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INVITED REVIEW ARTICLE

Gene therapy for hearing loss

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Abstract

Sensorineural hearing loss (SNHL) is the most common sensory disorder. Its underlying etiologies include a broad spectrum of genetic and environmental factors that can lead to hearing loss that is congenital or late onset, stable or progressive, drug related, noise induced, age related, traumatic or post-infectious. Habilitation options typically focus on amplification using wearable or implantable devices; however exciting new gene-therapy-based strategies to restore and prevent SNHL are actively under investigation. Recent proof-of-principle studies demonstrate the potential therapeutic potential of molecular agents delivered to the inner ear to ameliorate different types of SNHL. Correcting or preventing underlying genetic forms of hearing loss is poised to become a reality. Herein, we review molecular therapies for hearing loss such as gene replacement, antisense oligonucleotides, RNA interference and CRISPR-based gene editing. We discuss delivery methods, techniques and viral vectors employed for inner ear gene therapy and the advancements in this field that are paving the way for basic science research discoveries to transition to clinical trials.

Introduction

Hearing loss is the most common sensorineural deficit. It affects approximately 466 million people worldwide, 34 million of whom are children (http://www.searo.who.int/bangladesh/infographicworldhearingday2018.pdf?ua=1). By 2030, this number will have increased to nearly 630 million people, and by 2050, over 900 million people will have some degree of hearing loss (http://www.searo.who.int/bangladesh/infographicworldhearingday2018.pdf?ua=1). Its underlying etiologies are not surprisingly varied and, with advancing age, become increasingly more complex. By way of example, both genetic and environmental factors can lead to hearing loss that is congenital or late onset, stable or progressive, drug related,

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noise induced, age related, traumatic or post-infectious. On the otherwise healthy newborn with hearing loss, in which noise-induced, age-related and traumatic deafness do not occur, the etiology is most often genetic or infectious secondary to a prenatal cytomegalovirus infection.

Genetic hearing loss impacts about 1 in 500 newborns and over the course of a lifetime predisposes to or is directly responsible for ∼50–60% of all deafness, with percentages higher in developed countries (1). In the pre-lingual and young pediatric population, hearing loss has a profound impact on communication and language acquisition and carries a social stigma that leads to economic and educational disadvantages and isolation throughout life. Early diagnosis and intervention remediate much of this effect, and as a result, universal physiologic newborn hearing screening has been widely implemented across the United States in order to identify deaf or hard-of-hearing newborns and reduce time-to-diagnosis and intervention (2).

In the past decade, genetic testing has emerged as the most important diagnostic test for evaluation of children with deafness. Establishing a genetic diagnosis provides essential information for understanding the underlying pathophysiology, and with the advent of comprehensive genetic testing, it is trivial to screen all genes known to be implicated in hearing loss simultaneously (3,4). The accumulation of genetic, genomic and clinical data is readily accessible through extensive databases such as ClinVar and Human Gene Mutation Database, both of which continuously curate the rapidly increasing volume of reported genetic variants, and the Deafness Variation Database, a deafness-specific open-access resource that integrates all available genetic, genomic and clinical data, together with expert curation to generate a single resource for clinical and research use (5).

Spurred by advances in our genetic understanding of sensorineural hearing loss (SNHL) and the notable successes in other specialties, interest in gene therapy for hearing loss has grown. Current habilitation options focus on hearing aids and cochlear implants, both of which bypass the biologic deficit by amplifying sounds in the case of hearing aids or by encoding them as electrical impulses that are transmitted to the auditory nerve through an electrode array in the case of cochlear implants. While these devices are very effective, they do not actually restore 'normal' hearing, making the development of novel therapeutics to restore or prevent hearing loss an important goal to enhance quality of life (6).

Genetic hearing loss is viewed as a relatively circumscribed and comparatively straightforward therapeutic 'target', as an established genetic diagnosis defines both the underlying pathophysiology and the essential problem to the 'corrected'. In addition, the inner ear is an isolated site that can be accessed safely surgically and into which therapeutics can be delivered with minimal off-target systemic effects. In this review, we focus on recent advances in inner ear gene therapies for SNHL.

Methods for gene therapy—gene specific

Gene replacement

Gene replacement is arguably the most 'straight forward' form of gene therapy (Table 1) (7–26). Based on identifying and replacing the defective gene with a normal or wild-type copy, notable successes have been achieved treating patients with Leber's congenital amaurosis and with hemophilia (27,28). Perhaps portending eventual success in the treatment of persons with hearing loss are a number of studies on murine models of Usher syndrome (12–15,17,18,29), Jervell and Lange–Nielsen syndrome (23) and a type of hearing loss caused by absence of vesicular glutamate transporter-3 (*VGLUT3*) (26).

The first successful inner-ear gene therapy study treated mice homozygous for the targeted deletion of *VGLUT3*. These mice are born deaf but exogenous replacement of *VGLUT3* and its overexpression in inner hair cells (IHCs) mediated by adeno-associated virus 1 (AAV1) leads to sustained hearing recovery, partial restoration of ribbon synapse morphology and a startle response. Interestingly, although transgene expression of *VGLUT3* within the inner ear was not specific solely to IHCs, the observed phenotypic rescue reflected improved IHC function, suggesting that cell-specific transduction may not be an absolute necessity in all instances. Two important limitations of this study are the fact that autosomal recessive non-syndromic hearing loss caused by *VGLUT3* has not yet been described in humans. The observed utility of this approach, which was successful at P1 for the duration of the study but showed a variable level of rescue at later time points such as P10–12, remains to be established. This temporal difference is relevant because, in the P1 mouse, inner ear maturation is occurring and auditory function does not fully mature until about P15. In contrast, humans are born with mature inner ears. This difference confounds any inferences murine results may have for human deafness unless gene therapy is delivered to the mature murine ear.

