Review Article

Dual-AAV delivery of large gene sequences to the inner ear

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ABSTRACT

Adeno-associated viruses (AAVs) are preferred vectors for gene replacement therapy, as they are non-pathogenic, non-inflammatory, induce stable transgene expression in terminally differentiated cells, and a series of natural and engineered capsid proteins can be employed to target the vectors to specific cells. Only one feature of AAVs is limiting: the low cargo capacity for foreign DNA, restricting their application to coding sequences of <4 kb. In the last decade, splitting larger cDNAs into two AAVs and co-transducing tissue with such dual-AAV vectors has shown to result in the expression of the full-length protein in different tissues like retina, muscle and liver. This is due to the intrinsic capability of the AAV genomes to undergo homologous recombination and/or head-to-tail multimerization in nuclei of target cells. Recently, two groups independently found that a dual-AAV approach successfully delivered the 6 kb full-length otoferlin cDNA into inner hair cells of otoferlin knock-out mice and restored hearing. These pioneering studies pave the way for gene therapeutics that use dual-AAV vectors to restore hearing in different forms of deafness caused by mutations in large genes.

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1. Introduction

The replacement of a defective gene requires a shuttle that is able to bring the coding sequence (CDS) for the missing protein into the nucleus of a target cell. For inner ear cells, the adeno-associated virus (AAV) turned out to be the vector of choice for replacing genes with CDS <4 kb for several reasons: First, AAVs are non-pathogenic and are the least immunogenic of all viral vectors known to date. Second, several natural and engineered capsid proteins have been found to transduce diverse types of inner ear cells that are involved in different forms of genetic hearing loss, including inner and outer...
2. Mini-genes

In order to circumvent the ~5 kb packaging limit of AAV genomes, several groups tried to use a truncated cDNA, motivated by the idea that a minimal protein fragment might be able to fulfill the gene function at least in part. One hint for this strategy came from the observation that mutations leading to a truncated dystrophin fragment resulted in a less severe muscular dystrophy phenotype than frameshift or premature stop mutations (Love et al., 1990; Wang et al., 2000). Targeted assessment of dystrophin fragments indeed suggested that at least a partial functional rescue could be achieved (Wang et al., 2000; Harper et al., 2002; Liu et al., 2005). Similarly, a truncated cystic fibrosis transmembrane conductance regulator (CFTR) could rescue Cl− conductance via AAV transduction of airway epithelia (Ostedgaard et al., 2005, 2003), and a miniature human Factor VIII heavy chain transferred by AAVs to liver cells ameliorated the hemophilia A phenotype in mouse models (Chao et al., 2000).

It is self-evident, however, that it needs to be assessed individually whether or not a protein fragment can sufficiently rescue the function of a protein encoded by a mutated gene. In the inner ear, this has been tested with varying degrees of success for otoferlin, an IHC protein that when mutated causes profound congenital hearing loss (DFNB9) not only in humans, but also in zebrafish and mouse models. In zebrafish with a morpholino-induced knock-down of the two otoferlin isoforms, mouse cDNA constructs containing at least the C2F domain of otoferlin seemed to rescue the formation of the swim bladder (Chatterjee et al., 2015). In addition, the C-terminal otoferlin fragments increased the motility of zebrafish larvae within the first 10 s after an acoustic startle signal. This was interpreted as a rescue of the acoustic startle reflex, although this is typically recorded in much shorter time windows, since the fast and slow components of the zebrafish startle response have latencies of 5.3 ± 2 ms and 28 ± 9 ms, respectively (Burgess and Granato, 2007). Notably, even untreated otoferlin morpholino knock-down zebrafish display a reflex in the first second after the acoustic stimulus, indicating a partial function of the incompletely suppressed otoferlin.

In contrast to the proposed rescue by mini-otoferlin in zebrafish, several mammalian mini-otoferlin variants only partially restored otoferlin function in otoferlin knock-out (Otof−/−) mice (Tertrais et al., 2019). Despite the observation that otoferlin protein fragments were detected in IHCs by immunohistochemistry, none of the mini-otoferlins reinstated hearing after injection into cochlea of Otof−/− mice. Cellular electrophysiological recordings of transduced IHCs revealed that fast exocytosis could be rescued by different mini-otoferlins, but not sustained exocytosis, which is also required for hearing (Pangrsic et al., 2010). Since the individual functions of the multiple domains in otoferlin have not been unraveled to date, it is currently rather unlikely that a fully functional mini-otoferlin with <4 kb coding sequence will be designed. Nevertheless, aiming to design truncated proteins that could be transduced by AAVs might be a way to treat some forms of deafness, especially if the coding sequence is only slightly above the cargo limit.

