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(54) Title: DUAL RECOMBINANT AAV8 VECTOR SYSTEM ENCODING ISOFORM 5 OF OTOFERLIN AND USES THEREOF

(57) Abstract: The present invention is based on the observation that a dual AAV vector strategy encoding isoform 5 of otoferlin cDNA that has been split into two expression cassettes both packaged in - and delivered by - an AAV8 capsid can efficiently deliver the otoferlin cDNA to the inner hair cell (IHC). Moreover, the inventors highlighted that the use of the CMV promoter in one of the two AAV8 vectors provides a significant expression of otoferlin in these particular cells. As the AAV serotype and the type of promoter used are two key elements that have a significant effect on the transduction efficiency, the development of the vector system of the invention is going to provide optimal therapeutic benefit in patients suffering from DFNB9 deafness. To further improve this therapeutic effect, the inventors finally tested some particular otoferlin-encoding dual vector constructs to identify enhanced transfection rate and a very effective in vitro and in vivo otoferlin expression in mature cochlea of DFNB9 mice models, leading to the restoration of their hearing.

Dual recombinant AAV8 vector system encoding isoform 5 of Otoferlin and uses thereof

SUMMARY OF THE INVENTION

The present invention is based on the observation that a dual AAV vector strategy encoding isoform 5 of otoferlin cDNA that has been split into two expression cassettes both packaged in - and delivered by - an AAV8 capsid can efficiently deliver the otoferlin cDNA to the inner hair cell (IHC). Moreover, the inventors highlighted that the use of the CMV promoter in one of the two AAV8 vectors provides a significant expression of otoferlin in these particular cells. As the AAV serotype and the type of promoter used are two key elements that have a significant effect on the transduction efficiency, the development of the vector system of the invention is going to provide optimal therapeutic benefit in patients suffering from DFNB9 deafness. To further improve this therapeutic effect, the inventors finally tested some particular otoferlin-encoding dual vector constructs to identify enhanced transfection rate and a very effective *in vitro* and *in vivo* otoferlin expression in mature cochlea of DFNB9 mice models, leading to the restoration of their hearing.

BACKGROUND OF THE INVENTION

More than half of the cases of nonsyndromic profound congenital deafness have a genetic cause, and most (~ 80%) are autosomal recessive (DFNB) forms (Duman D. & Tekin M, Front Biosci (Landmark Ed) 17:2213-2236 (2012)). Genetic diagnosis of deafness provides essential information for cochlear gene therapies, and rapid progress has been made in both the accuracy and accessibility to genetic testing in the last few years. Identification of mutations in syndromic deafness genes could be done many years before the emergence of symptoms in patients, giving time for planning disease management.

Deafness genes encode proteins with a wide range of molecular functions vital for cochlear functioning, such as development of the sensory organ, sound transduction in the stereocilia of hair

cells, maintenance of the endocochlear potential (EP) and high concentration of extracellular potassium, and synaptic neurotransmission between hair cells and spiral ganglion neurons (SGNs). Major proteins made from deafness genes include ion channels and transporters, gap junctions and tight junctions, protein subunits in cytoskeleton and molecular motors, and transcription factors transiently expressed in cochlear development. Whether a mutation affects early cochlear development and leads to a significant cellular degeneration is a major factor in determining the "treatment time window", which is a crucial problem in this therapeutic field.

Prosthetic cochlear implants are currently used for rehabilitation (Kral A & O'Donoghue GM N Engl J Med 363(15):1438-1450 (2010)), but hearing recovery is far from perfect, particularly for the perception of speech in noisy environments or of music, because of their inherent limitation of frequency resolution as imposed by inter-channel electrical interference.

A primary motivation in developing biological treatments is to restore hearing without the implantation of any prosthetic device, and to achieve sound resolution quality and unit cost that is much better than what is currently achievable with cochlear implants. In particular, gene therapy with local Adeno-associated virus (AAV)-mediated gene therapy has already been proposed for treating human forms of deafness (Zhang et al, Frontiers in Molecular Neuroscience, vol.11, Art.221, 2018). This approach is now being tested for several inherited disorders, including Parkinson's disease, visual impairment and metabolism disorders, in various preclinical and clinical trials.

No such trials have yet been performed in humans for hearing loss, but the anatomy of the human inner ear is ideal for in vivo gene therapy approaches, as the relatively isolated fluid-filled compartments provide opportunities for local virus application with a low risk of dissemination.

In the last decade, it has been shown that the AAV8 serotype with a hybrid CMV enhancer/chicken β-actin promoter (CAG promoter) specifically targets the cochlear and vestibular hair cells (Emptoz et al. Proc Natl Acad Sci U S A. 2017 Sep 5;114(36):9695-9700). With this AAV configuration, the hearing in a mouse model of DFNB59 was restored, and both hearing and balance in a mouse model of Usher 1G

and IIIA syndrome were improved (Delmaghani et al. Cell. 2015 Nov 5;163(4):894-906; Emptoz et al. Proc Natl Acad Sci U S A. 2017 Sep 5;114(36):9695-9700; Dulon et al. J Clin Invest. 2018 Aug 1;128(8):3382-3401). In addition, the first proof-of-principle that dual AAV gene therapy reverses the deafness phenotype in a mouse model for a form of profound deafness, DFNB9, was generated, raising hopes for future gene therapy trials in DFNB9 patients. Remarkably, the dual AAV therapy not only prevented these mutant mice from becoming deaf, but also restored hearing in the mice injected after the hearing onset. These results raise strong hopes for future gene therapy trials in DFNB9 (Akil et al. Proc Natl Acad Sci U S A. 2019 Mar 5;116(10):4496-4501).

