REVIEW

Gene transfer in inner ear cells: a challenging race

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Recent advances in human genomics led to the identification of numerous defective genes causing deafness, which represent novel putative therapeutic targets. Future gene-based treatment of deafness resulting from genetic or acquired sensorineural hearing loss may include strategies ranging from gene therapy to antisense delivery. For successful development of gene therapies, a minimal requirement involves the engineering of appropriate gene carrier systems. Transfer of exogenous genetic material into the mammalian inner ear using viral or non-viral vectors has been characterized over the last decade. The nature of inner ear cells targeted, as well as the transgene expression level and duration, are highly dependent on the vector type, the route of administration and the strength of the promoter driving expression. This review summarizes and discusses recent advances in inner ear gene-transfer technologies aimed at examining gene function or identifying new treatment for inner ear disorders.

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INTRODUCTION

Approximately 278 million people worldwide suffer from inner ear disorders.¹ The lack of effective treatments for many forms of hearing loss or balance disorders is complicated by the diversity of disease processes leading to functional hearing loss. The mature mammalian inner ear comprises one auditory and five vestibular organs: the cochlea, the utricle, the saccule and three semicircular canals. The sensory epithelia of these organs consist of sensory cells, non-sensory supporting cells (SCs) and nerve endings. Hair cells (HCs) and SCs arise from a common progenitor after proliferation and differentiation within the sensory epithelia. There are two types of HCs in the organ of Corti (OC), the inner HCs being the true sensory cell type, responsible for the main mechanotransduction mechanism, and the outer HCs used to enhance the performance of the cochlea.² Numerous disease processes, such as presbycusis, sudden neurosensory hearing loss, genetic diseases or Menière's disease, cause inner ear dysfunction. The greatest majority of these disorders result directly or indirectly from degeneration and death of cochlear sensory cells and/or their associated peripheral neurons (that is, spiral or vestibular ganglion neurons). Currently, therapeutic strategies to treat inner ear diseases are limited to systemic delivery of medications, surgical intervention or sound amplification (with hearing aids) with limited success. Recent technological advancements in gene therapy and local drug delivery shade a new light for inner ear diseases. Therefore, there is a growing interest in successfully transferring genes in OC or vestibular cells (HCs, SCs and progenitors) and spiral or vestibular ganglion neurons. There are currently two main technical approaches for gene transfer: viral and non-viral vectors (Table 1). Gene transfer using viruses is named transduction, whereas gene transfer using non-viral vectors is referred as transfection. Both approaches may be appropriate for inner ear gene modulation, depending on the experimental needs, especially which cell type should be targeted.

Part of the challenge for developing gene therapy for the inner ear is physical. The inner ear is separated from the general

circulation by a blood–cochlea barrier with similar properties to the blood–brain barrier, making systemic delivery less efficient.³ However, the inner ear is accessible via intratympanic delivery and diffusion through the round window membrane (RWM) or via a cochleostomy, through the stapes footplate, or through the endolymphatic sac⁴ (Figure 1). As described here, gene transfection or transduction via cochleostomy into the basal turn of the *scala media* was found to be more efficient than the RWM approach, mainly linked to the cell barrier and tight junctions present between the *scala tympani* and *scala media* (Tables 2–5). The inner ear is a relatively closed space, making gene or drug delivery very efficient as compared with systemic delivery, for which large doses would be needed to reach the inner ear.⁵

VIRAL VECTORS

Viral vectors are powerful tools for gene delivery *in vitro* and *in vivo*. For vector development, elements of the viral genome that contribute to replication, virulence and disease are deleted and replaced by genes of interest, but infectivity (*cis*-acting regulatory sequence) is retained. The deleted genes encoding proteins that are involved in replication or capsid/envelope proteins are included in a separate packaging construct to provide helper functions in trans. Non-replicating viral particles are generated by the introduction of the vector genome and packaging construct into a packaging cell line.⁶ One of the major advantages of viral vectors is their good efficiency of transfection and distinct tropism capacity, which can help restrict transgene expression to a subset of cells. The major drawbacks of these systems are their potential immunological and genetical toxicities, causing inflammation and insertional mutations, respectively. Six families of viruses have been engineered for inner ear transfection, both *in vitro* and *in vivo*: adenovirus, adeno-associated virus (AAV), lentivirus, herpes simplex virus, vaccinia virus and Sendai virus. They differ in

Table 1. Principal features of gene delivery systems

Vector	General characteristics	Main advantages	Main limitations
Adenovirus	Composed of a linear 35 Kb double-stranded DNA. Cloning capacity 8 Kb	Infects a wide variety of dividing and post-mitotic cells Easy to make Grow easily in culture and allow high titres of viral particle Long-term and high levels of expression of target gene Large insert capacity	Strong host antiviral response Transient expression
Adeno-associated virus	Replication-deficient member of the parvovirus family with a 4.7 kb single-stranded DNA. Cloning capacity 4–5 Kb	Infects a wide variety of dividing and post-mitotic cells Few immunogenicity Long-term gene expression	Difficult to produce at high titres Limited gene insert space (< 5 kb) Integration may occur
Herpes simplex virus	Complex double-stranded DNA virus surrounded by icosahedral capsid and an envelope. Cloning capacity 30 kb	Large gene capacity Neurotropic No integration	Potential of recombination Limited duration of transgene recombination Low transduction efficiency
Lentivirus	Single-stranded DNA virus. Cloning capacity 8 kb. Vectors based on the HIV	Highly effective in dividing and non-dividing cells Good transduction efficiency Large insert capacity Low immune response	Inefficient gene transfection of HCs Random integration
Sendai virus	Single-stranded RNA virus. Cloning capacity around 4–5 Kb	Non-integrative vector Rapid cellular uptake Good transduction efficiency	Limited gene insert space (< 5 kb)
Vaccinia virus	Linear, double-stranded DNA molecule of approximately 190 Kb. Cloning capacity of 25 Kb	Large gene capacity Protection against ligation with foreign DNA	Limited to non-small pox vaccinated individuals
Cationic liposomes	Phospholipid spherical vesicle fusing with cellular membrane, thanks to the cationic charge	Non-infectious Easy to make Large gene capacity	Low efficiency May provoke an acute immune response
Cationic non-liposomal polymers	Polycationic polymers attract negatively charged phosphates of DNA	Not immunogen Easy to prepare	Low efficiency Toxic to cells May provoke an acute immune response
Electroporation	Application of electric-field pulses creating transient aqueous pathways in lipidic membrane	Not immunogen High transfection efficiency	Tissue damage Gene transfer limited to targeted area Needs surgical procedure for internal organs
Biolistic	Bombardment of a targeted cellular surface by micro-sized DNA-coated gold particles, which are subjected to an acceleration by a pressure pulse of compressed helium gas	Not immunogen Good <i>in vivo</i> activity	Cannot efficiently transfect small cells and may cause significant tissue damage Gene transfer limited to targeted area Needs surgical procedures for internal organs

complexity of construction, expression time, potential side effects and carrying capacity.

