro models of GJB2-related hearing loss recapitulate Ca(2+) transients via a gap junction characteristic of developing cochlea. Stem Cell Rep. 7, 1023−1036. https://doi.org/10.1016/j.stemcr.2016.10.005.

Gao, X., et al., 2018. Treatment of autosomal dominant hearing loss by in vivo delivery of genome editing agents. Nature 553, 217−221. https://doi.org/10.1038/nature25164.

Gaudelli, N.M., et al., 2017. Programmable base editing of A•T to G•C in genomic DNA without DNA cleavage. Nature 551, 464−471. https://doi.org/10.1038/nature24644.

Géléoc, G.S.G., Holt, J.R., 2014. Sound strategies for hearing restoration. Science 344, 1241062. https://doi.org/10.1126/science.1241062.

Georgomanoli, M., Papapetrou, E.P., 2019. Modeling blood diseases with human induced pluripotent stem cells. Dis. Model Mech. 12. https://doi.org/10.1242/dmm.039321.

Gilbert, L.A., et al., 2013. CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. Cell 154, 442−451. https://doi.org/10.1016/j.cell.2013.06.044.

Gilbert, L.A., et al., 2014. Genome-scale CRISPR-mediated control of gene repression and activation. Cell 159, 647−661. https://doi.org/10.1016/j.cell.2014.09.029.

Halpin-Healy, T.S., Klompe, S.E., Sternberg, S.H., Fernández, I.S., 2020. Structural basis of DNA targeting by a transposon-encoded CRISPR-Cas system. Nature 577, 271−274. https://doi.org/10.1038/s41586-019-1849-0.

Hilton, I.B., et al., 2015. Epigenome editing by a CRISPR-Cas9-based acetyltransferase activates genes from promoters and enhancers. Nat. Biotechnol. 33, 510−517. https://doi.org/10.1038/nbt.3199.

Hosoya, M., et al., 2017. Cochlear cell modeling using disease-specific iPSCs unveils a degenerative phenotype and suggests treatments for congenital progressive hearing loss. Cell Rep. 18, 68−81. https://doi.org/10.1016/j.celrep.2016.12.020.

Hou, Z., et al., 2013. Efficient genome engineering in human pluripotent stem cells using Cas9 from *Neisseria meningitidis*. Proc. Natl. Acad. Sci. USA 110, 15644. https://doi.org/10.1073/pnas.1313587110.

Hu, J.H., et al., 2018. Evolved Cas9 variants with broad PAM compatibility and high DNA specificity. Nature 556, 57−63. https://doi.org/10.1038/nature26155.

Jeong, M., et al., 2018. Generating inner ear organoids containing putative cochlear hair cells from human pluripotent stem cells. Cell Death Dis. 9, 922. https://doi.org/10.1038/s41419-018-0967-1.

Jinek, M., et al., 2012. A programmable dual-RNA−guided DNA endonuclease in adaptive bacterial immunity. Science 337, 816. https://doi.org/10.1126/science.1225829.

Kearns, N.A., et al., 2015. Functional annotation of native enhancers with a Cas9-histone demethylase fusion. Nat. Methods 12, 401−403. https://doi.org/10.1038/nmeth.3325.

Kim, K., et al., 2017. Highly efficient RNA-guided base editing in mouse embryos. Nat. Biotechnol. 35, 435−437. https://doi.org/10.1038/nbt.3816.

Kleinstiver, B.P., et al., 2015. Engineered CRISPR-Cas9 nucleases with altered PAM specificities. Nature 523, 481−485. https://doi.org/10.1038/nature14592.

Kleinstiver, B.P., et al., 2016. High-fidelity CRISPR−Cas9 nucleases with no detectable genome-wide off-target effects. Nature 529, 490−495. https://doi.org/10.1038/nature16526.

Koehler, K.R., et al., 2017. Generation of inner ear organoids containing functional hair cells from human pluripotent stem cells. Nat. Biotechnol. 35, 583−589. https://doi.org/10.1038/nbt.3840.