The development of vectors with optimized properties, including enhanced targeting specificity to ensure specific infection of defective cells, and high level of expression of the affected protein in these particular cells, now appears to be an essential step in the development of curative gene therapy for inherited inner ear defects.

DESCRIPTION OF THE INVENTION

In this context, the present inventors have designed new therapeutic recombinant vectors that can be used in DFNB9 preclinical trials. These vectors differ from those of the prior art in that they express the isoform 5 of the human otoferlin protein, placed under the control of the CMV promoter optionally followed by an intronic sequence, and are packaged in an AAV8 capsid, which specifically target inner hair cells (IHCs). Their comparative results identified particular constructs encoding efficiently the isoform 5 of the Otoferlin protein in the right place, at the right level, at the right time, leading to an optimal therapeutic effect.

It is well-known that the otoferlin cDNA sequence (6kb) exceeds the packaging capacity of AAV (5kb). Therefore, a dual AAV vector strategy was adopted, in a similar manner to the one successfully used for the previous mouse studies (Akil et al. Proc Natl Acad Sci U S A. 2019 Mar 5;116(10):4496-4501).

The predicted cochlear isoforms of human otoferlin cDNA (isoform 5 and new isoforms) were split into two expression cassettes, both delivered by an AAV8 vector. As the efficacy of the dual AAV transfer is likely to be affected by the split site within the otoferlin cDNA, several cutting sites between exons encoding the otoferlin transcript were investigated. The corresponding 5' and 3' portions of the human otoferlin cDNA were cloned into a shuttle vector with AAV inverted terminal repeats (ITRs), and the ubiquitous CMV promoter was inserted upstream of the 5' human otoferlin cDNA, said promoter being optionally followed by an intronic sequence. Then, the different dual plasmids were tested *in vitro* by transfecting HEK293 cells using liposome as carrier and OTOF expression was assessed 48 hours post transfection, using immunocytochemistry and Western-Blot (figures 3, 5, and 7). The recombination efficacy of the various dual AAV OTOF vectors to produce the full-length protein was furthermore investigated by RT-PCR (figure 7A). The dual vector showing the best transfection rate and the most effective *in vitro* protein expression was furthermore investigated by verifying the accuracy of the recombined region that produced the full-length protein. Then, the dual expression cassettes were packaged in the AAV8 capsid and *in vivo* delivered to cochlea of the DFNB9 mouse model. Immunoconfocal microscopy was used to determine whether the otoferlin protein was properly targeted to the IHC after cochlear AAV delivery. Hearing restoration in the mice was assessed by auditory-evoked brainstem response recordings at different stages after AAV delivery.

Otoferlin is abundantly expressed in sensory IHCs of the cochlea. It is also expressed in other cells of the central nervous system. It plays a key role in the final steps of synaptic vesicle fusion at cochlear hair cell synapses with afferent spiral ganglion neurons. More precisely, it is important for exocytosis at the auditory ribbon synapse (Roux et al, Cell 127(2):277-89, 2006). In human beings, mutations affecting the Otoferlin gene ("OTOF gene") lead to severe non-syndromic bilateral loss of hearing that occurs after birth but before the acquiring of language. Some of them also lead to a temperature-sensitive nonsyndromic auditory neuropathy, that is triggered when the body temperature increases importantly (for example in case of fever, see Marlin S. et al, Biochemical and Biophysical Research Communications, 394 (2010) 737-742; Varga R. et al, J. Med. Genet 2006; 43:576-581; Zhang Q. et al,

Hearing research, Volume 335, May 2016, Pages 53-63; Starr A. et al, Brain, Volume 119, Issue 3, June 1996, Pages 741–753).

At least 75 mutations have been identified so far, among which 7 are known to be thermosensitive (P.Q994VfsX6, P.I515T, p.G541S, PR1607W, p.E1804del, c.2975_2978delAG/c.4819C>T, c.4819C>T (c.R1607W) reviewed in Pangrsic T. et al, Trends in Neurosciences, 2012, col.35, No.11. These deafness phenotypes (constitutive and inducible) are found all over the world and known as the "Deafness, Autosomal Recessive 9" or "DFNB9" deafness. DFNB9 deafness accounts for up to 10% of autosomal recessive non-syndromic hearing loss, thereby residing within the top five of genetic hearing disorders that still require a therapeutic intervention.

Importantly, the present inventors have shown that AAV8 vectors containing the 5' portions of the human otoferlin cDNA under the transcriptional control of the CMV promoter are optimal and efficient in the target cells. Therefore, the present inventors have set up a dual-AAV vector system containing the identified promoter and two half-portions of the Otoferlin gene to IHCs, where a trans-plicing and/or homologous recombination occur leading to the expression of protein full-length.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. For purposes of the present invention, the following terms are defined below.