Gene suppression—antisense oligonucleotides

Antisense oligonucleotides (ASOs) are modified nucleic acid sequences that bind to complementary RNA sequences by Watson–Crick base pairing. They regulate gene expression by two primary mechanisms that are dependent on their chemical properties and target (30). The first, ASO knock down, occurs with ribonuclease-H (RNase-H) cleavage of the RNA strand from the RNA–DNA duplex, with resultant degradation of the mRNA. The second, splice site switching, occurs when ASOs interfere with alternative splicing by targeting splice sites, exons or introns, resulting in exclusion or inclusion of specific exons (31). To date, five ASOs are approved by the US Food and Drug Administration and many clinical trials are underway. The first-approved ASO, fomivirsen, is used for the treatment of cytomegalovirus-induced retinitis in patients with acquired immune deficiency syndrome (32).

Lentz *et al*. (16) have reported on the utility of ASO treatment in the murine inner ear to rescue the *USH1C* 216G>A (216A) mutation in a mouse model of USH1C. This founder mutation in the Acadian population leads to a cryptic 5′ splice site, which is used in preference of the authentic 5′ splicing site of exon 3. The result is a frameshift and truncated harmonin protein. The Usher syndrome mouse model used in the study was a knock-in based on the human 216A mutation—*Ush1c* c.216G>A. The group designed an ASO-29 to redirect cryptic splicing of 216A pre-mRNA to the authentic site.

ASO-29 injected into homozygous (216AA) and heterozygous (216GA) knock-in mice intraperitoneally at P3 and P10 led to near-normal hearing thresholds in broad band noise and 8 and 16 kHz pure tones in 216AA mice treated between P3 and P5. Hearing threshold at 32 kHz could not be rescued, and over the course of 3 months there was a gradual decline in thresholds at 8 and 16 kHz. The vestibular dysfunction was also rescued. 216AA Mice treated at P10 showed significantly higher thresholds than animals treated at P3–5 but significantly better thresholds than untreated or control-ASO-treated animals in both broad band

Table 1. Gene-specific reports of gene therapy in mouse models of deafness

Gene	Gene therapy method	Genotype	Age at intervention	Length of follow up	Age at best ABR results	Click-evoked best ABR results	Tone-burst best ABR results				Reference
							8kHz	16kHz	24kHz	32kHz	
Tmc1	GR	Homo	P0–2	P84–90	P28–30	NR	65	65	85	100	Nist-Lund *et al.*, 2019 (7)
		Homo	P0–2	P60	P25–30	NR	85	100	120	120	Askew *et al.*, 2015 (8)
	RNAi	Hetero	P15–16	P140–146	P56–62	40	50	85	NR	90	Yoshimura *et al.*, 2019 (9)
		Hetero	P0–2	P245–251	P28	40	20	30	NR	90	Shibata *et al.*, 2016 (10)
	CRISPR/ Cas9	Hetero	P1	P28–34	P28–34	NR	55	60	65	80	Gao *et al.*, 2018 (11)
Clrn1	GR	Homo	P1	P28–34	P28–34	NR	85	90	95	95	Gyorgy *et al.*, 2019 (12)
		Homo	P1–3	P60	P22–24	NR	30	30	35	50	Dulon *et al.*, 2018 (13)
		Homo	P1–3	P70	P22	45	35	35	NR	40	Geng *et al.*, 2017 (14)
Ush1c c.216G>A	GR	Homo	P1	P168–174	P42–48	NR	50	50	80	95	Pan *et al.*, 2017 (15)
	ASOs	Homo	P5	P84–90	P28–34	45	45	45	NR	70	Lentz *et al.*, 2013 (16)
Whrn	GR	Homo	P1–5	P90	P30–90	NR	95	95	NR	95	Chien *et al.*, 2016 (17)
		Homo	P1–5	P120	P30	NR	60	80	NR	80	Isgrig *et al.*, 2017 (18)
Otof	GR	Homo	P10	P210–216	P28–34	35	65	65	NR	60	Akil *et al.*, 2019 (19)
		Homo	P6–7	P30	P23–30	50	70	90	90	90	Al-Moyed *et al.*, 2019 (20)
Lhfpl5	GR	Homo	P0–1	P28–34	P28–29	NR	70	90	85	100	Gyorgy *et al.*, 2017 (21)
MsrB3	GR	Homo	E12.5	P56–62	P28	25	25	30	40	40	Kim *et al.*, 2016 (22)
Kcnq1	GR	Homo	P0–2	P210–216	NR	45	55	65	75	80	Chang *et al.*, 2015 (23)
Gjb2	GR	Homo	P0	P84–90	P70–84	NR	NR	NR	65	NR	Iizuka *et al.*, 2015 (24)
		Homo	P0–1	P28–34	P28–34	NR	85	85	85	85	Yu *et al.*, 2014 (25)
Vglut3	GR	Homo	P1	P483–489	P50–52	40	65	55	NR	65	Akil *et al.*, 2012 (26)

ABR, auditory brainstem response; GR; gene replacement; NR, not recorded/no report.

noise and at 8 kHz. Histological assessment of ASO-29-treated inner ears demonstrated rescue of outer hair calls (OHCs) and IHCs at P1 but not at P5 (33).