ADENOVIRUS

Adenovirus is a non-enveloped, double-stranded DNA virus. The viral linear DNA genome coding region contains five early transcription regions (E1A, E1B, E2, E3 and E4), which have regulatory functions, and one late transcription region, encoding structural proteins.⁶ Adenoviruses exist as over 50 different serotypes, but only a few of them have been modified to generate vectors. Most emphasis has been placed on serotype 5 (Ad5).⁷ Adenoviral vectors infect a large variety of non-dividing cells. They grow easily in cell lines, allowing for production of high

viral titres ($> 10^{11}$ viral particles per ml). To provide additional cloning space, the E1 and E3 early regions of the adenovirus are usually deleted to enable the insertion of an expression cassette of up to 7.5 Kb. Long-term and high expression levels of target genes are possible with this vector type; however, despite a good efficiency of transduction, its injection can provoke strong immune responses responsible for damage in recipient cells.^{8–10}

The first generation of replication-deficient adenovirus vectors (deleted for E1A, E1B and a portion of E3 region) was based on the human Ad5 and has been widely used to transduce different cell types of inner ear explants in culture (Table 2). HCs, SCs and spiral ganglion neurons (SGNs) were successfully targeted by low titres of viral particles without any cell damage or defect in neurite extension.^{11–14} However, transduction currents evoked by hair

Figure 1. Schematic illustration of the inner ear and of a cochlear turn cross-section. The *scala vestibuli* (SV) and *scala tympani* (ST) are filled with perilymph fluid, whereas the *scala media* (SM), containing the OC, is filled with endolymph. The OC comprises the non-sensory supporting cells as well as the sensory inner HCs (IHCs) and outer HCs (OHCs), contacted by spiral ganglion neuron fibres. Various routes of administration have been tested in the inner ear for gene delivery. Gene transfection or transduction in the inner ear could be performed via intratympanic delivery through the round window membrane (a), after cochleostomy and injection in the ST (b) or in the SM (c), or through the endolymphatic sac (d).

bundle displacement cannot be recorded after 36 h, despite a cell body appearing healthy.¹³

Cochlear cells have also been efficiently transduced *in vivo* by this first generation of adenoviral vectors. Ad5-RSV-LacZ (Ad5 expressing beta-galactosidase under the control of the Rous sarcoma virus—RSV—promoter) was injected through the round window into the *scala tympani* of the guinea pig cochlea. This led to a successful transduction of neural, epithelial and connective tissues of the cochlea.^{8,15} Indeed, almost 50% of the cells in the spiral ganglion were efficiently infected, as well as the cells lining the perilymphatic space, including the cells of Reissner's membrane and mesothelial cells (that is, fibroblasts that form a monolayer of specialized pavement-like cells that line the cavity of the *scala tympani*). However, this viral vector failed to transduce cells of the OC. In contrast, Ad5 vectors with the cytomegalovirus promoter driving green fluorescent protein (GFP) or LacZ expression have been shown to efficiently transduce pillar cells and, to a lesser extent, HCs when used at high titre ($>10^8$ pfu per cochlea).^{10,16} The use of alternative *in vivo* administration routes, through the endolymphatic sac or the *scala media*, enlarged the panel of transfected cells to *stria vascularis* cells and other types of SCs.^{17–19}

To date, adenoviral vectors have proven their therapeutic potential in protecting inner ear cells from ototoxic damages. Delivery of glial cell-derived neurotrophic factor (*GDNF*) or transforming growth factor beta (*TGFβ*) genes to the mesothelial or Reissner's membrane cells promoted survival of HCs and/or SGNs after aminoglycoside administration.^{20–24} However, the major limitation of this first generation of adenovirus vectors is that they usually trigger strong immune responses.^{13,25} *Ex vivo* experiments with inoculation of fibroblasts, previously transduced with adenoviral vectors (Ad5-RSV-LacZ), into the perilymph, were performed to avoid an inflammatory response. Unfortunately, T-cell infiltration was not reduced using this approach.⁹ Therefore, new vector constructs were developed by retaining the E3 region, but deleting the E1 and E4 region.²⁶ Various results were obtained using these new constructs (Table 2). Intact hair bundles of GFP-positive cells after exposure to high titres (10^7 particles per ml) of

the defective vector were seen *in vitro*,²⁷ whereas no difference in transfection efficiency and toxicity was observed in another study.¹² *In vivo* experiments demonstrated that the second generation of adenoviruses were efficiently transduced in the OC after *in utero* injection. However, both early and advanced generation of adenoviruses induced toxicity when delivered to cells of the developing murine cochlea.²⁸ E1/E3/E4 (Ad.11D)-deleted vectors were used to transduce *Atoh1* gene, encoding a bHLH transcription factor involved in the generation of HCs in the mature guinea pig cochlea.²⁹ Overexpression of *Atoh1* resulted in the production of new HCs, both in physiological and pathological (destruction of HCs by aminoglycosides) conditions.^{29,30} Ad5 vectors expressing catalase and Mn superoxide injected through the round window in the *scala tympani*, protects HCs against aminoglycoside-induced oxidative stress.³¹ The same Ad5 vector expressing neurotrophins transduced into the *scala media* in guinea pigs allowed short-term survival of SGNs after the degeneration of HCs, even if the vectors were not directly transduced into the neurons.³² Moreover, gain-of-function of *Atoh1* with Ad28 vector in vestibular SCs (Ad28-GFAP-*Atoh1*) was accompanied by *de novo* hair-cell generation and restoration of balance function following drug-induced hair-cell loss in mice.³³