Definitions

As used herein, the terms "nucleic acid" and "nucleotide sequence" and "polynucleotide sequence" refer to a deoxyribonucleotide or ribonucleotide polymer in either single or double stranded form, and unless otherwise limited, encompass known analogues of natural nucleotides that can function in a similar manner as naturally occurring nucleotides.

As used herein, the term “Otoferlin” designates the Otoferlin polypeptide. It is herein abbreviated as “OTOF”. This polypeptide is also known as “AUNB1”, “DFNB6”, “DFNB9”, “NSRD9” and “FER1L2”.

This polypeptide is a member of the Ferlin family of transmembrane proteins, which has C2 domains as synaptotagmins, PKC and PLC (Yasunaga S et al, J Hum Genet. 2000 Sep;67(3):591-600). This long form contains six C2 domains. As mentioned above, it is involved in synaptic vesicle fusion between cochlear hair cell and afferent spiral ganglion neurons (Roux et al, Cell 127(2):277-89, 2006; Michalski et al, Elife, 2017 Nov 7;6 e31013).

As used herein, the term “Otoferlin polypeptide” designates the isoform 5 (variant e) of the wild-type human Otoferlin polypeptide of SEQ ID NO:5 (corresponding to Genbank number NP_001274418) and homologous sequences. It is encoded for example by the cDNA sequence NM_001287489.1 (SEQ ID NO:91, wherein the coding sequence of said isoform starts at nucleotide 186) and by SEQ ID NO:15 (corresponding to the coding sequence of said isoform).

Are herewith encompassed the homologous polypeptide thereof whose amino acid sequence shares at least 70% identity and/or similarity with SEQ ID NO:5, that retain at least one biological function of the Otoferlin polypeptide of SEQ ID NO:5. For example, this biological function is related to the modulation of vesicles fusion at the cochlear inner hair cell ribbon synapses that activate the primary auditory neurons (Michalski et al, Elife, 2017 Nov 7;6 e31013). This modulation could be assessed with classical ex vivo electrophysiological measures. Said homologous sequence more preferably shares at least 75%, and even more preferably at least 80%, at least 85%, or at least 90% identity and/or similarity with SEQ ID NO:5. When the homologous polypeptide is much shorter than SEQ ID NO:5, then local alignment can be considered.

Said homologous polypeptide can have for example the amino acid sequence presented in SEQ ID NO:1 (corresponding to Genbank number NP_919224.1). Said sequence characterises the isoform a (variant 1) of the wild-type human Otoferlin polypeptide. This variant has an alternate in-frame exon in the 3'

coding region, as compared to SEQ ID NO:5. It furthermore contains a distinct C-terminus as compared to SEQ ID NO:5 (but its N-terminal part is the same).

Said homologous polypeptide can also have the amino acid sequence presented in SEQ ID NO:2 (corresponding to Genbank number NP_004793.2) or the amino acid presented in SEQ ID NO:3 (corresponding to Genbank number NP_919303.1) corresponding to the short isoforms b and c (variants 2 and 3) respectively. More precisely, SEQ ID NO:2 represents the isoform b (variant 2, also called 'short form 1') which has a shorter N-terminus and lacks a segment compared to SEQ ID NO:1. On another hand, SEQ ID NO:3 represents the isoform c (variant 3, also called “short form 2”), which differs in the 5' UTR and coding sequence compared to variant 1 (SEQ ID NO:1) because it has a shorter and distinct C-terminus compared to SEQ ID NO:1.

Said homologous polypeptide can also have the amino acid sequence presented in SEQ ID NO:4 (corresponding to Genbank number NP_919304.1) corresponding to the isoform d (variant 4). This variant differs in the 5' UTR and coding region, as well as in the 3' coding region, compared to variant 1. The resulting isoform (d) has a shorter N-terminus and a distinct C-terminus compared to isoform a of SEQ ID NO:1. It is encoded by SEQ ID NO:14 (corresponding to GenBank number NM_194323.3).

In an embodiment, the vector system of the invention can allow for the expression of a functional fragment of the Otoferlin polypeptide of SEQ ID NO:5. The term “functional fragment” herein designates any fragment of the human Otoferlin polypeptide or any fragment of a polypeptide having a homologous sequence as defined above, wherein said fragment retains at least one biological function of the Otoferlin polypeptide that is of interest in the present context. For example, this biological function is related to the modulation of vesicles fusion at the cochlear inner hair cell ribbon synapses that activate the primary auditory neurons (Michalski et al, Elife, 2017 Nov 7;6 e31013). This modulation could be assessed with classical ex vivo electrophysiological measures.

In another embodiment, the vector system of the invention can allow for the expression of three particular homologous proteins of the variant 5 (see the Example 2 and associated Figure 6). These

three alternative OTOF isoforms have the amino acid sequences of SEQ ID NO:6, SEQ ID NO:7 or SEQ ID NO:8. They can be encoded by the cDNA sequences of SEQ ID NO:16, SEQ ID NO:17 and SEQ ID NO:18, respectively. It is thus preferred to use in the vector system of the invention any of these new isoforms, as they are thought to have a potential to restore hearing in humans.