3. The dual-AAV method - trans-splicing

How could the cargo limit of <4.9 kb be overcome? One observation was vital to develop a novel approach: Upon transduction, AAV genomes in the nuclei of host cells undergo intramolecular circulation and intermolecular head-to-tail connection resulting in circular episomes, which are called concatemers. Over the course of several weeks, these episomes get larger, comprising more and more single vector genomes, each connected over an ITR.
to the neighboring virus genomes. Based on this discovery, it was hypothesized that two different AAV vectors would be able to form heteromeric episomes, assembling the two vector genomes in random orientation. In an attempt to employ such concatemers in a targeted manner, transcriptional enhancers transduced via one AAV could enhance transcription of an expression cassette co-transduced with a second viral vector (Nakai et al., 2000). Moreover, by means of splice signal sequences, even coding sequences split in two halves and transported by two AAVs could be co-transported and re-assembled. In this so-called “trans-splicing” strategy, dual-AAV half-vectors contain a splice donor site downstream of the 5′ cDNA in the first half-vector, and a splice acceptor site just after the ITR in the 3′ half-vector, upstream of the CDS. Here, endogenous splice signals and introns can be used, the efficiency of which might differ and seems worth to test (Lai et al., 2005). Alternatively, synthetic splice signal sequences or introns from other genes might be used, which need less effort for screening of the best endogenous intron, but are not necessarily better than endogenous splice sites (Lai et al., 2006). Only the plasmid for the 5′ virus contains a promoter sequence, whereas downstream of the cDNA in the 3′ virus a polyadenylation signal is included. After concatenization in the nuclei of the cells, the mRNA transcription starts at a promoter sequence and ends at the next poly-adenylation signal (Fig. 1A). Sequences between the first splice donor (SD) and the subsequent splice acceptor (SA) site will be excised by the endogenous spliceosome of the target cell. That way, any time a 5′ and a 3′ vector connect to come in this order, a full-length mRNA will be produced (Fig. 1A). In the event that two 5′ vectors connect, or if the attachment of a 3′ vector is in wrong orientation, the mRNA polymerase will continue transcription until the next polyA signal, and the endogenous spliceosome will splice out any sequence between the first SD site and the subsequent SA site (Fig. 1B). That way, incorrectly connected virus genomes might be spliced out, thereby increasing the chance of a correct full-length mRNA in arbitrarily connected half-virus genomes (Fig. 1B). Whether, and how efficiently, such a skipping of vector genomes occurs will need more experimental testing. The proof-of-principle that the trans-splicing approach leads to re-assembly of a split coding sequence was demonstrated with several reporter genes (Sun et al., 2000; Yan et al., 2000). Moreover, the suitability of the trans-splicing dual-AAV approach in therapeutic applications was proven, first for erythropoietin (Yan et al., 2000) and Factor XIII heavy chain (Chao et al., 2002).

4. The dual-AAV overlap strategy

Another way to expand the cargo capacity of AAVs relies on the observation that viral DNA can recombine with homologous sequences, thus it was supposed that recombination could also occur with a homologous DNA fragment transported by a second AAV half vector. To make use of recombination of overlapping sequences, Duan et al. (2001) cloned two overlapping fragments of β-galactosidase cDNA for AAV production. They demonstrated that the two half vectors, one encoding the 5′ cDNA, one the 3′ cDNA, both including a 1 kb overlap, indeed built a functional β-galactosidase enzyme in transfected cells. While the first study comparing the overlap-dual-AAV approach found a rather low efficiency of recombination and a lower transgene expression than is achieved with trans-splicing vectors (Duan et al., 2001), other studies reported higher levels of expression for overlapping dual-AAVs when compared with other approaches (Halbert et al., 2002; Pryadkina et al., 2015).

Refining the overlap-dual-AAV approach for an individual target gene can require major effort, as the cDNA region that has the highest potential for homologous recombination needs to be experimentally tested for each gene, and there remains debate over the optimal length of overlapping sequences (i.e., are longer sequences generally superior to shorter overlapping sequences?) (Carvalho et al., 2017; McClements et al., 2019). Nonetheless, because the individual vector halves maintain a cargo capacity of 4.7−5 kb, the maximum length of overlapping sequences is limited by the size of the cDNA, and the only feasible overlapping segments are restricted to the central part when genes are close to the maximum length of 8−8.5 kb.

5. The Dual-AAV hybrid strategy

As an alternative, the so-called “hybrid” strategy was proposed, making use of a universal overlapping DNA sequence for homologous recombination that is later spliced out from the mature mRNA. In this method, highly recombinogenic sequences (e.g. from phage DNA) are used in addition to the splice signal sequences. This strategy was expected to result in higher protein expression levels because i) the highly recombinogenic sequences should reinforce the correct orientation of the two virus halves, and ii) full-length mRNA transcription is also possible through trans-splicing in the event that no homologous recombination happens (Chosh et al.,

![Fig. 1. Potential episomal combinations of 5′ and 3′ half vectors in the trans-splicing approach.](image)
experiments discovered that none of the viral particles contained trans- splice and overlap vectors (Ghosh et al., 2008). In a follow-up study, the authors determined a minimal recombinogenic fragment of AP of 270 bp, which was as efficient as the longer AP overlap (Ghosh et al., 2010). Later, Trapani et al. (Trapani et al., 2015, 2014) demonstrated that a 77 bp sequence from the Fl phage genome (AK) was highly efficient in driving the expression of three different genes from hybrid-dual-AAV vectors in HEK293 cells.