Adenoviruses bind cells primarily via the coxsackievirus and adenovirus receptor through the knob of the viral fibre. Adenovirus entry occurs through clathrin-mediated endocytosis after binding of the penton via its arginyl-glycyl-aspartic acid motif to $\alpha V\beta 3$ and $\alpha V\beta 5$ integrins.³⁴ Therefore, the expression of coxsackievirus, adenovirus receptor and integrins on the host cells are a prerequisite to efficient transduction. Indeed, SGNs do not express the coxsackievirus and adenovirus receptor, and therefore, are not targeted by adenoviral vectors.³⁵ Despite the presence of coxsackievirus and adenovirus receptor and integrins on other cochlear cells, poor transduction efficiency can be observed because of limited accessibility of those receptors. Different strategies to improve the binding of the vector to the cells have been developed. Incorporation of a small targeting ligand within the vector, such as an arginyl-glycyl-aspartic acid peptide specific to integrin receptor (Ad-arginyl-glycyl-aspartic

Table 2. Principal adenoviral vectors used to insert genes into the inner ear

Virus type	Titre	Targeted cells	Route of administration	References
Ad5-RSV-LacZ	10 ⁷ pfu per cochlea	SGN, cells lining perilymphatic space	Perilymphatic	8, 43
Ad5-CMV-GFP + Ad5-CMV-LacZ	> 10 ⁸ pfu per cochlea	SCs and HCs of the OC	Perilymphatic	10, 25
Ad5-CMV-GDNF + Ad5-CMV-LacZ	10 ¹⁰ VP per ml	Utricle, mesothelial cells of the <i>scala vestibuli</i> in the basal turn of the cochlea	<i>Scala vestibuli</i>	21
Ad5-RSV-LacZ	10 ¹⁰ VP per ml	Cells lining fluid space, Reissner's membrane, SCs of the OC (Hensen cells), Spiral ligament	Injection into the endolymphatic sac	17
Ad5-CAG-GDNF	10 ¹⁰ VP per ml	Mesothelial cell layer of the OC, Reissner membrane, spinal ganglion and cells around HCs of the OC	Round window (<i>scala tympani</i>)	24
Ad5-CMV-GDNF	2 × 10 ¹⁰ VP per ml	Mesothelial cells in the perilymphatic space	Round window (<i>scala tympani</i>)	22
Ad5-CMV-TGF-β	10 ⁸ –10 ⁹ VP per ml	HCs and SCs of the saccule, SCs of the utricle	Round window (<i>scala tympani</i>)	33
Ad28-CMV-GFP + Ad28-GFAP-Atoh1	10 ¹¹ VP per ml	Hensen cells, inner sulcus areas and the interdental cell area	Cochleostomy (<i>scala media</i>)	29
Ad5-CMV-Math1.11D	10 ¹¹ VP per ml	Non-sensory cells of the OC	Cochleostomy (<i>scala media</i>)	30
Ad5-CMV-Atoh1-GFP	10 ¹² VP per ml	Cells lining the <i>scala media</i> , SCs of the OC and satellite cells surrounding the SGNs	Cochleostomy (<i>scala media</i>)	35
Ad5-CMV-GFP	3 × 10 ¹¹ pfu per cochlea	SCs, mesothelial cells of the <i>scala tympani</i> and <i>scala vestibuli</i> , cells of the Reissner's membrane	Cochleostomy (<i>scala media</i>)	19
Ad5-CMV-LacZ	10 ¹¹ VP per ml	SCs, cells lining the <i>scala media</i> and <i>scala vestibuli</i> , inner and outer sulcus, Reissner's membrane	Cochleostomy (<i>scala media</i>)	18
Ad5-CMV-GFP-Neurotrophin	10 ¹⁰ VP per ml	HCs and SCs of the OC, interdental cells of the spiral limbus, <i>stria vascularis</i> and Reissner's membrane	Cochleostomy (<i>scala media</i>)	32
Ad5.11D-CMV-GFP/LacZ	5 × 10 ¹¹ –2 × 10 ¹² VP per ml	<i>Stria vascularis</i> , SGN, HCs and SCs	Cochleostomy (<i>scala media</i>)	36
Ad5.2K-CMV-GFP/LacZ				
Ad5.RGD-CMV-GFP/LacZ				
Ad5.11D-CMV-GFP	9.6 × 10 ¹¹ VP per ml	OC region (no intense staining) difficult to identify cell types transduced	Otocyst <i>in utero</i> injections (E11–E12)	28
Ad5-CMV-LacZ	10 ¹⁰ pfu per ml	HCs, SCs of the OC, mesothelial cells lining the perilymphatic space	OC explants (P1, P3, P5)	12
Advanced generation (deleted for E1A, E1B, E3, E4)				
Ad5-CMV-Bgal	10 ⁴ –10 ⁹ pfu per ml	SGN, HCs of the OC	Rat OC and spiral ganglion explants	11
Ad5-CMV-GFP + Ad5-CMV-Kir2	10 ⁴ –10 ¹¹ pfu per ml	Fibroblasts, HCs of the OC and the utricle	Utricle and OC explants	13
Ad5-CMV-LacZ	10 ¹⁰ pfu per ml	HCs, SCs of the OC	OC explants	12
Ad5-CMV-Kir2.1-Ires-GFP	7 × 10 ⁸ pfu per ml	SGN, HCs of OC	OC and spiral ganglion explants	14
Ad5-CMV-GFP	10 ¹² VP per ml	Mesothelial cells lining the <i>scala tympani</i> and the <i>scala vestibuli</i> , Reissner's membrane, SCs of the OC	OC explants	35

Abbreviations: Ad5, adenovirus 5; CMV, cytomegalovirus; GFP, green fluorescent protein; GDNF, glial cell-derived neurotrophic factor; HCs, hair cells; SCs, supporting cells; OC, organ of Corti; RGD, arginyl-glycyl-aspartic acid; RSV, Rous sarcoma virus; SGN, spiral ganglion neurons; TGF-β, transforming growth factor beta; VP, viral particles; pfu, plaque-forming unit.

acid) or seven lysine residues (Ad-F2K), greatly improves inner ear transduction.³⁶ Searching for improving the yield of cochlear cell transfection with adenoviral vectors, Taura *et al.*³⁷ recently discovered that histone deacetylase inhibition enhances adenoviral vector transduction in cochlear explants, and particularly in HCs and SCs.