After recombination, these new isoforms may encode *in situ* the proteins of SEQ ID NO:6, SEQ ID NO:7 and/or SEQ ID NO:8 having a potential to restore hearing in humans, in addition to the current human isoform 5 transcript.

In a particular embodiment, the vector system of the invention therefore allows for the expression of SEQ ID NO:6, SEQ ID NO:7 or SEQ ID NO:8, or for a functional homologous polypeptide thereof, that retain the activity of these new isoforms and/or of SEQ ID NO:5. These functional homologous are the ones whose amino acid sequence shares at least 70%, at least 75%, at least 80%, at least 85%, or at least 90% identity and/or similarity with SEQ ID NO:6, SEQ ID NO:7 or SEQ ID NO:8. When the homologous polypeptide is much shorter than SEQ ID NO:6, SEQ ID NO:7 or SEQ ID NO:8, then local alignment can be considered.

The invention provides systems encoding homologous amino acid sequences that are “similar” to these sequences, as defined above. In this case, they contain a coding sequence that can be for example the long cDNA sequence NM_194248.3 (isoform a or variant 1, SEQ ID NO:11), the shorter cDNA sequence NM_004802.4 (isoform b or variant 2, SEQ ID NO:12), the cDNA sequence NM_194322.3 (isoform c or variant 3, SEQ ID NO:13), or the cDNA sequence NM_194323.3 (isoform d or variant 4, SEQ ID NO:14). Said coding sequence can also have the sequence SEQ ID NO:16, 17 or 18 corresponding to the cDNAs of new isoforms of the OTOF gene, as explained below.

In a preferred embodiment, said coding sequence is derived from the human Otoferlin gene of SEQ ID NO:91 (NM_001287489.1) encoding the transcript variant 5 (which coding sequence begins at nucleotide 186). It is more preferably as disclosed in SEQ ID NO:15.

Thus, in the vector system of the invention, the coding sequence is preferably SEQ ID NO:15. It is also possible to use any homologous sequence thereof, having a sequence identity of at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% with SEQ ID NO:15.

In the context of the invention, the identity percentage between two homologous sequences is preferably identified by a global alignment of the sequences in their entirety when the sequences are of about the same size. This alignment can be performed by means of an algorithm that is well known by the skilled person, such as the one disclosed in Needleman and Wunsch (1970). Accordingly, sequence comparisons between two amino acid sequences or two nucleotide sequences can be performed for example by using any software known by the skilled person, such as the "needle" software using the "Gap open" parameter of 10, the "Gap extend" parameter of 0.5 and the "Blosum 62" matrix.

When local alignment of the sequences is to be considered (e.g., in case of homologs that have a smaller size than the sequences of the invention), then said alignment can be performed by means of a conventional algorithm such as the one disclosed in Smith and Waterman (J. Mol. Evol. 1981; 18(1) 38-46).

"Similarity" of two targeted amino acid sequences can be determined by calculating a similarity score for the two amino acid sequences. As used herein, the "similarity score" refers to the score generated for the two sequences using the BLOSUM62 amino acid substitution matrix, a gap existence penalty of 11, and a gap extension penalty of 1, when the two sequences are optimally aligned. Two sequences are "optimally aligned" when they are aligned so as to produce the maximum possible score for that pair of sequences, which might require the introduction of gaps in one or both of the sequences to achieve that maximum score. Two amino acid sequences are substantially similar if their similarity score exceeds a certain threshold value. The threshold value can be any integer ranging from at least 1190 to the highest possible score for a particular reference sequence (e.g., SEQ ID NO:15). For

example, the threshold similarity score can be 1190, 1200, 1210, 1220, 1230, 1240, 1250, 1260, 1270, 1280, 1290, 1300, 1310, 1320, 1330, 1340, 1350, 1360, 1370, 1380, 1390, 1400, 1410, 1420, 1430, 1440, 1450, 1460, 1470, 1480, 1490, 1500, or higher. If in a particular embodiment of the invention, the threshold score is set at, for example, 1300, as compared with a reference sequence, then any amino acid sequence that can be optimally aligned with said reference sequence to generate a similarity score of greater than 1300 is "similar" to said reference sequence. Amino acid substitution matrices and their use in quantifying the similarity between two sequences are well-known in the art and described, e.g., in Dayhoff et al. (1978), "A model of evolutionary change in proteins", "Atlas of Protein Sequence and Structure," Vol. 5, Suppl. 3 (ed. M. O. Dayhoff), pp. 345–352. Natl. Biomed. Res. Found., Washington, D.C. and in Henikoff et al. (1992) Proc. Natl. Acad. Sci. USA 89:10915–10919. While optimal alignment and scoring can be accomplished manually, the process is facilitated by the use of a computer-implemented alignment algorithm, e.g., gapped BLAST 2.0, described in Altschul et al., (1997) Nucleic Acids Res. 25:3389–3402, and made available to the public at the National Center for Biotechnology Information website. To generate accurate similarity scores using NCBI BLAST, it is important to turn off any filtering, e.g., low complexity filtering, and to disable the use of composition based statistics. One should also confirm that the correct substitution matrix and gap penalties are used. Optimal alignments, including multiple alignments, can be prepared using, e.g., PSI-BLAST, available through the NCBI internet site and described by Altschul et al., (1997) Nucleic Acids Res. 25:3389–3402.