6. Overlap versus oversize

To test the limits of foreign DNA packaging into AAVs, Grieger and Samulski (2005) analyzed virions made from recombinant DNA of up to 6 kb, and they concluded that packaging of DNA sequences of this size might be possible. Subsequently, Allocco et al. (2008) subcloned ABCA4 cDNA of ~7.3 kb length, added a promoter and polyA signals, summing to 8.9 kb between the ITRs, and produced an AAV (with serotype 5; AAV5) vector from that DNA. Murine retinal cells transduced by these viral particles were found to produce full-length ABCA4 protein, and vision impairment was ameliorated in ABCA4 deficient mice. The authors first proposed that in this so-called “oversize” approach, AAVs are indeed capable of packaging more than 5 kb of foreign DNA, at least for AAV5. Shortly thereafter, several groups repeating oversize packaging experiments discovered that none of the viral particles contained more than ~5 kb (Dong et al., 2010; Lai et al., 2010; Wu et al., 2010). A closer analysis of the viral genome revealed that the cDNA of the oversize gene had been fragmented for packaging. AAVs contained either the 5’ part of the CDS, or the 3’ part of it, depending on from which site the packaging had been started. To date, it is still under debate whether overlap-dual-AAVs recombine via the same intracellular mechanism as the viral particles generated during packaging of oversize plasmids. One hypothesis is that after entry of single stranded viral DNA molecules in the nucleus, respective overlaps from one plus and minus strand anneal and are completed by cellular DNA polymerases to produce double stranded DNA. Here, it is unclear how the ITR sequences could be removed in order to allow a respective second–strand synthesis primed by the complementary vector half (McClements and MacLaren, 2017). Alternatively, respective plus and minus virus genomes could first be completed to double stranded viral genomes, which undergo homologous recombination, making use of intrinsic DNA repair enzymes. More studies will be required to determine whether the recombination mechanism depends on the length and sequence of the overlapping DNA, and potentially whether full-length DNA completion can be forced.

Compared to the overlapping dual-AAV strategy, a major drawback of the oversize approach is that viral particles are highly variable in size, thereby giving rise to a series of protein fragments that can be expressed from single or arbitrarily recombinated viruses. A clinical application of the oversize strategy would require a detailed assessment and proof that none of these fragments has a deleterious influence, like forming dysfunctional protein complexes that exhibit a dominant negative effect. As comprehensive testing of this would be laborious if not even practically impossible, a therapeutic use of oversize vectors is unlikely, especially since the dual-AAV strategies achieve the same — packaging of large genes in AAVs — but make use of homogenous viral particles. Nevertheless, the expression of protein fragments cannot be excluded in the dual-AAV approach. In contrast to the oversize approach, however, these fragments are predictable and can be analyzed for their potential to interfere with the physiological function of the full-length protein. The weak intrinsic effect of the ITR to induce transcription typically leads to very low expression levels of the C-terminal fragment from an uncombined 3’ half-virus (Trapani et al., 2015). Conversely, the amount of N-terminal protein fragments expressed from the 5’ half-vector is typically larger, but it can be suppressed by efficient recombination with a 3’ vector. Moreover, since it will add the poly-adenyl-tail and potentially other mRNA stabilizing sequences like WPRE to the 3’ end of the mRNA, the correct recombination will increase the lifetime of the full-length mRNA. Because a polyA tail is missing from the mRNA transcribed from an uncombined 5’ virus, such mRNA is supposed to be quickly degraded. Indeed, in most dual-AAV experiments, the full-length protein was more strongly expressed than the 5’ fragment (Trapani et al., 2015). Moreover, the dual-AAV design allows to include sequences for efficient degradation of a protein fragment translated from an uncombined 5’ vector, e.g. the 16 amino acid CL1 degron (Trapani et al., 2015).

7. Triple-AAV transduction

With a maximum cargo capacity of ~8—9 kb, dual-AAV vectors are still not able to transduce the largest genes, like cadherin-23, causing DFNB12 and USH1D when mutated. In an attempt to increase the length of the transported coding sequence even further, Maddalena et al. (2018b) tested a triple-AAV approach, where they split the coding sequence of cadherin-23 and the gene defective in Alström syndrome type 1 (ALMS1) to three separate AAVs which they injected into mouse retinas. To force correct recombination, they employed AK overlaps at one and AP overlaps at the other split site. Interestingly, when they tested any possible recombination of the three vector genomes by real-time PCR, an effect on favoring the correct assembly was not apparent, indicating that the ability of these sequences to induce homologous recombination might be underestimated.