Koffler, T., Ushakov, K., Avraham, K.B., 2015. Genetics of hearing loss: syndromic. Otolaryngol. Clin. North Am. 48, 1041−1061. https://doi.org/10.1016/j.otc.2015.07.007.

Kokotas, H., Petersen, M.B., Willems, P.J., 2007. Mitochondrial deafness. Clin. Genet. 71, 379−391. https://doi.org/10.1111/j.1399-0004.2007.00800.x.

Komor, A.C., Kim, Y.B., Packer, M.S., Zuris, J.A., Liu, D.R., 2016. Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. Nature 533, 420−424. https://doi.org/10.1038/nature17946.

Konermann, S., et al., 2015. Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex. Nature 517, 583−588. https://doi.org/10.1038/nature14136.

Koonin, E.V., Makarova, K.S., Zhang, F., 2017. Diversity, classification and evolution of CRISPR-Cas systems. Curr. Opin. Microbiol. 37, 67−78. https://doi.org/10.1016/j.mib.2017.05.008.

Lahlou, H., Lopez-Juarez, A., Fontbonne, A., Nivet, E., Zine, A., 2018. Modeling human early otic sensory cell development with induced pluripotent stem cells. PLoS One 13, e0198954. https://doi.org/10.1371/journal.pone.0198954.

Lee, J.K., et al., 2018. Directed evolution of CRISPR-Cas9 to increase its specificity. Nat. Commun. 9, 3048. https://doi.org/10.1038/s41467-018-05477-x.

Litsiou, A., Hanson, S., Streit, A., 2005. A balance of FGF, BMP and WNT signalling positions the future placode territory in the head. Development 132, 4051. https://doi.org/10.1242/dev.01964.

Liu, X.S., et al., 2016. Editing DNA methylation in the mammalian genome. Cell 167, 233−247.e217. https://doi.org/10.1016/j.cell.2016.08.056.

Liu, M., et al., 2018a. Methodologies for improving HDR efficiency. Front. Genet. 9, 691. https://doi.org/10.3389/fgene.2018.00691.

Liu, X.S., et al., 2018b. Rescue of fragile X syndrome neurons by DNA methylation editing of the FMR1 gene. Cell 172, 979−992.e976. https://doi.org/10.1016/j.cell.2018.01.012.

Lopez-Juarez, A., et al., 2019. Engraftment of human stem cell-derived otic progenitors in the damaged cochlea. Mol. Ther. J 27, 1101−1113. https://doi.org/10.1016/j.ymthe.2019.03.018.

Ma, H., et al., 2016. Multiplexed labeling of genomic loci with dCas9 and engineered sgRNAs using CRISPRainbow. Nat. Biotechnol. 34, 528−530. https://doi.org/10.1038/nbt.3526.

Makarova, K.S., Wolf, Y.I., Koonin, E.V., 2018. Classification and nomenclature of CRISPR-Cas systems: where from here? CRISPR J. 1, 325–336. https://doi.org/10.1089/crispr.2018.0033.

Mali, P., et al., 2013. RNA-guided human genome engineering via Cas9. Science 339, 823. https://doi.org/10.1126/science.1232033.

Martin, K., Groves, A.K., 2006. Competence of cranial ectoderm to respond to Fgf signaling suggests a two-step model of otic placode induction. Development 133, 877. https://doi.org/10.1242/dev.02267.

Mertens, J., Reid, D., Lau, S., Kim, Y., Gage, F.H., 2018. Aging in a dish: iPSC-derived and directly induced neurons for studying brain aging and age-related neurodegenerative diseases. Annu. Rev. Genet. 52, 271–293. https://doi.org/10.1146/annurev-genet-120417-031534.

Michalski, N., Petit, C., 2019. Genes involved in the development and physiology of both the peripheral and central auditory systems. Annu. Rev. Neurosci. 42, 67–86. https://doi.org/10.1146/annurev-neuro-070918-050428.