These observations may reflect two important limitations of ASO at least in this animal model and for this indication—a therapeutic time window in the *USH1C* mouse before which therapy must be delivered to be effective and lack of a sustained response.

Gene suppression—RNA interference

RNA interference (RNAi) is a biological process in which RNA molecules inhibit gene expression or translation by neutralizing specifically targeted messenger RNA (mRNA) molecules. Its therapeutic use typically focuses on one of two types of small RNA molecules—small interfering RNA (siRNA) or microRNA (miRNA)—which represent a novel class of therapeutic agents with the potential to treat a wide range of disorders, including cancers and infections. Several clinical trials using siRNA- and miRNA-based drugs have been initiated, and one RNAi-based therapeutic is now available for the treatment of hereditary transthyretin-mediated amyloidosis in adults (34).

While siRNAs and miRNAs share many similarities—for example, both are short duplex RNA molecules that exert gene silencing effects at the post-transcriptional level—their mechanisms of action and clinical applications are different. siRNAs tend to be more specific than miRNAs. Once an siRNA enters a cell, it associates with a ribonucleoprotein complex known as RISC (RNA-induced silencing complex), which uses one strand of the siRNA molecule as a template to recognize a specific mRNA transcript. The identified mRNA molecule is then cleaved by Argonaute 2, a protein in the RISC complex, thereby preventing it from serving as a translational template (35,36).

miRNAs are in general less specific and typically regulate the expression of multiple genes (37). They are derived from a non-coding RNA primary transcript (pri-miRNA) that is processed in the cell nucleus into a stem-loop pre-miRNA structure by Drosha, an RNase III enzyme, and DGCR8, a double-stranded RNA-binding protein. The pre-miRNA is then exported to the cytoplasm by Exportin 5 using GTP bound to the Ran protein. In the cytoplasm, the dsRNA portion of pre-miRNA is cleaved by Dicer to produce a mature miRNA molecule that can be integrated into the RISC complex. At this point, miRNA and siRNA share the same downstream intracellular machinery.

The breadth of miRNA activity is derived from its partially complementarity to multiple mRNAs (37–39). However, artificial miRNAs can be designed to base pair perfectly with selected RNA targets, thereby inducing cleavage of specific mRNA molecules. These artificial miRNAs, like designer siRNAs, provide RNAi-based gene-specific and even allele-specific mRNA silencing (40). In proof-of-concept studies relevant to hearing loss, Maeda *et al.* (41) used siRNAs to suppress expression of an exogenous deafness-inducing pathogenic variant of *GJB2* thereby preventing hearing loss. Building on these results, Shibata *et al.* (10) designed an artificial miRNA to specifically target the mutation-carrying *TMC1* allele in the *Beethoven* mouse, a murine model of human autosomal dominant non-syndromic hearing loss at the DFNA36 locus, and slowed the progressive hearing-loss phenotype in mice treated at P1–2. A follow-up study by Yoshimura *et al.* (9) treated older animals, and although progression of hearing loss could also be slowed in animals treated at P15 and P30, the results were not as dramatic, and in animals treated at P60, no effect was observed. These findings suggest that, for *TMC1*-related deafness, the opportunity to intervene using RNAi is temporally defined and, beyond a specific time point, targeted allele suppression has no effect.

Gene editing: CRISPR/Cas9

Targeted genome editing has emerged as a powerful tool for biological research. Although there are three major programmable nucleases—ZFNs (zinc finger nucleases), TALENs (transcriptional activator-like effector nucleases) and CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 (CRISPR-associated protein 9)–ZFNs, and TALENs require large DNA fragments (500–1500 bp), while Cas9 can identify a target sequence with only 20 bp of guide RNA (gRNA). CRISPR/Cas9 can also be used for multiplexing by delivering multiple gRNAs to target multiple genes in the same cell at the same time (42). This efficiency and flexibility has propelled CRISPR/Cas9 to the forefront as a tool for prevention and treatment of human disease.

Recognized challenges include off-target effects, mutations, editing efficiency and uncertainty of single-site target selectivity in complex genomes; however, several methods have been developed to address these challenges (43–49). For example, Hsu *et al.* (44) have demonstrated that the concentration of single-gRNA and Cas9 can be limited to improve the on-target effect and Kim *et al.* (47) have developed RNA-guided engineered nucleases to reduce the frequency of producing off-target indels.

As applied to auditory research, Gao *et al.* (11) injected Cas9–gRNA–lipid complexes targeting the mutant *TMC1* allele into P1 *Beethoven* mice and substantially reduced progression of hearing loss. Significant hearing preservation was detected from 8 to 23 kHz with average ABR thresholds 15 dB lower for treated ears as compared to untreated contralateral ears. This study was the first of its kind to demonstrate the potential of CRISPR/Cas9 for the treatment of autosomal dominant hearing loss related to hair cell dysfunction.