Because transduction efficiency is expected to be poor overall with a triple-AAV approach, and due to the numerous possibilities of incorrect recombinations, a rather low level of protein expression from triple-AAVs was expected. Indeed, transduction efficiency was low after this subretinal injection in mice. Nevertheless, 102% ± 55% of cadherin-23 transcript levels were detected in triple-AAV transduced retinas when compared to wild-type levels. Cells with successful transduction and recombination exhibit a 30-fold higher mRNA transcript level as wild-type cells, although this is attributable to the low endogenous mRNA levels, since only few
copies of cadherin-23 are expressed in mouse photoreceptor cells. Moreover, the authors could detect full-length cadherin-23 protein in western blots from 11 out of 15 triple-AAV transduced CDH23  1/− eyes. Thus, for large proteins with rather low level expression, triple-AAV approaches might be a reasonable therapeutic option. Unfortunately, CDH23  1/− mice exhibit no retinal phenotype, so no therapeutic effect could be demonstrated in this case. For the inner ear, a triple-AAV has not been tested to date, which might be worth trying, especially for cadherin-23 which is an essential component of hair cell stereocilia tip-links.

For triple-AAV transduction of ALMS1, the number of successfully transduced cells and the protein levels achieved were presumably too low (7% ± 2% of endogenous levels for mRNA from whole retina) to measurably improve the vision impairment in ALMS1 −/− mice. Apparently, it will be a major effort to enhance protein expression from triple-AAVs in such cases where substantial protein levels are required.

8. Lessons from other tissues, most importantly: the eye

The three aforementioned dual-AAV strategies have been tested in cultured cells, in muscle, airway and liver tissue and in the retina. The optimal dual-AAV approach differs according to the tissue and gene, which has been reviewed elsewhere (Chamberlain et al., 2016; McClements and Maclaren, 2017; Trapani, 2019). In general, heterogeneous results point out that differences in cell types and the nature of the coding sequence make it unlikely that the best approach for a novel application can simply be predicted.

Nevertheless, as is the case for the inner ear, the eye is a volume constricted compartment with low immunogenic responses, which is why observations made in the eye will probably be helpful for the design of dual-AAV approaches for the inner ear.

Trapani et al. (2014) systematically tested the three different dual-AAV strategies encoding either ABCA4, a protein expressed in photoreceptor cells and localized in their outer segments, or Myo7a which is highly expressed in the retinal pigment epithelium (RPE) and weaker in photoreceptor cells of the eye. They observed that it makes a remarkable difference if dual-AAVs are applied to HEK293 cells, which are proliferating epithelial cells, or to terminally differentiated retinal cells in mouse or pig retinas. In HEK293 cells, oversized AAV vectors resulted in much weaker expression of ABCA4 and Myo7a compared to dual-AAV strategies. Of these, overlap worked best for ABCA4, and hybrid (AK) vectors were superior for Myo7a. For subretinal injections, overlap AAVs transduced the RPE but not photoreceptors, whereas trans-splicing and hybrid strategies resulted in full-length ABCA4 and Myo7a expression in both photoreceptors and the RPE with comparable efficiency. By dual-AAV mediated transduction of Myo7a into the RPE and photoreceptors of the respective USH1B mouse model (sh1  1/−), 19−21% of wild-type protein levels could be achieved with trans-splicing and hybrid vectors, which was sufficient to significantly improve the trafficking of melanosomes into RPE microvilli. Based on this study, clinical trials have been started, which will give important insights how successfully dual-AAV gene therapies perform in humans (Trapani, 2019).

Likewise, ABCA4 transduction of ABCA4  1/− retinas by dual-AAV vectors ameliorated the retinal phenotype through reduction of lipofuscin accumulation and improved recovery from light desensitization (Trapani et al., 2015, 2014). Notably, ABCA4  1/− mice show no photoreceptor degeneration or impairment of electroretinogram (ERG) signals, meaning that the phenotype of the human Stargardt disease (STGD) phenotype is only partially mimicked in this model and thus a therapeutic effect on vision can only indirectly be assessed in mice. More recently, McClements et al. (2019) optimized overlap dual-AAVs, and found ABCA4 to be well expressed in the outer segments of mouse photoreceptor cells from dual-AAV pairs, with an optimal overlap length in this case of 200−500 bp. Next to a moderate reduction in toxic bis-retinoids, they found the pathologic increase in retinal autofluorescence to be attenuated. Importantly, they compared a natural AAV8 capsid with an engineered variant, AAV8-Y733F (Petrus-Silva et al., 2009), and found the YF-capsid to increase the number of ABCA4 transcripts in the retina ∼30-fold upon dual-AAV transduction. This remarkable difference points towards a bottleneck in the intracellular processing of viral particles, which differs between capsid serotypes and seems to be especially limiting for dual-AAV transduction: Given that a serotype mediates a tight attachment of viral particles to the surface of a target cell, most cells incorporate the viruses through endocytosis. In hepatic cells for example, AAVs entered the nuclei of almost all cells, but only 5% stably expressed the transgene (Wang et al., 2007). After endocytosis, the virus particles escape the endosomes and traffic through the cytoplasm, where they get attached to the nuclear membranes. In this phase, the viruses are most susceptible to degradation. Different tyrosine kinases phosphorylate viral capsid proteins, which are subsequently ubiquitinated and undergo proteasomal degradation (Duan et al., 2000; Zhong et al., 2007, 2008b). This degradation could be overcome by inhibiting the postphosphorylation of capsid proteins, either by applying kinase inhibitors, or by substitution of tyrosine residues at the surface of capsid proteins to abolish their phosphorylation, thereby augmenting AAV transduction (Maddalena et al., 2018a; Petrus-Silva et al., 2009; Zhong et al., 2008a, 2008b, 2007). In a study comparing the transduction efficiency of engineered AAV2 capsid proteins that replaced one to seven tyrosine (Y) residues at the outer surface of the virus particle with phenylalana-nine (F) residues, the 3YF construct seemed to be most efficient in terms of viral transduction rate, the spread within the tissue, and the intensity of eGFP fluorescence in the mouse retina (Petrus-Silva et al., 2011). Recently, Maddalena et al. (2018a) found tyrosine kinase inhibitors not only to increase AAV transduction for single AAVs, but also to substantially enhance transduction and protein expression from dual-AAV vectors.