It is also possible to use the sequences of the murine Otoferlin gene, as shown in example 1 below, notably SEQ ID NO:79 and SEQ ID NO:80 encoding the N-terminal and C-terminal part of the isoform 1 of the mouse Otoferlin gene (NM_001100395.1).

Vector system of the invention

In a first aspect, the invention relates to a vector system comprising at least two different AAV particles, namely:

a) at least one AAV8 particle comprising a first polynucleotide comprising an inverted terminal repeat at each end of said polynucleotide, and, between the said inverted terminal repeats, from 5' to 3': a CMV promoter sequence followed by a partial coding sequence that contains the N-terminal coding part of the Otoferlin gene, and

b) at least one AAV8 particle comprising a second polynucleotide comprising an inverted terminal repeat at each end of said polynucleotide, and, between the said inverted terminal repeats, from 5' to 3': a partial coding sequence that contains the C-terminal coding part of the Otoferlin gene, optionally followed by a polyadenylation sequence,

wherein the first and second polynucleotides comprise a recombinogenic polynucleotide sequence,

and wherein the coding sequences in the first and second polynucleotides when combined encode the isoform5 of the Otoferlin polypeptide, an homologous or a functional fragment thereof, as defined above.

The vector system of the invention contains at least one AAV8 particle containing the polynucleotide as defined in a) (i.e., encoding the N-terminal coding part of Otoferlin), and at least one AAV8 particle containing the polynucleotide as defined in b) (i.e., encoding the C-terminal coding part of Otoferlin). In other terms, said vector system contains said first and second polynucleotides, each polynucleotide being preferably contained in separate AAV8 particles. The two different types of AAV8 particles can be contained within the same composition or within different compositions and may be administered together or separately.

It is herein understood that "first" and "second" are not meant to imply a particular order or importance. What is necessary however is that the vector system of the invention contains two

different recombinant AAV vectors, one comprising the above-mentioned polynucleotide a) and the other comprising the above-mentioned polynucleotide b), so that the two polynucleotides are simultaneously present in the target cells, and the Otoferlin polypeptide can be generated *in situ*.

AAVs are small replication-deficient adenovirus-dependent viruses from the *Parvoviridae* family. They have an icosaedrical capsid of 20–25 nm in diameter and a genome of 4.7 kb flanked by two inverted terminal repeats (ITRs). After uncoating in a host cell, recombinant AAV genome can persist in a stable episome state by forming high molecular weight head-to-tail circular concatamers providing long-term and high-level transgene expression. AAV8, which is the preferred AAV serotype in the context of the present invention, is currently tested *in vivo*.

In order to increase the efficacy of gene expression, and prevent the unintended spread of the virus, genetic modifications of AAV8 can be performed. These genetic modifications include the deletion of the E1 region, deletion of the E1 region along with deletion of either the E2 or E4 region, or deletion of the entire adenovirus genome except the cis-acting inverted terminal repeats and a packaging signal. Such modified vectors are advantageously encompassed in the present invention.

Moreover, it is also possible to use genetically modified AAV8 having a mutated capsid protein so as to direct the gene expression towards a particular tissue type, e.g., to auditory cells. In this aim, AAV8 vectors in which tyrosine residues in the viral envelope are substituted for alanine residues can be used. For example, tyrosine 733 can be substituted with an alanine residue (AAV8-Y733A). By using AAV8-Y733A, it is possible to increase gene transfer by up to 10,000 fold, decreasing the amount of AAV necessary to infect the sensory hair cells of the cochlea. It is also possible to use AAV8 vectors in which any tyrosine residues in the viral envelope are substituted by alanine residues. In addition, the efficacy of AAV8 serotype can be further improved using peptide ligand insertion as disclosed in Michelfelder, PLoS One. 2011; 6(8): e23101.

Methods for preparing viruses and virions comprising a heterologous polynucleotide or construct are known in the art. In the case of AAV, cells can be coinfected or transfected with adenovirus or

polynucleotide constructs comprising adenovirus genes suitable for AAV helper function. Examples of materials and methods are described, for example, in U.S. 8,137,962 and 6,967,018. It is routine for the skilled person to generate the AAV particles that are essential to the vector system of the invention, based on the information provided herein and his / her common knowledge.

As used herein, the term "promoter of the invention" designates the CMV promoter having the SEQ ID NO:9 and homologous sequences thereof that retain the promoter function of SEQ ID NO:9 on the Otoferlin polypeptide. It is indeed possible to use any homologous sequence of SEQ ID NO:9, having a sequence identity of at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% with SEQ ID NO:9.

In particular, it is possible to insert an intronic sequence downstream of the CMV promoter, to stabilise the mRNAs, improve cytoplasmic export and therefore enhance the efficiency of said CMV promoter. This additional sequence can be for example the sequence of SEQ ID NO:10, said sequence representing a chimera between introns from human beta-globin and immunoglobin heavy chains.