Similar to the aforementioned gene therapy methods, the therapeutic time window in this mouse model is in the early postnatal period for this therapy to be effective. Further longitudinal studies will be necessary to assess the longevity of the treatment effect.

Methods for gene therapy—non-specific

Cell replacement: stem cell-based therapy

While mammalian cochlear hair cells lack regenerative capacity, avian cochlea hair cells do not and following acoustic trauma can be replaced by mitosis of supporting cells (50–53) (Table 2) (50,51,54–75). Attempts to regenerate hair cells using progenitor cells have explored trans-differentiation of supporting cells into HCs and mitosis of supporting cells (54,55). Both strategies depend on the condition of the extant supporting cells, with concomitant intracochlear drug delivery required to direct outcome (76,77). Studies in newborn mice suggest that 'naïve' cells can be used to 'jump-start' the formation of supporting cells and their transformation into hair cells, as naïve cells respond better to molecular cues; however, the potential for trans-differentiation is temporally limited (78,79). Hair cell loss months or years earlier precludes this possibility perhaps in part due to loss of molecular interactions between hair cells and supporting cells (80).

As an alternative, stem cell transplantation has been explored using embryonic stem cells (ESCs), adult-derived stem cells (ASCs) or induced pluripotent stem cells (iPSCs) as seeds from which to generate HCs. ESCs have the advantage of

Table 2. Stem-cell based therapies

Methods	Sub methods	Cells used	End result	*In vivo* experiment	References
Inner ear stem cell induction	Direct transdifferentiation	SCs	1 HC		Adler *et al.*,1997 (54); Roberson *et al.*, 2004 (55)
	Mitosis of SCs	SCs, daughter cells	1 SC and 1 HC		Corwin and Contanche, 1988 (50); Ryals and Rubel, 1988 (51)
Cell transplantation	Embryonic stem cells	mESCs	Inner HCLC		Li *et al.*, 2003 (56)
		mESCs	Mechanosensitive inner ear HCLC		Oshima *et al.*, 2010 (57)
		mESCs	SCs, mechanosensitive HCLC, neurons		Koehler *et al.*, 2013 (58)
		mESCs	iHCs with HC specific markers and protrusions reminiscent of stereociliary bundles		Costa *et al.*, 2015 (59)
		hESCs	ONPs, OEPs	Transplanted into adult gerbils; ABR improved	Chen *et al.*, 2012 (60)
		hESCs	iHCs with HC specific markers and protrusions reminiscent of stereociliary bundles		Ronaghi *et al.*, 2014 (61)
		hESCs, hiPSCs	Neurons, SCs, functional HCLCs in 3D culture		Koehler *et al.*, 2017 (62)
	ASCs	BM-MSCs	SGNs	Transplanted into adult gerbils	Matsuoka *et al.*, 2007 (63)
		BM-MSCs	Fibrocyte-like cells	Transplanted into young and adult mice; migrated MSCs seen only in young mice	Kasagi *et al.*, 2013 (64)
		Adult human MSCs	SGNs		Bas *et al.*, 2014(65)
		HUMSCs	Unknown	Transplanted into albino deaf pig; new ABR waves appeared after injection	Ma *et al.*, 2016 (66)
		ratMSCs	HCs	Pre-injected by deferoxamine to home MSCs in noise induced injured cochlea	Peyvandi *et al.*, 2018 (67)

Continued

Table 2. (Continued)

Methods	Sub methods	Cells used	End result	*In vivo* experiment	References
		BM-MSCs	Assess adverse effect	No effect on ABR or DPOAE in rats	Mittal *et al.*, 2019 (68)
		HL-MSCs	Cochlear nerves	Transplanted into intracranial cochlear nerve trunk; ABR improved	Chen *et al.*, 2019 (69)
	Induced pluripotent stem cells	miPSCs	SGNs	Transplanted into mouse cochlea	Nishimura *et al.*, 2009 (70)
		miPSCs	Mechanosensitive inner ear HCLC		Oshima *et al.*, 2010 (57)
		hiPSCs	HCLCs		Ohnishi *et al.*, 2015(71)
		hiPSCs from human MYO15A patients	HCLCs	Corrected mutation by CRISPR/Cas9; functional and morphological rescue	Chen *et al.*, 2016 (72)
		hiPSCs from human MYO7A patients	HCLCs	Same as above	Tang *et al.*, 2016 (73)
		miPSCs	Outer sulcus cell-like cells	Transplanted into mouse otocysts at E11.5	Takeda *et al.*, 2017 (74)
		hiPSCs	OEPs, HCLCs	Transplanted into mouse cochlea; synaptic connection with SGNs	Chen *et al.*, 2018 (75)

SC, Supporting cell; HC, Hair cell; HCLC, Hair cell-like cell; mESC, mouse embryonic stem cell; hESC, human embryonic stem cell; iHC, induced hair cell; ONP, otic neural progenitor; MSC, mesenchymal stem cell; BM-MSC, bone marrow-derived MSC; HL-MSC, human limbus-MSC; HUMSC, human umbilical cord MSC; ABR, auditory brainstem response; DPOAE, distortion product otoacoustic emissions; PSC, pluripotent stem cells; hiPSC, human-induced PSC; miPSC, mouse-induced PSC.