McClents et al. (2019) tested more variants of dual-AAVs for their impact on dual-AAV mediated protein expression. Codon optimization, which is supposed to reduce limits in translation, turned out to increase ABCA4 protein levels when expressed from a plasmid, but decreased expression from dual-AAV overlap vectors. Potentially, the modifications of the coding sequence might have impaired its ability to undergo homologous recombination. Further, they found out that the presence of out-of-frame ATG codons behind the ITR in the downstream vector strongly decreases the presence of protein fragments expressed from the 3' half AAV. This example shows that substantial effort to optimize a dual-AAV system for a certain gene can definitively improve protein expression and increase the chance of therapeutic success. However, such optimization will likely be required for each individual gene and might necessitate further modifications for different species.

Regarding viral injections to the retina, several studies observed major differences in transduction rates for mice, pigs and rats. For sub-retinal injection, which implies a high local virus concentration in a small volume, Colette et al. (2014) found two independent AAVs to co-transduce 74% of photoreceptor cells in the pig retina, but only 24% in mice. For dual-AAVs injected sub-retinally, the same group showed 6% of co-transduced cells in mouse retina, but only 24% in mice. For dual-AAVs injected sub-retinally, the same group showed 6% of co-transduced cells in mouse retina, but only 24% in mice. For dual-AAVs injected sub-retinally, the same group showed 6% of co-transduced cells in mouse retina, but only 24% in mice. For dual-AAVs injected sub-retinally, the same group showed 6% of co-transduced cells in mouse retina, but only 24% in mice. For dual-AAVs injected sub-retinally, the same group showed 6% of co-transduced cells in mouse retina, but only 24% in mice.
encoded eGFP expression after intravitreal injection only in mice, but not in rats (Dias et al., 2019). The group of A. Auricchio proposed that thicker physical barriers in pigs than in mice might have hindered AAV diffusion after subretinal injection and concentrating the viral particles in a small volume, which will favor cotransduction. Likewise, such stronger barriers might have impaired AAV trafficking to the target cells after intravitreal injection into rat eyes.

Also for the inner ear, different outcomes for different species should be expected, and the best route of administration in mice might not necessarily the best in other species.

9. Dual-AAV-application to the inner ear

The possibility that with the dual-AAV technique, coding sequences of more than 4 kb can be transduced into the inner ear by a non-integrating vector now opens up the possibility of a gene replacement for a series of hereditary deafness forms. For example, the genes underlying the recessively inherited forms of deafness DFNB2, DFNB9, DFNB16, DFNB18B, DFNB21, DFNB23, DFNB53, DFNB77, DFNB84A, and DFNB84B, as well as DFNX6 could theoretically be replaced by a dual-AAV approach (www.hereditaryhearingloss.org; Van Camp and Smith, n.d.).

Recently, two groups independently succeeded in restoring hearing in Otof+/− mice by transducing IHCs with full-length otoferlin cDNA by means of dual-AAV gene transfer (Al-Moyed et al., 2019; Akil et al., 2019). The attempt to target DFNB9 due to mutations in the gene OTOF seems to be an obvious choice for several reasons. First, the organ of Corti, and within it the inner and outer hair cells, can develop in absence of otoferlin (Roux et al., 2006), which will presumably allow for recovery by gene therapy in postnatal humans. Second, hearing impairment caused by OTOF mutations is in most cases profound and congenital (Yasunaga et al., 1999; Migliosi et al., 2002; Rodríguez-Ballesteros et al., 2003, 2008), and Otof+/− mice are profoundly deaf (Roux et al., 2006; Reisinger et al., 2011), such that any rescue of hearing can be clearly distinguished from non-treated controls. Moreover, in contrast to degenerative forms of hearing loss, a therapeutic effect is detectable in relatively short durations. Third, the function of otoferlin has been studied in detail and can be quantitatively assessed by cell physiological capacitance recordings (Roux et al., 2006).

Most of the work for the development of a dual vector system was done by the group of A. Auricchio (Roux et al., 2006). They used the otoferlin gene, which has a very low expression in other tissues. Otoferlin is expressed in the auditory system, and although it is not expressed in the sensory hair cells, it could still be used as a therapeutic target due to its potential role in the functional regeneration of hair cells. Therefore, the authors proposed a dual vector system to transduce otoferlin in the auditory system. They designed two pairs of plasmids for AAV packaging, one for trans-splicing and one for the hybrid strategy (Fig. 2A, B, Supplementary Table 1).