Mittal, R., et al., 2017. Recent advancements in the regeneration of auditory hair cells and hearing restoration. Front. Mol. Neurosci. 10, 236. https://doi.org/10.3389/fnmol.2017.00236.

Myers, S.A., et al., 2018. Discovery of proteins associated with a predefined genomic locus via dCas9-APEX-mediated proximity labeling. Nat. Methods 15, 437–439. https://doi.org/10.1038/s41592-018-0007-1.

Ohnishi, H., et al., 2015. Limited hair cell induction from human induced pluripotent stem cells using a simple stepwise method. Neurosci. Lett. 599, 49–54. https://doi.org/10.1016/j.neulet.2015.05.032.

Paquet, D., et al., 2016. Efficient introduction of specific homozygous and heterozygous mutations using CRISPR/Cas9. Nature 533, 125–129. https://doi.org/10.1038/nature17664.

Park, S.E., Georgescu, A., 2019. Organoids-on-a-chip. Science 364, 960–965. https://doi.org/10.1126/science.aaw7894.

Qi, L.S., et al., 2013. Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. Cell 152, 1173–1183. https://doi.org/10.1016/j.cell.2013.02.022.

Ran, F.A., et al., 2015. In vivo genome editing using *Staphylococcus aureus* Cas9. Nature 520, 186–191. https://doi.org/10.1038/nature14299.

Rees, H.A., et al., 2017. Improving the DNA specificity and applicability of base editing through protein engineering and protein delivery. Nat. Commun. 8, 15790. https://doi.org/10.1038/ncomms15790.

Rios, A.C., Clevers, H., 2018. Imaging organoids: a bright future ahead. Nat. Methods 15, 24–26. https://doi.org/10.1038/nmeth.4537.

Roccio, M., Edge, A.S.B., 2019. Inner ear organoids: new tools to understand neurosensory cell development, degeneration and regeneration. Development 146. https://doi.org/10.1242/dev.177188.

Ronaghi, M., et al., 2014. Inner ear hair cell-like cells from human embryonic stem cells. Stem Cells Dev. 23, 1275–1284. https://doi.org/10.1089/scd.2014.0033.

Sakagami, M., et al., 2019. Differentiation of embryonic stem cells into inner ear vestibular hair cells using vestibular cell derived-conditioned medium. Biochem. Biophys. Rep. 19, 100649. https://doi.org/10.1016/j.bbrep.2019.100649.

Sanjurjo-Soriano, C., et al., 2020. Genome editing in patient iPSCs corrects the most prevalent USH2A mutations and reveals intriguing mutant mRNA expression profiles. Mol. Ther. Methods Clin. Dev. 17, 156−173. https://doi.org/10.1016/j.omtm.2019.11.016.

Schaefer, S.A., et al., 2018. From otic induction to hair cell production: Pax2(EGFP) cell line illuminates key stages of development in mouse inner ear organoid model. Stem Cells Dev. 27, 237−251. https://doi.org/10.1089/scd.2017.0142.

Sharma, A., Wu, J.C., Wu, S.M., 2013. Induced pluripotent stem cell-derived cardiomyocytes for cardiovascular disease modeling and drug screening. Stem Cell Res. Ther. 4, 150. https://doi.org/10.1186/scrt380.

Shmakov, S., et al., 2017. Diversity and evolution of class 2 CRISPR-Cas systems. Nat. Rev. Microbiol. 15, 169−182. https://doi.org/10.1038/nrmicro.2016.184.

Sinkunas, T., et al., 2011. Cas3 is a single-stranded DNA nuclease and ATP-dependent helicase in the CRISPR/Cas immune system. EMBO J. 30, 1335−1342. https://doi.org/10.1038/emboj.2011.41.

Slaymaker, I.M., et al., 2016. Rationally engineered Cas9 nucleases with improved specificity. Science 351, 84−88. https://doi.org/10.1126/science.aad5227.