Table 3. AAV transduction *in vivo*

Reference	Mouse model	AAV model	Delivery method	Age at delivery	Transduction rate of IHCs (%)			Transduction rate of OHCs (%)		
					Apex	Middle	Base	Apex	Middle	Base
Gyorgy *et al.*, 2019 (12)	BL6	9PHP	RWM	P1	70	70	50	40	35	35
			CAA	4 weeks		NR		0	0	0
	CD1		RWM	P1	70	60	70	50	40	40
	Clrn				63	54	39	36	31	9
Gu *et al.*, 2019 (100)	ICR	2/2	CO	P2–3		Few			few	
		2/9			39.6 ± 16.3	52.7 ± 5.7	78.3 ± 7.2	14.4 ± 0.9	15.0 ± 3.0	100
		Anc80			100	100	100	93.5 ± 7.2	95.6 ± 4.4	81.6 ± 11.4
Kim *et al.*, 2019 (101)	ICR	2/DJ	RWM	P2	52	41	37	90	88	37
		2/DJ8			59	24	10	<10	<10	<10
		2PHP			86	81	62	63	31	16
Akil *et al.*, 2019 (19)[a]	*Otof* $^{-/-}$	AAV2 quadY-F capsid	RWM	P2		78 ± 6			0	
				P10		64 ± 6			0	
				P17		82 ± 9			0	
				P30		85 ± 7			0	
Isgrig *et al.*, 2019 (102)	CBA	2.7m8	CAA	P0–5		84.1 ± 5.66			83.1 ± 6.17	
				1–6 months		84.5 ± 4.91			74.9 ± 6.53	
		8BP2		P0–5		55.7 ± 9.53			44.1 ± 7.94	
Akil *et al.*, 2019 (103)	FVB	5	RWM	P1–3		80			<1	
Yoshimura *et al.*, 2018 (104)	C3H	2/9	RWM +CF	P15–16	94.6	96.8	94.2		NR	
				P56–60	89.7	92.2	98.1		NR	
				P15–16	16.7	17.4	18		NR	
		Anc80			84.5	90.8	91.9		NR	
Tao *et al.*, 2018 (105)	BL6	1	CO	10 weeks	7.9 ± 2.0	11.8 ± 4.7	7.0 ± 0.6	0	0	0
		2			95.4 ± 8.0	86.5 ± 13.9	85.5 ± 17.0	12.1 ± 12.3	7.1 ± 3.0	1.6 ± 1.6
		6.2			11.9 ± 1.7	3.1 ± 0.8	2.6 ± 0.6	0	0	0
		8			61.0 ± 18.9	NR	72.6 ± 27.5	0	0	0
		9			58.8 ± 7.6	61.8 ± 19.0	49.3 ± 11.9	0	0	0
		rh.39			42.3 ± 12.0	36.2 ± 4.7	61.1 ± 7.9	0	0	0
		rh.43			93.2 ± 3.1	92.7 ± 6.1	94.3 ± 3.9	0	0	0
		Anc80			98.4 ± 2.7	98.4 ± 2.7	89.3 ± 18.5	67.2 ± 28.5	39.7 ± 31.7	10.4 ± 18.1
Shibata *et al.*, 2017 (106)	C3H	2/9	Systemic injection	P0–1	70–90	40–70	20–40			
					20–30	5–20	0–5		sparse	
					70	NR	10			
		1			3	NR	3			
Landegger *et al.*, 2017 (107)	BL6	Anc80	RWM	P1	100	NR	100	95	NR	95
Suzuki *et al.*, 2017 (108)	CBA	Anc80	CAA	7 weeks	100	100	100	80–90	35–75	20–35
Gyorgy *et al.*, 2017 (21)	CD1	2/1	RWM	P0–1	50	70	70	10	10	20
			CO		25	30	45	10	10	25
		e2/1	RWM		85	90	90	30	25	25
			CO		50	65	70	15	20–30	40
		2/9	CO			NR			NR	
		e2/9				60			25	
	Lhfpl5	e2/1	CO			>95			>85	
Pan *et al.*, 2017 (15)[a]	BL6	Anc80	RWM	P1	69 (Total of all IHCs and OHCs)				65 (co-transfection of total of all IHCs+OHCs)	
		Anc80			74 (total of all IHCs and OHCs)					

Continued

Table 3. (continued)