This promoter (optionally followed by the intronic sequence) can be incorporated into the vectors of the invention using standard techniques known in the art. It has to be located upstream of the first exon of the Otoferlin gene. In one embodiment, the promoter (and optionally the intronic sequence) is positioned about the same distance from the transcription start site as it is from the transcription start site in its natural genetic environment. Yet, variation in this distance is permitted without substantial decrease in promoter activity. A transcription start site is typically included in the vector.

The polynucleotides included in the vector system of the invention contain the N- or the C-terminal coding part of the Otoferlin gene, encoding, when recombined, the isoform5 of the Otoferlin polypeptide of SEQ ID NO:5 (corresponding to Genbank number NP_001274418), or functional fragments and homologous sequences thereof, as defined above.

In one preferred embodiment, the polynucleotides included in the vector system of the invention contain a part of the cDNA sequence NM_001287489.1 (isoform 5 or variant e, SEQ ID NO:91), more preferably the coding part thereof whose sequence is displayed on SEQ ID NO:15.

In another preferred embodiment, the polynucleotides included in the vector system of the invention contain a part of the cDNA sequence of SEQ ID NO:16, SEQ ID NO:17 and SEQ ID NO:18, allowing for the expression of three particular homologous proteins of the isoform 5 (see the Example 2 and associated Figure 6) of SEQ ID NO:6, SEQ ID NO:7 or SEQ ID NO:8 respectively.

Cutting sites

A dual-vector approach is advantageous to split a long coding sequence into two parts, in order to be packaged more easily into virions having a limited packaging capacity. As AAVs capsids are herein used, it is preferred to use polynucleotides that contain an OTOF coding sequence that contains no more than 5 kilobases, preferably no more than 4,7 kilobases.

The vector system of the invention should therefore contain two distinct polynucleotides each containing parts of the coding sequence of an Otoferlin gene that encodes the Otoferlin polypeptide described above. Said coding sequence is for example the one displayed in SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17 or SEQ ID NO:18, or any homologous sequence thereof, having a sequence identity of at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% with SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17 or SEQ ID NO:18.

The partial coding sequences contained in the polynucleotides described herein are designed so that, upon delivery of the polynucleotides, the partial coding sequences are joined together, e.g., through homologous recombination, and form a complete coding sequence (also referred to as “Otoferlin gene”) that encodes an Otoferlin polypeptide as defined above.

In a preferred embodiment, said coding sequence (or "Otoferlin gene") has the sequence displayed in nucleotides 186-6179 of SEQ ID NO:91, or it has the sequence displayed in SEQ ID NO:15, SEQ ID NO:16, SEQ ID NO:17 or SEQ ID NO:18 or an homologous sequence thereof, as defined above.

The coding sequence of the human OTOF gene is preferably cut at a natural splicing site.

For example, the human OTOF gene isoform 5 of SEQ ID NO:15 can be split between exons 18 and 19 into a N-terminal coding part having the nucleotides 1-2214 of SEQ ID NO:15 and a C-terminal coding part having the nucleotides 2215-5991 of SEQ ID NO:15.

Alternatively, the human OTOF gene isoform 5 of SEQ ID NO:15 can be split between exons 20 and 21 into a N-terminal coding part having the nucleotides 1-2406 of SEQ ID NO:15 and a C-terminal coding part having the nucleotides 2407-5991 of SEQ ID NO:15.

Alternatively, the human OTOF gene isoform 5 of SEQ ID NO:15 can be split between exons 21 and 22 into a N-terminal coding part having the nucleotides 1-2523 of SEQ ID NO:15 and a C-terminal coding part having the nucleotides 2524-5991 of SEQ ID NO:15.

Alternatively, the human OTOF gene isoform 5 of SEQ ID NO:15 can be split between exons 22 and 23 into a N-terminal coding part having the nucleotides 1-2676 of SEQ ID NO:15 and a C-terminal coding part having the nucleotides 2677-5991 of SEQ ID NO:15.

Also, the human OTOF gene isoform 5 of SEQ ID NO:15 can be split between exons 24 and 25 into a N-terminal coding part having the nucleotides 1-2991 of SEQ ID NO:15 and a C-terminal coding part having the nucleotides 2992-5991 of SEQ ID NO:15.

Finally, the human OTOF gene isoform 5 of SEQ ID NO:15 can be split between exons 25 and 26 into a N-terminal coding part having the nucleotides 1-3126 of SEQ ID NO:15 and a C-terminal coding part having the nucleotides 3127-5991 of SEQ ID NO:15.

The same cutting sites can be used for splitting the new isoforms of SEQ ID NO:16-18 into two parts, thereby allowing an easy encapsulation in AAV8 capsids and favouring an easy recombination *in situ*.