The two groups used differing dual-AAV approaches to express otoferlin in IHCs of Otof+/− mice (Fig. 2 and Supplementary Table 1). The success of their strategies, despite the differences, indicates that the effect in rescuing auditory function seems to be robust and not dependent on a specific otoferlin isoform, plasmid design, AAV serotype, or mouse strain. In addition, differences in the outcome might be linked to differences in experimental design and may help to guide future developments of dual-AAV gene therapies (Fig. 2).

Al-Moyed et al. (2019) designed two pairs of plasmids for AAV packaging, one for trans-splicing and one for the hybrid strategy (Fig. 2A, B, Supplementary Table 1). For the overlapping sequence in the hybrid vectors, they applied the highly recombinogenic sequence derived from the F1 phase (AK) (Trapani et al., 2014). Akil et al. (2019) designed vectors for a hybrid approach, with alkaline phosphatase (AP) cDNA as the overlapping sequence to induce recombination (Fig. 2G). AK and AP are comparable in their efficiency to induce recombination of dual-AAV genomes, causing no obvious differences in ABCA4 protein levels in dual-AAV transduced retinas (Trapani et al., 2015).

As promoters to drive mRNA transcription, Al-Moyed et al. (2019) employed a human β-actin promoter and a CMV enhancer, while Akil et al. (2019) subcloned a cytomegalovirus immediate early/chicken β-actin chimeric promoter to drive expression of otoferlin. Both promoters are known to drive strong expression of downstream genes ubiquitously in all cell types. In Al-Moyed et al., a cDNA encoding eGFP and a P2A peptide encoding sequence followed the promoter sequence, and downstream of that was the 5′ part of otoferlin cDNA. The P2A peptide causes ribosome skipping at the third last codon, and thereby the transcription leads to separate upstream (eGFP) and downstream (otoferlin) proteins (Kim et al., 2011).

To rescue Otof gene function, Al-Moyed et al. (2019) cloned otoferlin variant 4 (NM_001313767), with amino acids 1–840 encoded by the 5′ AAV and amino acids 841–1977 by the 3′ vector. Akil et al. (2019) employed otoferlin isoform 1 (NM_001100395), which was divided into a 5′ fragment comprising the cDNA for amino acids 1–816 and a 3′ fragment encoding amino acids 817–1992. The two otoferlin variants differ in the alternative use of exon 6, encoding 15 amino acids (AA169–183 in variant 1) (Strenzke et al., 2016). While for mouse inner ears, variant 4 was found to be predominantly expressed in IHCs (Strenzke et al., 2016), it is still unclear which isoform is precisely expressed in human inner ears, but the respective 15 amino acids are absent in isoform e (NP_001274418), which is the current reference sequence for human geneticists. In order increase the life-time of the mRNA, Al-Moyed et al. subcloned both a bovine growth hormone polyadenylation signal and a WPRE downstream of the 3′cDNA, while Akil et al. used solely a poly-adenylation signal.

Even of more relevance for impact on gene rescue than the differences in plasmid design might be the differences in virus packaging. Al-Moyed et al. (2019) found the natural serotype 6 capsid to efficiently target IHCs when injected into mice at postnatal days 5–8. A control AAV encoding only eGFP transduced up to 99% of IHCs. For the dual-AAV vectors transduction rates of up to 51% (average: 30 ± 4%, n = 10 animals for trans-splicing, and 19 ± 3%, n = 9 animals for hybrid), were found for full-length otoferlin transduction along the length of the organ of Corti, and if the apical turn was considered separately, even up to 70% of IHCs expressed otoferlin. However, the group quantified otoferlin immunofluorescence levels in individual transduced Otof+/− IHCs, they did not reach more than ~35% of protein levels compared to wild-type IHCs (Fig. 2D). This is about as much as in a mouse line with the p.ile51T thr mutation in Otof, which correlated with auditory fatigue in mice and impaired speech perception in humans (Varga et al., 2006; Wynne et al., 2013; Strenzke et al., 2016). How could the protein levels be increased? The hybrid approach, offering two possibilities of recombination of half-vectors, did not lead to significantly higher protein levels than trans-splicing in this experiment (Al-Moyed et al., 2019). More viral particles entering the cells might be beneficial, however, an experimental approach in the retina found that higher virus titers or capsid serotypes with higher transduction rates like the ancient AncSOL65 serotype (Lanegger et al., 2017; Suzuki et al., 2017) did not result in higher protein levels in dual-AAV transduced retinal cells (Carvalho et al., 2017). Rather, mechanisms increasing the number of virus particles entering the nucleus, unpacking viral
DNA, and promoting the formation of stable concatemers are expected to increase protein levels, like strategies interfering with virus phosphorylation. In Akil et al. (2019), a 4 YF capsid variant was applied, by means of which the authors found 78 ± 6% of IHCs to express eGFP from a single AAV. By co-injecting the dual-AAV half-vectors at P10, 64 ± 6% (n = 3 cochleae) of IHCs expressed full-length otoferlin, and even more (at P17: 82 ± 9%, n = 5; at P30: 85 ± 7%, n = 3) for later injections. This is in line with the finding of McClements et al. (2019) for retina photoreceptors indicating that YF-capsids might be beneficial to increase the rate of dual-AAV transduced cells. It will be important to study if also the levels of proteins expressed from such YF-dual-AAV vectors will be higher than when using natural AAV capsids.