Reference	Mouse model	AAV model	Delivery method	Age at delivery	Transduction rate of IHCs (%)			Transduction rate of OHCs (%)		
					Apex	Middle	Base	Apex	Middle	Base
Isgrig *et al.*, 2017 (18)	*Whrn*	2/8	CA	P1–5	71.7 ± 26.0	81.2 ± 15.3	75.2 ± 17.6	10.4 ± 6.38	8.64 ± 13.2	3.21 ± 5.95
Chien *et al.*, 2016 (17)	*Whrn*	2/8	RWM	P1–5	15.3	16.2	11.8	0	0	0
Kim *et al.*, 2016 (22)	ICR *MsrB3*	2/1	Trans uterine injection	E12.5		>90 89–91			83 84–92	
Shu *et al.*, 2016 (109)	CD1	1	CO	P1–2	2.6 ± 0.6	9.6 ± 4.1	14.9 ± 2.6	6.3 ± 1.1	15 ± 6.3	18.3 ± 6.5
		2			11.8 ± 2.1	15 ± 2.9	22.6 ± 4.8	4.1 ± 1.1	28 ± 4.5	39.5 ± 8.5
		5			0	11.2 ± 2.9	28.1 ± 3.4	0	0	0
		6.2			0	0	0	0	0	0
		7			3.1 ± 0.8	16.2 ± 2.6	20.5 ± 2.5	0	0	0
		8			4.2 ± 0.9	14.2 ± 2.1	15 ± 3.1	6.1 ± 1.3	18.7 ± 1.7	21 ± 2.5
		9			0	0	0	4.2 ± 0.9	16.2 ± 2.3	21 ± 3.1
		rh.10			8.1 ± 2	24 ± 4.7	34 ± 5.7	0	0	0
		rh.39			NR	NR				
		rh.43			0	3 ± 1.1	5.3 ± 2.1	0	0	0
	CBA	1	CO	6 weeks	12.2 ± 2.3	24.1 ± 6.2	45.8 ± 7.3			
		2			13.2 ± 2.1	27.2 ± 4.5	35.2 ± 6.3			
		5			NR					
		6.2			10.5 ± 1.5	18 ± 2.1	28 ± 4.8			
		7			NR					
		8			NR				NR	
		9			9.1 ± 1.4	35.1 ± 3.2	61.6 ± 8			
		rh.8			NR					
		rh.10			NR					
		rh.39			5.6 ± 1.6	15 ± 2.3	20 ± 4.7			
		rh.43			NR					
Chien *et al.*, 2015 (110)	CBA	2/8	RWM CO	1– 2 months	12 4.3	12 5.3	31 28		NR NR	
Askew *et al.*, 2015 (8)	BL/6	2/1	RWM	P0–2		59 ± 2 70 ± 9			NR NR	
Yu *et al.*, 2014 (25)	*Gjb2*	2/1	RWM	P1	Apex:13 ± 2, Middle:32 ± 4, Base:44 ± 3 (total of all IHCs and OHCs)					
Wang *et al.*, 2013 (111)	BL6	2/1	CO	P1	NR	NR	0	NR	NR	0
		2/7					10.2 ± 1.9 82.1 ± 9.3			32 ± 4.3 0
Akil *et al.*, 2012 (26)	*VGLUT3*	1	RWM	P1-P3	100	100	100		NR	
				P10–12	40	40	40		NR	
				P1–3		NR			NR	
			CO	P10–12	100	100	100		NR	
				P10–12	100	100	100		NR	
				P10–12	40	40	40		NR	
Xia *et al.*, 2012 (112)	BL6	8	RWM RWMPD	P7		58 ± 4 47 ± 7			19 ± 4 17 ± 4	
Kilpatrick *et al.*, 2011 (113)	CBA	1 2 5 6 8	CO	P7	AAV2 and AAV8 had robust transduction			AAV8 had robust transduction		

Continued

Table 3. (continued)

Reference	Mouse model	AAV model	Delivery method	Age at delivery	Transduction rate of IHCs (%)			Transduction rate of OHCs (%)		
					Apex	Middle	Base	Apex	Middle	Base
Bedrosian *et al.*, 2006 (114)	BALB/c	2/1	Ex-utero injection	E12	82.54	NR	79.4	63.7	NR	64.67
		2/2				NR			NR	
		2/5				0			0	
		2/6				0			0	
		2/7				0			0	
		2/8				Second best			Second best	
		2/9				0			0	
	CBA	2/1	Trans uterine injection	E12		Best		Best		
		2/2				Second best			Second best	
		2/5			0	0				
		2/6			0	0				
		2/7			0	0				
		2/8				Second best			Second best	
		2/9			0	0				
Liu *et al.*, 2005 (115)	BL6 and ICR	3	RWM	4	100	100	0		0	

Anc80, AAV2/Anc80l65; 2/DJ, rAAV2/DJ; 2/DJ8, rAAV2/DJ8; 2.7m8, AAV2.7m8; 8BP2, AAV8BP2; 2PHP, rAAV2/PHP.B; 9PHP, AAV9/PHP.B; rh.39, AAVrh.39; rh.43, AAVrh.43; e2/1, exo-AAV2/1; e2/9, exo-AAV2/9; rh.8, AAVrh.8; rh.10, AAVrh.10; BL6, C57BL/6; C3H, C3HeB/FeJ; CBA, CBA/CaJ; RWM, round window membrane; RWM + CF, round window membrane combined with canal fenestration; RWMPD, round window membrane partial digestion; CA, canalostomy; CAA, canalostomy to ampulla; CO, cochleostomy

[a]They injected dual vectors.

pluripotency and can be differentiated into hair cell-like cells (57), otic sensory neurons (81) and spiral ganglion neuron (SGN)-like cells (82); however, the available pool is limited and their use raises ethical issues. Although ASCs obviate these ethical considerations, they are of limited pluripotency, and as a result, most studies have focused on iPSCs.

Several laboratories have reported stunning successes using iPSCs to generate hair cells that are even responsive to mechanical stimulation. Chen *et al.* (75), for example, used human iPSCs to generate otic epithelial progenitors (OEPs) and investigated their migration, differentiation and synaptic connections in mouse cochlea. *In vitro*, OEP-derived hair cell-like cells formed synaptic connections with SGNs in co-culture. *In vivo*, a few OEPs migrated into the organ of Corti and differentiated into hair cells that established connections with native SGNs (75).