ADENO-ASSOCIATED VIRUS

AAVs are replication-deficient members of the parvovirus family with a 4.7Kb single-stranded DNA genome. The viral genome flanked by two inverted terminal repeat sequences contains two open reading frames, *rep* and *cap*, and alternative splicing signals. The *rep* open reading frame encodes four proteins for replication, whereas the *cap* open reading frame encodes three structural proteins that form the capsid. A total of 12 human serotypes of

AAV (AAV-1 to AAV-12) and more than 100 serotypes from non-human primates have been discovered to date. The use of the different AAV serotypes in a pseudotyping approach (creating hybrid AAV with the genome of one inverted terminal repeat sequence serotype being packaged into a different serotype capsid) has broadened tissue tropism.^{38–40} AAV is unique among viruses that are being developed for gene therapy in that the wild-type virus is not responsible for human disease. Therefore, AAV are able to transduce a wide range of cells and tissues, and are devoid of adverse consequences, such as immunogenicity, preventing cell clearance and thus allowing long-term gene expression. One major drawback of AAV is that the vector capacity is limited to 4–5 Kb, making it unsuitable for transfer of large genes.

Numerous publications have reported AAV transduction of the rodent inner ear through different routes of administration,

Table 3. Main adeno-associated viral vectors used to insert genes into the inner ear

Type	Titre	Targeted cells	Route of administration	References
AAV1-5, 7, 8-CAG-GFP	5×10^{10} genomic copies per ml	IHCs (1-3, 5, 7, 8), SGN (5), spiral ligament (1, 7), SCs (5, 8)	Round window (<i>scala tympani</i>)	44
AAV1-CAG-GFP, AAV1-Myo-GFP AAV1-CMV-GFP AAV1-EF1-GFP AAV1-NSE-GFP AAV1-RSV-GFP AAV1-CMV-GFP	6×10^{12} - 8×10^{13} genomic copies per ml	IHCs (CAG, CMV, Myo), SGN, spiral ligament and mesenchymal cells (CAG, CMV, NSE, EF1), Reissner membrane (CAG, CMV, NSE) inner sulcus cells (CMV, CAG)	Round window (<i>scala tympani</i>)	51
AAV1-2, 4, 5-CMV-GFP	1.4×10^{13} particle per ml	IHCs, SCs	Cochleostomy + RWM (<i>scala tympani</i>)	19
AAV1, 2, 5-CBA-GFP	10^9 genomic copies per ml	HCs (1, 2, 5), SCs (1, 5), fibroblasts (2, 4)	Cochleostomy (<i>scala media</i>)	45
AAV1, 2, 5-GFAP-GFP	10^{10} genomic copies per ml	HCs, SCs (1, 2)	Cochlear explants (P0, E13)	41
AAV1, 2, 3, 5, 6, 8-CMV-GFP	10^{10} genomic copies per ml	SCs (1, 2, 5)	Cochlear explants (P0, E13)	41
AAV2-CAG-GDNF-EGFP	6.7×10^{12} - 1×10^{13} genomic copies per ml	IHCs (2, 5, 6, 8), SCs (2, 6, 8), Rosenthal canal (2), spiral ligament (2, 8), SGN, (1, 3, 5, 6)	Cochleostomy (<i>scala media</i>)	49
AAV2-CMV-Bgal	5×10^{10} genomic copies per ml	SGNs, IHCs	RWM (<i>scala tympani</i>)	55
AAV2-CMV-GFP	10^5 VP per ml	OC, spiral ligament, spiral limbus and the spiral ganglia	Cochleostomy	48
AAV2-CMV-GFP	1.68×10^{12} particle per ml	Interdental cells, inner sulcus (SM), Mesothelial cells lining the perilymphatic space (ST)	Round window (<i>scala tympani</i>) + cochleostomy (<i>scala media</i>)	56
AAV2-CMV-GFP	2×10^{11} pfu per ml	No targeted cells	Gelfoam on RWM	42
AAV2-CMV-GFP	1×10^{11} - 2×10^{11} pfu per ml	SGNs, nerve fibres projecting towards the OC, <i>stria vascularis</i>	Round window (<i>scala tympani</i>)	43
AAV2-PDGF-GFP, AAV2-NSE-GFP	2×10^8 VP per ml	Nerve fibres (NSE), cochlear blood vessels (PDGF), non-interdental cells of the spiral limbus of the cochlea (EF1alpha)	Round window (<i>scala tympani</i>)	16
AAV2-EF1alpha-GFP	3×10^{12} - 1×10^{13} genomic copies per ml	HCs, SCs (AAV2/8, AAV2/1)	Ex utero microinjections (E11-E12.5) + transuterine microinjection	47
AAV2/1, 2/2, 2/5, 2/6, 2/7, 2/8, 2/9-CMV-GFP	1×10^{10} genomic copies per ml	IHCs, spiral limbus, spiral ligament (all), OHCs and Hensen cells (2/1, 2/2, 2/9)	Round window (<i>scala tympani</i>)	46
BAAV-CAG-GFP or AAV2, 4, 5-CAG-GFP	10^9 - 10^{11} VP per ml	IHCs (BAAV, AAV2), OHCs, SCs, vestibular HCs (BAAV)	Rat culture explants of OC and SGN (P2, P10)	53
BAAV-CMV-Cx30-GFP	10^{11} particle per ml	Non-sensory cells of the OC	Cochlear explants (P5)	57
BAAV-CMV-GFP	5×10^{11} VP per ml	SCs, HCs, SGN, <i>stria vascularis</i> , Reissner membrane	Transuterine otocyst microinjection (E11-E12)	28
BAAV-β-actin-GFP	10^{12} VP per ml	SCs, Reissner membrane, OHCs	Cochleostomy (<i>scala media</i>) + RWM (<i>scala tympani</i>)	54