In both studies, the viral vectors were injected through the round window membrane. Since the start of their projects, an improved injection methods has been proposed (Yoshimura et al., 2018), which will be worth testing, since a reliable and efficient injection method will potentially further increase transduction.

Fig. 2. Comparison between the dual-AAV approaches to rescue hearing in Otof<sup>-/-</sup> mice
A, trans-splicing and B, hybrid virus genomes from Al-Moyed et al. (2019) with otoferlin variant 4 (Otof_v4; NM_001313767) as coding sequence, and the AK hybridization site (from F1 phage). eGFP was separated from otoferlin by a P2A sequence. WPRE, Woodchuck Hepatitis Virus Posttranscriptional Regulatory Element; SD, splice donor site, SA, splice acceptor site (see also Supplementary Table 1 for comparison of the different approaches). C-F, read-out from Al-Moyed et al. (2019) including C, immunohistochemistry, D, quantification of immunofluorescence with two different antibodies and for comparison otoferlin protein levels from Otof<sup>I515T/I515T</sup> IHCs from Strenzke et al. (2016) (*; grey). E, ABR recordings in response to click stimuli (P23-30) and F, patch-clamp cellular recordings, in the latter exocytosis for Otof<sup>I515T/I515T</sup> IHCs (grey) and their wild-type littermates were replotted from Strenzke et al. (2016). G, hybrid strategy from Akil et al. (2019) with otoferlin variant 1 (Otof_v1; NM_001100395) and the alkaline phosphatase sequence fragment (AP) to induce homologous recombination. H, immunohistochemical assay for otoferlin and I, ABR recordings for click stimuli (3 weeks after injection) from Akil et al. (2019) exemplified for the P10 injected mice.
rates. The advancement of the Yoshimura et al. method lies in an additional fenestration in the posterior semicircular canal, which allows a pressure release when solutions are injected through the round window membrane. Besides, Suzuki et al. (2017) successfully injected inner ears of adult mice through the semicircular canal, causing less harm to auditory tissue than the round window membrane injection. Such alternative injection ways might be considered in future studies, despite no injection trauma was described in Akil et al. (2019), and only minor effects of injection on inner ear function were reported in Al-Moyed et al. (2019).

The Otof−/− mouse strains in which the viral vectors were injected are of different origin and background: The mouse line generated in Roux et al. (2006), backcrossed to FVB mice, was the subject for dual-AAV application in Akil et al., while Al-Moyed et al. used an independently generated Otof−/− mouse line (Reisinger et al., 2011), which has a similar genetic deletion of exons 14 and 15 as the other mutant (Supplementary Table 1). Al-Moyed et al. injected the dual-AAV vectors into F1 offspring from CD1 females and C57Bl6 males, in which the deletion has been backcrossed. At least for young age, the FVB and the F1CD1Bl6 mixed background mice should have no additional hearing impairment, thus it is unlikely that hearing differences after dual-AAV treatment are due to background strain differences. However, otoferlin mRNA expression has been discovered in different mouse strains, which could be unwarped in a side-by-side experimental testing of different capsids in the same strains and vice versa. In Al-Moyed et al., injections were performed at P6–P7 due to the focus on testing the function of otoferlin by means of patch-clamp electrophysiology, while Akil et al. assessed if the rescue of hearing by dual-AAV injection depends on the maturation stage of the inner ear, which is why they injected at P10, P17 and P30.