While these results offer the potential promise for cell-based therapies for hearing loss in the future, there are major challenges to consider. First, regenerated hair cells more closely resemble vestibular rather than cochlear hair cells (58,62, 83–86); second, after culture, the cells must be introduced into the inner ear and then self-insert into the proper location in the membranous labyrinth (87,88); and third, the carcinogenic potential of stem cells must be carefully followed (89,90). To put these challenges into perspective, it is worth noting that mice are deaf even if all hair cells form but are disorganized.

Goals and timing of therapy

The goal of gene therapy is hearing preservation or restoration. To achieve this goal, an exquisite understanding of normal and abnormal inner ear development and function is required. Included in this understanding is cross-talk between HCs and SGNs, the complexities of which are only beginning to be appreciated. Sun *et al.* (91), for example, have recently shown that active mechanotransduction channels in HCs are critical to shape the spontaneous firing patterns in SGNs prior to the hearing onset. This activity is initiated at least in part by the spontaneous release of ATP from supporting cells, which causes HCs to depolarize and release glutamate, triggering discrete bursts of action potentials in primary auditory neurons. Subtype specification of SGNs is thus initiated in the pre-hearing period coincident with the timeframe in which spontaneous activity in SGNs is observed. Refinement of SGN subtypes continues into the fourth week after birth, suggesting that sensory input drives some aspects of SGN specification.

Maturation of ribbon synapses also continues until the fourth postnatal week (92–94), overlapping the period when murine SGNs refine their firing properties from an immature to a mature state in which there is a range of fibers with different spontaneous firing rates (95), indicating an intricate interplay between molecular and functional diversification (91). This study raises questions about cochlear-directed gene therapy and whether studies focused solely on the organ of Corti may fail because of an unrecognized need to address SGN function. This interrelationship is especially apt to be important with age-related and noise-induced hearing loss.

Viral vectors

Viral vectors are the workhorse for cochlear gene therapy, with studies exploring the use of adenovirus (96–99), AAV (7–9,12,15,17–23,25,77,100–117). helper-dependent adenovirus (118), herpes simplex virus (119–121), vaccinia virus (121), sendai virus (122) and lentivirus (111,114,123,124). Of all these choices, AAV has emerged as the most attractive vector for cochlear gene delivery. Belonging to the Parvoviridae family genus *Dependovirus*, AAV is a small virus (25 nm) that lacks pathogenicity and has minimal immunogenicity (125). It transduces both non-dividing and dividing cells to provide stable

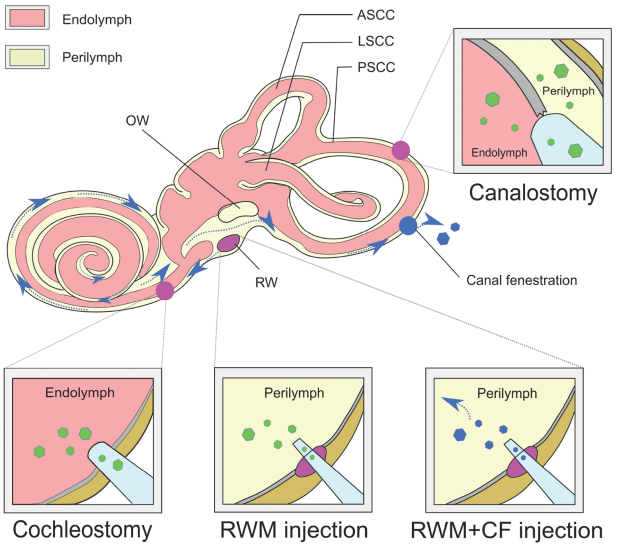

Figure 1.. Inner ear delivery of gene therapy. Shown are the major routes by which gene therapy can be delivered to the inner ear. With the RWM approach, a small needle is used to pierce the RWM and deliver vector directly into the perilymph. A 'canalostomy' is done by drilling a hole into a semicircular canal (usually the posterior canal) and inserting a canula into the hole. Vector is delivered into both endolymph and perilymph in one of two directions depending on the orientation of the cannula. A cochleostomy requires a hole in the bony labyrinth between round window and basal turn of cochlea. Vector is delivered into endolymph. The RWM + CF technique combines RWM injection with a CF, which functions as a vent to allow egress of fluid (red, endolymph; yellow, perilymph; blue arrow, expected flow of injected vectors through cochlea when RWM + CF is done; PSCC, posterior semicircular canal; ASCC, anterior semicircular canal; LSCC, lateral semicircular canal; OW, oval window; RW, round window).

long-term gene expression by persisting as an episome without chromosomal integration (125).

The major disadvantage of AAV is its low viral capacity (4.7 kb), which is halved when using a self-complementary AAV (125). This limitation can be overcome by dual injection methods at the expense of transduction efficiency (116). In a recent report, this approach was used to deliver *Otof* (6 kb) into the inner ear of mice lacking this gene, with restoration of hearing (19).

In the inner ear, AAVs demonstrate broad tropism, stable gene expression and little to no ototoxicity (126). Transgene expression is affected by several factors including serotype, age at treatment, method of delivery, titer, promoter type and the presence or absence of enhancers (such as WPRE). Significant effort has defined the tropism of AAV subtypes relative to different cell types in the murine cochlea (Table 3).