Abbreviations: AAV, Adeno-associated virus; BAAV, bovine AAV; CMV, cytomegalovirus; GDNF, glial cell-derived neurotrophic factor; GFP, green fluorescent protein; HCs, hair cells; IHCs, inner HCs; SCs, supporting cells; OC, organ of Corti; OHC, outer HCs; SGN, spiral ganglion neurons; VP, viral particles; pfu, plaque-forming unit.

targeting a range of cell types with variable efficiencies (Table 3). Different serotypes of AAV, with different cell tropisms, have been tested in the inner ear to specifically target HCs or neurons. Among them, AAV-2 showed the ability to transduce HCs and SCs *in vitro*,⁴¹ although it can only infect the spiral limbus, spiral ligament and spiral ganglion cells *in vivo*, following injection through the RWM.^{16,42,43} The same results were obtained using AAV-1,⁴¹ whereas AAV-5 could not transduce HCs either *in vitro* or *in vivo*.^{41,44} However, AAV5 was efficient for transducing cells of the spiral ganglion, inner sulcus cells and Claudius cells.^{44,45}

In addition to these three serotypes, other AAV serotypes (AAV-3, -4, -7, -8) were tested *in vivo* by injection through the round window into the mouse cochlea. The AAV-3 vector was shown to be the most efficient in transducing cochlear HCs, whereas AAV-1, -2 and -7 could also transfect efficiently the SGNs.⁴⁴ To further increase the AAV potential for inner ear

gene delivery, pseudotyped vectors derived from AAV-2 serotype were constructed. The distribution of six serotypes (AAV2/1, -2/2, -2/5, -2/7, -2/8 and -2/9) following injection into the perilymph after cochleostomy was studied. All these AAV serotypes displayed tropism to HCs, with AAV2/2 being the most efficient.^{46,47}

In addition to AAV serotype, successful transduction *in vivo* is also dependent on the route of administration. In 1998, Lalwani et al.⁴⁸ tried to transduce cochlear cells through a cochleostomy rather than through the round window. Sensory cells were not transduced by this approach, whereas spiral ganglion cells and spiral limbus cells were moderately transduced. Recently, AAV was directly injected into the *scala media* through a cochleostomy, providing direct access to the OC. Serotypes -1, -2, -5, -6 and -8 were successfully transduced into sensory and non-sensory cells of the OC.^{19,49} However, this type of surgery does not guarantee hearing preservation.

Table 4. Principal lentiviral or HSV vectors used to insert genes into the inner ear

Virus type	Titre	Targeted cells	Route of administration	References
<i>Lentiviral vectors</i>				
Lenti-GFP	10^4 – 10^5 pfu	SGN and SCs surrounding the sensory cells	OC explants	59
Lenti-GFP	10^6 pfu	Cells bordering the periphery of the perilymphatic space, spiral ligament	Cochleostomy	59
Lenti-GFP	2×10^8 pfu	<i>Stria vascularis</i> , basement membrane	Round window (<i>scala tympani</i>)	61
Lenti-HOX-GFP	8×10^6 – 10^7 pfu	The structural lining cells of the perilymphatic space of the cochlea, the epithelial cells surrounding the <i>scala vestibuli</i> and <i>scala tympani</i>	Round window (<i>scala tympani</i>)	60
Lenti-VSVG-GFP	3×10^{12} – 1×10^{13} VP	HCs and SCs of the OC	Transuterine microinjections (E12)	47
<i>pHSV vectors</i>				
pHSVnt-3myc/ sv40lacZ	2.7×10^5 IU	SGNs	Lateral wall <i>scala vestibuli</i>	66
pHSVnt-3myc	2.7×10^5 IU per explant for 1 h	SGNs	<i>In vitro</i> explant	63
pHSV-LacZ	2×10^7 pfu per cochlea	Cells lining perilymphatic space, SCs, <i>stria vascularis</i> (fibrocytes)	Round window (<i>scala tympani</i>)	69
pHSV-LacZ	10^4 – 10^5 pfu per cochlea	SGNs and <i>stria vascularis</i>	Round window (<i>scala tympani</i>)	67
pHSV-BDNF/ sv40lacZ	10^5 pfu per cochlea	SGNs	Round window (<i>scala tympani</i>)	68
pHSV-bcl-2	10^6 IU per explant for 12 h	HCs area	OC <i>in vitro</i> explant	64
pHSV-BDNF-LacZ	10^4 IU per explant for 4 h	SGNs	<i>In vitro</i> explant	65

Abbreviations: BDNF, brain-derived neurotrophin factor; GFP, green fluorescent protein; HCs, hair cells; HSV, herpes simplex virus type 1; IU, infectious unit; SCs, supporting cells; OC, organ of Corti; SGN, spiral ganglion neurons; VSVG, vesicular stomatitis virus glycoprotein; VP, viral particles; pfu, plaque-forming unit.

AAV transduction efficiency into the inner ear also varies upon the promoter type driving the transgene expression. Most of the studies have used the human cytomegalovirus immediate-early promoter to carry out transgene expression. Nevertheless, the hybrid CAG promoter sequence from the chicken β -actin promoter and the cytomegalovirus immediate-early enhancer^{41,50,51} seems to be, to date, the most efficient promoter-mediating expression in HCs and neurons of the inner ear. More recently, delivery of AAV vectors to mouse embryonic otocysts *in vivo* allows the targeting of cochlear progenitors, and therefore HCs, SCs and SGNs.⁴⁷

One drawback of using the AAV vectors is that the efficiency of gene transduction could be diminished in patients who possess AAV neutralizing antibodies. Indeed, AAV infection is common in childhood, leading to the acquisition of immunity against this virus. Thus, a new generation of AAV has been used: the bovine AAV, which has been shown to be serologically distinct from AAV serotypes 1 to 4.⁵²