In the vector system of the invention, the N-terminal coding part of the Otoferlin gene contained in one of the two polynucleotides preferably consists in: the nucleotides 1-2214, the nucleotides 1-2406, the nucleotides 1-2523, the nucleotides 1-2676, the nucleotides 1-2991, or the nucleotides 1-3126 of the Otoferlin gene of SEQ ID NO:15. And the C-terminal coding part of the Otoferlin gene contained in the other polynucleotide consequently preferably consists in: the nucleotides 2215-5991, the nucleotides 2407-5991, the nucleotides 2524-5991, the nucleotides 2677-5991, the nucleotides 2992-5991, or the nucleotides 3127-5991 of the Otoferlin gene of SEQ ID NO:15.

Exemplary polynucleotides that can be used as first and second polynucleotide in the vector system of the invention are for example SEQ ID NO: 47&48 or 47&49, 50&51 or 50&52, 53&54 or 53&55, 56&57 or 56&58, 59&60 or 59&61, and 62&63 or 62&64, each SEQ ID NO: of said pair containing the CMV promoter of SEQ ID NO:9, and the sequence encoding respectively the N-terminal & the C-terminal part of the isoform 5 of the Otoferlin human protein mentioned above, in a hybrid AP vector. SEQ ID NO:48, 51, 54, 57, 60 and 63 contain the WPRE sequence of SEQ ID NO:23, whereas SEQ ID NO:49, 52, 55, 58, 61 and 64 don't.

It is also possible to use as first polynucleotide of the vector system of the invention the nucleotides of SEQ ID NO:70-75, containing the CMV promoter of SEQ ID NO:9, the intronic sequence SEQ ID NO:10, and respectively the nucleotides 1-2214, the nucleotides 1-2406, the nucleotides 1-2523, the nucleotides 1-2676, the nucleotides 1-2991, or the nucleotides 1-3126 of SEQ ID NO:15, thereby encoding N-terminal parts of the isoform 5 of the human Otoferlin (SEQ ID NO:15).

It is also possible to use as first polynucleotide of the vector system of the invention a polynucleotide whose sequence is SEQ ID NO:73, containing the CMV promoter of SEQ ID NO:9, the intronic sequence of SEQ ID NO:10, and the N-terminal part of the isoform 5 of the Otoferlin human protein, more precisely the nucleotides 1-2676 of SEQ ID NO:15.

It is also possible to use as first polynucleotide of the vector system of the invention a polynucleotide containing the AAV cassette of SEQ ID NO:90, containing the CMV promoter of SEQ ID NO:9, the

intronic sequence of SEQ ID NO:10, and the N-terminal part of the isoform 5 of the Otoferlin human protein, more precisely the nucleotides 1-2676 of SEQ ID NO:15.

Other exemplary polynucleotides that can be used as first and second polynucleotide in the hybrid AP vector system of the invention are for example SEQ ID NO: 79&80, SEQ ID NO:79 containing the CMV promoter of SEQ ID NO:9, and the sequence encoding the N-terminal part of the isoform 1 of the Otoferlin murine protein, in a hybrid AP vector. SEQ ID NO: 80 contains the sequence encoding the C-terminal part of the isoform 1 of the Otoferlin murine protein, without the WPRE sequence.

Other components of the vectors of the invention

As explained in WO 2013/075008, the first and second polynucleotides used in this particular embodiment should contain specific genetic components (inverted terminal repeats, polyadenylation sequence, recombinogenic region, etc) in order to induce the appropriate recombination and expression of the Otoferlin protein in the target cells.

More specifically, these genetic components are the following:

- ITRs

The vector system of the invention can contain wild-type ITR sequences or engineered ITR sequences. The skilled person well knows which ITR can be used advantageously in a dual approach AAV system.

If AAV8 vector are used, the ITR sequences of a polynucleotide described herein can be derived from any AAV serotype (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10) or can be derived from more than one serotype. In some embodiments of the polynucleotide provided herein, the ITR sequences are derived from AAV8. ITR sequences and plasmids containing ITR sequences are known in the art and commercially available.

An exemplary AAV8 ITR sequence for flanking the 5' end of an expression construct comprises the sequence SEQ ID NO:19. An exemplary AAV8 ITR sequence for flanking the 3' end of an expression construct comprises the sequence SEQ ID NO:20.

It is also possible to use any homologous sequence having a sequence identity of at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% with SEQ ID NO:19 and/or SEQ ID NO:20.

- A recombinogenic region

The two polynucleotides of the invention (first and second polynucleotides) also comprise a so-called "recombinogenic region" which can promote recombination, including homologous recombination, between the two polynucleotides once delivered to a cell, so as to produce the entire coding sequence of the OTOF polypeptide and expression thereof in the transfected inner hair cells (see, e.g., Ghosh et al. Hum Gene Ther. 2011 Jan;22(l):77-83).

This recombinogenic region may typically consists in a first region of the first polynucleotide that has an homologous region in the second polynucleotide, or vice versa. The two regions preferably have a threshold level of sequence identity with each other of at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% identity, as defined above.

This recombinogenic region has preferably a size comprised between 50 and 500, 50 and 400, 50 and 300, 100 and 500, 100 and 400, 100 and 300, 200 and 500, 200 and 400, or 200 and 300 nucleotides.

In a preferred embodiment, the two regions are identical and have a size comprised between 200 and 300 nucleotides.