In general, AAV1, 2, 5, 6, 8, 9 and rh10 consistently transduce IHCs in both neonatal and adult mice; however, transduction of OHCs and supporting cells is variable and may be impacted by route of delivery. For example, when AAV1 is injected into the endolymph in neonatal mice, it transfects supporting cells (i.e. Deiters and Hensen's cells) but not when it is injected into the perilymphatic space (111). Likewise, when AAV8 is injected into the endolymph in adult mice, OHCs, IHCs and supporting cells are transduced, but when injected into the perilymphatic space, transduction is limited to IHCs (113,115). Most studies have utilized ubiquitous promoters such as CMV and CBA; however, cell-specific promoters permit cell-specific expression, which may reduce the risk of off-target effects (127).

New synthetic vectors have emerged as an alternative to conventional AAV vectors and have demonstrated superior transduction in the inner ear (15,21,100–102,104,105,107,108). Most widely used is AAV2/Anc80L65, a novel designer AAV in which the main capsid proteins approximate the imputed ancestral sequence of AAV1, 2, 8 and 9 (128). AAV2/Anc80L65 has shown promising potential to transduce IHCs and OHCs when injected through either perilymph or endolymph in both neonatal and adult mice (100,108).

Delivery of gene therapy

The membranous labyrinth, which includes the cochlear duct, semi-circular ducts, utricle and saccule, lies within the bony labyrinth in the temporal bone. It is relatively isolated, has minimal lymphatic circulation and is separated from blood by the blood–labyrinthine barrier, three factors that limit efficacious systemic delivery of therapeutics to only neonatal mice (106,129). Direct local injection of viral vectors into the inner ear is necessary to order to achieve viral titers appropriate for gene therapy. Established injection routes include (1) round window membrane (RWM), (2) canalostomy, (3) cochleostomy into either the endolymph or perilymph and (4) RWM combined with canal fenestration (CF) (Figure 1).

Round window membrane

The RWM is a three-layered membranous opening into the perilymphatic space of the scala tympani. This approach is well established and used clinically for cochlear implantation and, to date, is the most commonly utilized method of introducing transgenes into the inner ear in animal models (Table 1). Concerns for hearing loss secondary to perilymphatic leakage have been raised but can be obviated by plugging the RWM perforation with fascia (10,104). One disadvantage of the RWM approach is that distribution of the viral vector throughout the cochlear duct is challenging and, as a result, transduction tends to occur in a base-to-apex gradient in adult mice (104).

Canalostomy

Kawamoto *et al.* (130) developed the canalostomy approach for mouse cochlear gene therapy as an alternative to RWM injection. They injected an adenovirus expressing bacterial lacZ through a fenestration in the posterior semicircular canal, directing the cannula toward the crus commune. Hearing was preserved, with transduction mostly restricted to vestibular organs; minimal cochlear transduction was achieved. Suzuki *et al.* (108) modified the technique by using AAV2/Anc80L65 and targeting injection toward the ampulla. Hearing was not compromised, and cochlear transduction was 100% in IHCs and 80–90%, 35–70% and 20–35% in apical, mid and basal OHCs, respectively.

Cochleostomy

Cochleostomy directly delivers transgenes to the scala media, which can be accessed via a hole drilled through the basal portion of cochlea into the cochlear endolymphatic space near the round window. Chien *et al.* (110) compared cochleostomy to RWM injection in adult mice and demonstrated similar transduction efficiency although significant hearing loss was noted following the cochleostomy. In contrast, Kilpatrick *et al.* (113) showed that IHC and OHC transduction with AAV8 were superior with a cochleostomy and that hearing loss was minimal at high frequencies (≥32 kHz) and absent at low to middle frequencies (<32 kHz) 1 month after surgery. However, this approach is

technically challenging. Its clinical application may be as an approach for stem cell transplantation or hair cell regeneration.

RWM with CF

Both the RWM and canalostomy injections in adult mice demonstrate transduction biases in either a base-to-apex or apex-to-base gradient, which cannot be overcome without increasing injection volume (18,108,130). Unfortunately, increased injection volume leads to hearing loss. To improve injection efficiency while maintaining hearing, Yoshimura et al. modified the RWM approach by adding a CF (104). The fenestration serves as a vent and permits longitudinal flow throughout the cochlea resulting in even distribution of the injected vector. Delivery is into perilymph, hearing is preserved and near total IHC transduction is possible. One disadvantage of this approach is the short-lived vestibular dysfunction associated with creation of a venting hole in the posterior semicircular canal. Treated mice have nystagmus in the acute recovery phase, which abates by the next day, although they do not have abnormal circling behavior.

Conclusion

Rapid development in diagnostics and therapy for SNHL has been made in recent years, and there have been multiple reports describing variably successful gene therapy in neonatal and adult mice models of human deafness. In addition, a three-part, multicenter, open label, single dose study is listed under ClinicalTrials.gov (https://clinicaltrials.gov/ct2/show/NCT02132130) to assess the safety, tolerability and efficacy of intra-labyrinthine CGF166, a recombinant adenovirus 5 vector containing the human atonal transcription factor cDNA, in patients with severe-to-profound hearing loss. However, for inner-ear gene therapy to enter the clinical realm with the goal of preventing or restoring hearing, important questions remain to the addressed in both mouse models of deafness and in nonhuman primates.

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