Bovine AAV efficiently transduced auditory and vestibular HCs and SCs⁵³ *in vitro*. They also successfully transduced cells of the OC *in vivo*, both via trans-uterine microinjection to the embryonic E12.5 mouse otocyst,²⁸ or after injection into the *scala media* or the *scala tympani* in adult guinea pig cochlea.⁵⁴

One therapeutic perspective is to express large amounts of secretory proteins using AAV constructs. An AAV-1 vector containing the *GDNF* gene under the control of the CAG promoter has been injected into the rat RWM, and allowed protection of HCs and SGNs against aminoglycoside ototoxicity.⁵⁵ AAV-2 carrying the brain-derived neurotrophin factor (*BDNF*) gene has been successfully transduced into the basilar membrane area (epithelial and mesothelial cells) following aminoglycoside treatment. This resulted in robust regrowth of nerve fibres towards the cells that secreted the neurotrophin.⁵⁶ More recently, gene therapy has been successfully used *in vitro*. Indeed, the bovine AAV vectors delivering the *Cx26* gene restored gap-junction coupling in cochlear non-sensory cells from the *Cx26* conditional knock-out mice maintained in organotypic cultures.⁵⁷

LENTIVIRUS

Lentiviruses belong to a subclass of retroviruses. The one used for transduction are molecularly modified lentiviruses derived from HIV-1. The lentiviral vectors include a transgene construct in which the viral genes *gag*, *pol* and *env* have been replaced by promoter and transgene sequences and three helper (packaging) constructs expressing *gag/pol*, *env* and *rev* separately, to prevent homologous recombination. Typically, packaging cells (for example, the 293T human embryonic kidney cells) are co-transfected with a plasmid containing the vector genome and the packaging constructs to produce lentiviruses. Biosafety has been further increased by the development of self-inactivating vectors that contain deletions of the intrinsic promoter/enhancer activity of the HIV-1 long terminal repeat sequence, eliminating the transcription of the packaging signal that is required for vector mobilization.⁵⁸ Given the restricted host range of the HIV-1 *env* glycoprotein, lentiviral vectors are pseudotyped with the vesicular stomatitis virus glycoproteins. The main advantages of lentiviruses is that they offer the possibility of inserting long DNA fragments, and they are able to transduce non-dividing cells. Therefore, lentiviruses were considered as suitable candidates for inner ear gene therapy (Table 4). Second-generation self-inactivating vectors under the control of the human elongation factor-1 α promoter with a GFP reporter cassette have been transduced in the inner ear directly through the RWM. GFP transgene expression was restricted to the cells lining the perilymphatic space and lining cells of the *scala tympani*.^{59–61} Using trans-uterine microinjection, a low GFP expression level could be detected in outer HCs and SCs, but not in inner HCs.⁴⁷ *In vitro* infection of cochlear explants from neonatal rats allowed a successful transduction of SGNs and some SCs, but not of any HCs.⁵⁹ Another unique feature of lentiviruses is their capacity to integrate the host genome, thus allowing long-term transgene expression, but this advantage can turn out to be a serious limitation to the use of these vectors in

gene therapy, as it would be genetically deleterious for the recipient cells.

HERPES SIMPLEX VIRUS

The herpes simplex virus type 1 (HSV-1) is a human neurotropic virus with an overall diameter of 180–200 nm. It is a complex double-stranded DNA virus of about 150 Kb, encoding over 80 proteins.⁶² It is surrounded by an icosahedral capsid and an envelope. A similar strategy to the one used for producing adenoviral vectors has been applied to generate replication-defective HSV amplicon vectors. All or a combination of the five immediate-early genes (*ICP0*, *ICP4*, *ICP22*, *ICP27* and *ICP47*), which are required for lytic infection and expression of all other viral proteins, were removed. The interest of HSV-1 as a gene transfer vector stems from its ability to infect many different cell types, both quiescent and proliferating cells. In addition, its extensive transgene capacity make it suitable for gene therapy. Amplicons are capable of harbouring an insert gene up to 100 Kb, and it never integrates into cellular chromosomes thus avoiding the risk of insertional mutagenesis.

HSV-1 amplicon vectors have been employed *in vitro* in the OC and the spiral ganglion explants (Table 4). Indeed, HCs and neurons were successfully infected and thus protected against specific ototoxic injuries with the use of HSV vectors harbouring brain-derived neurotrophin factor, NT-3 or bcl-2 cDNA.^{63–65} Similar amplicons were injected *in vivo* through the round window or via the lateral wall of the *scala vestibuli*. Although very few cells of the OC were transduced, SGNs were highly infected.^{66–68} In addition, inoculation of HSV vector driving NT-3 or brain-derived neurotrophin factor expression was able to protect neurons from a toxic insult⁶⁶ or following hair-cell destruction.⁶⁸

OTHER VIRAL VECTORS

Vaccinia virus is a large, complex, enveloped virus belonging to the poxvirus family. Its genome consists of a linear, double-stranded DNA molecule of approximately 190 Kb. Vaccinia virus bearing the *lacZ* gene was tested for *in vivo* cochlear infection, following injection through the round window. Spiral ligament, Reissner's membrane, lining cells of the *scala vestibuli*, and occasionally the inner and outer HCs of the apical region of the OC were transfected.⁶⁹

Sendai virus, one of the members of *Paramyxoviridae*, is an enveloped virus with a negative-strand RNA genome of about 15 Kb. It causes severe respiratory disease in mice, but is non-pathogenic for humans.⁷⁰ Sendai virus vector has been developed and has been shown to have high gene transduction efficiency and protein expression in different cell lineages.⁷¹ One of the characteristics of this vector is that its genome is located exclusively in the cytoplasm of infected cells and never goes through a DNA phase, thus precluding the integration of its genetic information into the cellular genome. Sendai virus vectors were injected into the guinea pig cochlea through the *scala media* or via the *scala tympani*, and were able to transfect a huge variety of cells, including the *stria vascularis* cells, SGNs and sensory cells of the OC.⁷²

NON-VIRAL DELIVERY SYSTEMS

Another strategy for gene delivery is based on the use of natural or synthetic compounds (also named non-viral vectors) in which complexes of DNA, proteins, polymers or lipids are formed in particles capable of efficiently transferring genes into cells. Methods of non-viral gene delivery can be subdivided into physical (carrier-free gene delivery) and chemical approaches (synthetic vector-based gene delivery). Despite their relatively lower efficiency, non-viral approaches are emerging as simpler,

safer and more flexible alternatives to viral vectors. Methods of non-viral gene delivery to target the inner ear cells include chemical approaches using liposomes or polymers, and physical methods employing a physical force such as pressure or electric pulse. Noteworthy, these techniques are widely used as a tool of choice for research, allowing the functional characterization of numerous genes (Table 5), but less developed for gene therapy, as they are associated with a lack of specificity, a short-term transgene expression and cell toxicity.