To assess the effect of dual-AAV injection into the inner ear of Otof−/− mice, both groups recorded auditory brainstem responses (ABRs, Fig. 2E1), counted the number of transduced IHCs and synapses, and probed for full-length mRNA by means of PCR (Supplementary Table 1). In addition, Al-Moyed et al. validated the coding sequence by Sanger sequencing of these ampiclons, and they quantified the otoferlin protein levels by immunofluorescence analysis in 3D stacks from confocal images and correlated this with exocytosis recordings from transduced IHCs (Fig. 2D,F). Here, they found exocytosis to be wild-type-like for short depolarization stimuli of up to 20 ms, which reflects Ca2+ triggered fusion of the readily releasable pool of synaptic vesicles. Sustained exocytosis, for stimuli up to 100 ms, was restored to 35–50%. Notably, OtofP157I/G155V IHCs, having almost identical otoferlin protein levels as dual-AAV transduced Otof−/− IHCs, display undistinguishable exocytosis levels both for short and long stimuli as the trans-splicing dual-AAV transduced Otof−/− IHCs (Fig. 2F, patch-clamp recordings were done by the same researcher in both studies; Strenzke et al., 2016). Thus, the incomplete rescue of sustained exocytosis is attributed to the too low otoferlin levels expressed from dual-AAV vectors. Nevertheless, this correlation indicates that otoferlin transduced by dual-AAV vectors seems to be fully functional, which is in line with the sequencing results proving the absence of mutations at the assembly site of the split transcripts. Congruently, the authors found hearing to be partially restored, with thresholds of 50 dB for click stimuli (wild-type controls: 32 dB), but relatively small ABR wave amplitudes compared to controls (Fig. 2E). A better restoration of ABR amplitudes will likely be achieved with higher transduction rates: not only Al-Moyed et al. found that ABR wave amplitudes correlate with the rate of transduced IHCs in individual animals, also the study of Akil et al. with IHC transduction rates of ~64% (P10 injection) and ~82–85% (P17 and P30 injection) revealed a better restoration of ABR amplitudes. In Akil et al., ABR amplitudes of waves II-V seemed comparable to wild-type controls (Fig. 2I), while wave I reached 39 ± 7% to 47 ± 10% (4 or 20 weeks after P10 injection, similar for later injections) of wild-type values, which is remarkable given the ~40% reduced number of synapses in Otof−/− IHCs. Importantly, Akil et al. demonstrated that the restoration of hearing with thresholds of ~40 dB for click stimuli is stable over 30 weeks post injection, and that injection of dual-AAVs into mature ears of Otof−/− mice is at least as efficient to restore hearing as early postnatal injection. This is promising with respect to a future clinical application, since at the time of diagnosis in newborns, the inner ear is already mature.

Does dual-AAV mediated re-expression of otoferlin in Otof−/− IHCs prevent synapse loss? Here both studies came to different conclusions. In Al-Moyed et al., a ~25% higher number of synapses at P6 in otoferlin deficient IHCs indicated that otoferlin seems to play a role in early synaptic development. In the second and third postnatal week, synapse numbers decayed faster in Otof−/− IHCs than in wild-type controls, such that at P26–P29, Otof−/− IHCs contained ~40% fewer synapses compared to wild-type IHCs. The transduction of Otof−/− IHCs at P6–7 with either dual-AAV approach did not change the number of synaptic ribbons in comparison to the contralateral non-injected ear (10 ± 0.2 in trans-splicing (n = 59 IHCs), 10 ± 0.3 in OtofP157I/G155V IHCs). 10 ± 0.3 in non-treated controls (n = 46 IHCs), vs. 15 ± 0.3 in CD1Bl6F1 wild-type IHCs (n = 108 IHCs) and 15 ± 0.5 in B6 wild-type IHCs (n = 48 IHCs)), Thus, the authors concluded that re-expression of otoferlin at the end of the first postnatal week is already too late to prevent from synapse degeneration. In contrast, Akil et al. proposed that dual-AAV injection at P17 was able to induce synaptic regrowth (number of ribbons in untreated Otof−/− IHCs at P17: 8.2 ± 1.0; transduced at P17, analyzed at P80: 10.0 ± 1.3, non-transduced IHCs in the same cochlea: 5.8 ± 0.7, n = 48 IHCs in all conditions). Analysis of synapses at more time points will be required to find out if re-expression of otoferlin at any time point can prevent from a further loss of synapses, or if the newly gained exocytosis of IHCs can even induce the formation of new synaptic contacts, potentially by co-release of neurotrophic factors.

An interesting and so far enigmatic phenomenon has been observed in both studies: even though unspecific promoters were used, otoferlin was expressed strongly in IHCs, only very weakly in other cochlear cell types, since Al-Moyed et al. found eGFP expression, translated from the same mRNA as otoferlin, also in other cochlear cell types. Since Al-Moyed et al. found eGFP expression, translated from the same mRNA as otoferlin, also in other cochlear cell types, this indicates a likely posttranscriptional mechanism that restricts translation to IHCs. While the hair cell specific expression of otoferlin is beneficial regarding a future gene therapy, understanding this mechanism might be helpful for designing viral vectors to cure hearing impairment in other forms of deafness.

10. Conclusions and outlook

The two otoferlin dual-AAV studies unambiguously revealed that otoferlin expressed from split cDNA is fully functional. This indicates that the cellular repair mechanisms required for split virus genome assembly are intact, at least in IHCs. This dual-AAV strategy to regain hearing in DFNB9 will soon be translated to clinic, with three companies currently developing respective gene therapy products: (Decibel Therapeutics, Boston MA; Akouos Inc., Boston MA, Sensorion, France).

Because any cell type and any gene delivered so far behaves differently with respect to the most efficient strategy, dual-AAV approaches for other forms of deafness will require optimization. A series of different natural and engineered AAV capsid proteins that can target specific cell types in the cochlea are available, and
they will need to be evaluated for their suitability in dual-AAV applications in diverse species. With the molecular tools at hand, it seems plausible that for the aforementioned forms of deafness, a dual-AAV approach that can fully restore hearing function is within reach.

Declaration of competing interest

The author declares to be co-inventor on a patent for dual-AAV vectors to restore hearing. The former employer University Medical Center Göttingen has licensed the rights to these parts of the patent exclusively to Akous Inc., USA.

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Appendix A. Supplementary data

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