Takahashi, K., et al., 2007. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131, 861−872. https://doi.org/10.1016/j.cell.2007.11.019.

Takahashi, K., Yamanaka, S., 2006. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126, 663−676. https://doi.org/10.1016/j.cell.2006.07.024.

Takeda, H., Hosoya, M., Fujioka, M., 2018. Engraftment of human pluripotent stem cell-derived progenitors in the inner ear of prenatal mice. Sci. Rep. 8, 1941. https://doi.org/10.1038/s41598-018-20277-5.

Tanenbaum, M.E., Gilbert, L.A., Qi, L.S., Weissman, J.S., Vale, R.D., 2014. A protein-tagging system for signal amplification in gene expression and fluorescence imaging. Cell 159, 635−646. https://doi.org/10.1016/j.cell.2014.09.039.

Tang, Z.H., et al., 2016. Genetic correction of induced pluripotent stem cells from a deaf patient with MYO7A mutation results in morphologic and functional recovery of the derived hair cell-like cells. Stem Cells Transl. Med. 5, 561−571. https://doi.org/10.5966/sctm.2015-0252.

Tang, P.C., et al., 2019. Defective Tmprss3-associated hair cell degeneration in inner ear organoids. Stem Cell Rep. 13, 147−162. https://doi.org/10.1016/j.stemcr.2019.05.014.

Thakore, P.I., et al., 2015. Highly specific epigenome editing by CRISPR-Cas9 repressors for silencing of distal regulatory elements. Nat. Methods 12, 1143−1149. https://doi.org/10.1038/nmeth.3630.

Torres, R., et al., 2014. Engineering human tumour-associated chromosomal translocations with the RNA-guided CRISPR-Cas9 system. Nat. Commun. 5, 3964. https://doi.org/10.1038/ncomms4964.

Vakulskas, C.A., et al., 2018. A high-fidelity Cas9 mutant delivered as a ribonucleoprotein complex enables efficient gene editing in human hematopoietic stem and progenitor cells. Nat. Med. 24, 1216−1224. https://doi.org/10.1038/s41591-018-0137-0.

Venkatesh, M.D., Moorchung, N., Puri, B., 2015. Genetics of non syndromic hearing loss. Med. J. Armed Forces India 71, 363−368. https://doi.org/10.1016/j.mjafi.2015.07.003.

Wang, A., et al., 1998. Association of unconventional myosin MYO15 mutations with human nonsyndromic deafness DFNB3. Science 280, 1447−1451. https://doi.org/10.1126/science.280.5368.1447.

Wu, D.K., Kelley, M.W., 2012. Molecular mechanisms of inner ear development. Cold Spring Harb Perspect. Biol. 4, a008409. https://doi.org/10.1101/cshperspect.a008409.

Yamada, M., et al., 2017. Crystal structure of the minimal Cas9 from *Campylobacter jejuni* reveals the molecular diversity in the CRISPR-Cas9 systems. Mol. Cell 65, 1109e1103−1121e1103. https://doi.org/10.1016/j.molcel.2017.02.007.

Yang, L.Z., et al., 2019. Dynamic imaging of RNA in living cells by CRISPR-Cas13 systems. Mol. Cell 76, 981e987−997e987. https://doi.org/10.1016/j.molcel.2019.10.024.

Yeh, W.-H., Chiang, H., Rees, H.A., Edge, A.S.B., Liu, D.R., 2018. In vivo base editing of post-mitotic sensory cells. Nat. Commun. 9, 2184. https://doi.org/10.1038/s41467-018-04580-3.

Yin, X., et al., 2016. Engineering stem cell organoids. Cell Stem Cell 18, 25−38. https://doi.org/10.1016/j.stem.2015.12.005.

Yu, F., Hunziker, W., 2019. Engineering microfluidic organoid-on-a-chip platforms. Micromachines 10. https://doi.org/10.3390/mi10030165.

Zetsche, B., et al., 2015. Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. Cell 163, 759−771. https://doi.org/10.1016/j.cell.2015.09.038.