CATIONIC LIPOSOMES

Liposomes are spherical vesicles composed of concentric phospholipid bilayers enclosing an aqueous compartment. The lipid-based reagents used for transfection (that is, lipofection) are generally composed of synthetic cationic lipids that are often mixed with helper lipids such as DOPE (L- α -dioleoyl phosphatidylethanolamine) or cholesterol, which destabilizes the endosome following endocytosis, thus releasing DNA into the cytoplasm.⁷³ These lipid mixtures assemble in the liposomes or micelles with an overall positive charge at physiological pH, and are able to form complexes (lipoplexes) with negatively charged nucleic acids through electrostatics interactions. A broad range of liposomal reagents is commercially available and includes Lipofectamine 2000, Lipofectin (Invitrogen, Gent, Belgium), Fugene (Promega Benelux, Leiden, The Netherlands), Hifect (Lonza, Verviers, Belgium) and Trifectin (IDT, Leuven, Belgium). Cationic liposomes are easily prepared in large amounts, making them inexpensive to make and protect the DNA from nuclease-mediated degradation. In addition, liposomes overcome problems of immunogenicity. However, they present limitations, such as the absence of relative tissue specificity and low transfection efficiency compared to viral vectors. Numerous studies using cationic liposomes have been performed both *in vitro* and *in vivo* to mediate transgene expression in the inner ear (Table 4). Neonatal organs of Corti have been successfully transfected *in vitro* using commercially available reagents (Lipofectamine 2000 or Lipofectin) with plasmids containing *Math1*^(ref. 74) or with antisense oligonucleotides.⁷⁵ However, the transfection yield was low (around 3%) as compared with viral delivery approaches. Different formulations of liposomes have also been tested *in vivo*, principally following local application on the RWM.^{42,76–78} Analysis of cochlear sections from all liposomes-GFP or liposomes- β -galactosidase revealed transgene expression in various cell types, including SGNs, *spiral limbus* and spiral ligament cells. Transgene expression was persistent up to 14 days in certain cell populations, including SGNs.

CATIONIC NON-LIPOSOMAL POLYMERS

Synthetic and naturally occurring cationic polymers, in linear or branched configuration, constitute another category of DNA carriers that have been widely used for gene delivery. These include, among others, polyethylenimine, polyamidoamine, polypropyleneimine dendrimers, cationic dextran, chitosan and poly (lactic-co-glycolic) acid.⁷⁹ Although most cationic polymers condense DNA into small particles, forming polyplexes, and facilitate cellular uptake via endocytosis through charge-charge interaction with anionic cell surfaces, their transfection activity and toxicity differ dramatically.

In the cochlea, hyperbranched poly-L-lysine nanoparticles have been tested as delivery agents both *in vivo* and *in vitro*. These dendritic polymers with imperfectly branched or irregular structures, efficiently transfected spiral ganglion cells in cochlear organotypic cultures, although few HCs were targeted. Application of a gelatin sponge immersed in hyperbranched poly-L-lysine nanoparticles on the rat RWM *in vivo* favoured the targeting of HCs and SCs of the OC, cells of the *stria vascularis*, the spiral ligament and SGNs.⁸⁰ Polyethylenimine-mediated cochlear gene

Table 5. Principal non-viral vectors used to insert genes in the inner ear

Type of transfection	Treatment	Targeted cells	Route of administration	References
Liposomes	DDAB/cholesterol in 5% dextrose (12 nmol) with 1 µg DNA(beta-gal plasmid)	Spiral ligament, spiral limbus, OC, Reissner's membrane and SGNs	Injection through the RWM (10 µl in 20 min) or osmotic minipump after cochleostomy	76
Liposomes	POPC/CHIM/DDAB (7.2/4.8/6 nmol) mixed with 1 µg DNA (eGFP)	SGNs, HCs, spiral ligament, <i>stria vascularis</i>	Gelfoam on RWM	42, 77
Liposomes	10 ⁷ liposome (DOTMA/DOPE)/plasmid (pQI25-GFP) complexes	SGNs, SCs, <i>stria vascularis</i>	Mouse <i>scala tympani</i> injection	67
Lipofectin	Lipofectin-phosphorothioate antisense oligonucleotide	Unknown	E16 mouse or rat OC explants	75
Lipofectamine 2000	0.2 mM lipoplexes with pcDNA-GFP-Math1 (2:1 ratio lipid/DNA)	Numerous cells. Transfection efficiency: 2.9%	Newborn rat OC-dissociated cells	74
Lipid core nanocapsules (LNC)	Labrafac/lipoid/solutol(lipids)/DSPE/PEG2000 suspension solution (LNC) 2.95 ml with FITC (2 mg)	SGNs, HCs, spiral ligament	Gelfoam on RWM	78
Polybrene	6 µg ml ⁻¹ with 2 µg ml ⁻¹ oligonucleotide antisens	Unknown	OC-derived cell line (10 ⁴ cells per well, 24-well plate)	95
Dendritic polymers (HPNP)-FITC	10 ⁻³ M polyplex (1:1 HPNP:DNA, plasmid GFP)/cochlea	OC (few HCs) SGNs	Sponge on RWM	80
Hyperbranchedpolylysine nanoparticles-FITC	2.5 10 ⁻⁶ M with 2.5 µg DNA (plasmid GFP) for 24 h	Few HCs SGNs	<i>In vitro</i> rat P1 cochlear explants	80
PEI	PEI/DNA (EGF plasmid) at ratio 6:1	Fibrocytes lining the <i>scala vestibuli</i> and <i>scala tympani</i> , mesenchymal and epithelial cells of Reissner membrane, and fibrocytes of the spiral ligament	<i>Scala tympani</i> injection in guinea pig	81
PLGA nanoparticles	Rhodamine PLGA nanoparticles ratio 1:20	<i>Scala tympani</i>	Gelfoam on RWM	82
Gene gun	Gold particles (1 mg) + DNA (2 µg) pEGFP-MyoXVa	HCs (stereocilia)	P3-P5 mouse OC explants	93, 94
Electroporation	8 square-wave pulses (25 V, 50 ms) vector p-EGF-math1	Numerous GER cells and OC cells	E13.5-P0 mouse OC explants	85, 96
Electroporation	5 directional square-wave pulses (20 ms, 43 V) after injection of pEF1-math1-GFP (3 µg µl ⁻¹ , 10–15 injections at 15 psi)	OC cells including HCs and SCs	<i>In utero</i> (E11.5)	91, 97

Abbreviations: GFP, green fluorescent protein; eGFP, enhanced GFP; HPNPs, hyperbranched poly-L-lysine nanoparticles; POPC, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine; CHIM, cholesterylimidazole; DDAB, dimethyldioctadecylammonium bromide; DOPE, L-α-dioleoyl phosphatidyl-ethanolamine; DOTMA, N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride; DSPE, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine; PEG, polyethyleneglycol; RWM, round window membrane; PEI, polyethylenimine; PLGA, poly (lactic-co-glycolic) acid; SGNs, spiral ganglion neurons; GER, greater epithelial ridge; HC, hair cells.

transfer via cochleostomy and osmotic minipump was also tested.⁸¹ Mesothelial cells lining the *scala vestibuli* and *scala tympani*, mesenchymal and epithelial cells of the Reissner's membrane and fibrocytes in the supra-striatal zone of the spiral ligament were successfully targeted. Unfortunately, no transfected cells were observed in the OC or in the *stria vascularis*. Poly (lactic-co-glycolic) acid nanoparticles encapsulating rhodamine and placed locally on the RWM in adult guinea pigs allow gene transfer in cells bordering the *scala tympani* after 24 h.⁸² All these polymer-based vehicles for DNA delivery lack selectivity toward cells and present significant cytotoxicity, therefore largely limiting their application in clinical practice.

ELECTROPORATION

This technique is based on the application of electric field pulses that create transient pores in lipidic membrane, allowing uptake of large charged molecules, such as plasmid DNA. The permeability of the membrane quickly reseals. Gene delivery efficiency is determined by the pulse intensity, duration and frequency.⁸³ Electroporation works best on cells that are suspended in solution, but also works on solid tissue where electrodes can be applied.⁸⁴

Indeed, in 2000, Zheng and Gao⁸⁵ successfully transfected by electroporation postnatal rat cochlear explants with a pClig-GFP-Atoh1 vector *in vitro*. They showed that overexpression of Atoh-1 in the greater epithelial ridge and in the OC gave rise to new HCs. This technique has further been successfully used to transfect mouse explants at earlier developmental stages (starting at E13.5 in mice) and to silence specific gene expression by the use of short hairpin RNAs.^{86,87}

The choice of the vector promoter to drive gene expression is critical for the electroporation efficiency. Indeed, the use of the hybrid CAG promoter instead of cytomegalovirus alone results in a higher percentage of cells expressing the transgene to the sensory epithelia and the SGNs.^{88,89} Recently, the need of an adequate promoter driving transgene expression was circumvented by cloning the transgene into a particular plasmid that allows synthesis of stabilized mRNA.⁹⁰ Electroporation of this stable messenger greatly increases the number of cells expressing the transgene in the OC as compared with classical DNA plasmids.

Promising results were also obtained in the developing mouse inner ear *in vivo*. The DNA plasmid (pEF1-GFP) was microinjected through the uterine wall into the mouse E11.5 otic vesicle, and

electric pulses transferred it into otic epithelial progenitor cells.⁹¹ Robust GFP expression was observed in HCs and SCs in the OC at E18.5. However, this technique, so far, has not been successfully used to transfect postnatal mice *in vivo*.

BIOLISTIC

This method of gene delivery is also referred to as particle bombardment, micro-projectile gene transfer or gene gun. DNA is deposited on the coated surface of gold particles, which are then accelerated by high-voltage electronic discharge, spark discharge or helium pressure discharge onto cells or a tissue. Biolistic DNA injection also offers the capacity to deliver precise DNA dosages. Unfortunately, genes delivered by this method are expressed transiently, and there is considerable cell damage occurring at the centre of the discharge site. Early postnatal organs of Corti, were gene-gun transfected with pMYO15-EGFP-C2 previously coated with gold particles and were observed 24 h later. HCs were efficiently targeted, but the efficiency remains low (around three cells per explant).^{92–94}

CONCLUSION

Tremendous efforts have been made over the last decade to improve gene transfer technologies in the inner ear. Despite the fact that some of these are not suitable for gene therapy because of poor transfection efficiencies, immunogenicity or cellular toxicity, they all represent a tool of choice for basic research on the auditory system. In the absence of adequate mouse models, overexpressing or silencing a specific gene expression in a spatio-temporal controlled manner can be accomplished using these technologies without affecting other tissues, thus allowing for precise functional characterization of key actors in inner ear development, cell survival and cell regeneration. Nevertheless, the recent advances in gene delivery opens the door to future prospects in inner ear gene therapy. Among the available viral vectors, AAV is one of the most promising gene therapy vectors for human clinical trials. Other viruses are less efficient in transducing sensory cells or SGNs. Regarding non-viral systems, although the chemical and physical approaches showed efficient targeting of HCs, SCs and SGNs, the amount of transfected cells and the *in vivo* selectivity are not sufficient for gene therapy.

Because of recent work to improve cochlear transfection and/or transduction, gene therapy in the inner ear appears to be more accessible than in the past. Continuing efforts in improving cochlear gene delivery, crossed with improving the knowledge of molecular events driving development, may lead to successful establishment of gene therapy to treat deafness.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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