These recombinogenic sequences can also have sequences which are sufficiently homologous so as to permit hybridization with each other under standard stringent conditions and standard methods.

As used herein, "stringent" conditions for hybridization refers to conditions wherein hybridization is typically carried out overnight at 20-25°C below the melting temperature (T_m) of the DNA hybrid in 6x SSPE, 5x Denhardt's solution, 0.1% SDS, 0.1 mg/ml denatured DNA. The melting temperature is described by the following formula: Tm=81.5 C+16.6 Log[Na+]+0.41 (%G+C)-0.61 (% formamide)-600/length of duplex in base pairs. Washes are typically earned out as follows: (1) Twice at room temperature for 15 minutes in 1x SSPE, 0.1 % SDS (low stringency wash). 2) Once at Tm-20°C for 15 minutes in 0.2x SSPE, 0.1% SDS (moderate stringency wash).

In a preferred embodiment, the recombinogenic sequences, especially overlapping recombinogenic sequences, present in the two polynucleotides of the vector system of the invention are an exogenous fragment or a fragment of Otoferlin. Said recombinogenic sequence can be a fragment of an exogenous gene, coding or non-coding, or the ITRs present in the polynucleotide. In particular, it can be the sequence SEQ ID NO:69 (derived from the gene of the alkaline phosphatase AP), or an homologous sequence thereof, preferably having a sequence identity of at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% with SEQ ID NO:69.

Strategies to convey the fragments of OTOF

Several strategies are herein proposed by the inventors to convey the two parts of the Otoferlin polynucleotide and have it properly recombined in the target cell:

- A "trans-splicing strategy", in which a splice donor (SD) signal is placed at the 3' end of the 5'-half vector and a splice acceptor (SA) signal is placed at the 5'end of the 3'-half vector. Upon co-infection of the same cell by the dual AAV vectors, the inverted terminal repeat (ITR)-mediated head-to-tail concatemerization of the two halves induces trans-splicing of the two polynucleotide and results in the production of a mature mRNA and full-size protein of interest (Duan D. et al, Molecular Therapy 2001, vol. 4, N°4, pp. 383-391).

- An "overlapping strategy", where the recombinogenic sequence is part of the Otoferlin cDNA itself. Indeed, in this case, the two halves of a large transgene expression cassette contained in the dual AAV vectors include a homologous overlapping sequence (at the 3' end of the 5'-half vector and at the 5' end of the 3'-half vector, see bottom of figure 4), which will mediate reconstitution of a single large gene by homologous recombination (cf. WO 2013/075008). No splice site is required in this case (yet it is possible to use them in order to facilitate the recombination process).

- The "hybrid strategy", in which a highly recombinogenic sequence is added, potentially from an exogenous gene (for example alkaline phosphatase, AP) to the trans-splicing vector disclosed above. This second recombinogenic sequence is for example placed downstream of the SD signal in the 5'-half vector and upstream of the SA signal in the 3'-half vector in order to increase recombination between the dual AAVs (cf. Ghosh et al, Hum Gene Ther. 2011. 22:77–83).

In this latter strategy, the two exogenous recombinogenic sequences are preferably identical and have more preferably the sequence SEQ ID NO:69 (derived from the gene of the alkaline phosphatase AP), or an homologous sequence thereof, as defined above.

In the trans-splicing and hybrid strategy, the polynucleotides included in the dual-vector system of the invention comprise a splice donor or a splice acceptor site so that, once recombined *in vivo*, the exogenous recombinogenic region can be spliced out. In a preferred embodiment, the splice donor and/or splice acceptor sites contain splice consensus sequences. In a more preferred embodiment, the splice donor and/or splice acceptor sites carried by the polynucleotides included in the vector system of the invention contain splice consensus sequences derived from the alkaline phosphatase enzyme (see SEQ ID NO:21 and SEQ ID NO:22).

In a preferred embodiment, the polynucleotides included in the dual-vector system of the invention comprise SEQ ID NO:21 and/or SEQ ID NO:22 as splice donor and acceptor site respectively, or splice sites comprising a sequence having a sequence identity of at least 75%, at least 80%, at least 85%, at

least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% with SEQ ID NO:21 and/or SEQ ID NO:22.

The polynucleotides of the invention may contain several recombinogenic sequences, i.e., for example, splice donor / acceptor sites, and an exogenous recombinogenic sequence. The presence of two recombinogenic regions is indeed preferable in order to ensure a precise and accurate recombination, *in vivo*, with no remaining unwanted nucleotides left. Any appropriate combination or use of other recombinogenic sequences than those disclosed above can be contemplated, as soon as it enables efficient recombination in the target cells, such as inner hair cells.

The polynucleotide sequences present in the vector system of the invention may contain other regulatory components that are functional in the inner hair cells in which the vector is to be expressed. A person of ordinary skill in the art can select regulatory elements for use in human inner hair cells. Regulatory elements include, for example, internal ribosome entry site (IRES), transcription termination sequences, translation termination sequences, enhancers, and polyadenylation elements.

The polynucleotide sequences present in the vector system of the invention may for example contain the WHV Posttranscriptional Regulatory Element (WPRE) that can stabilize the mRNA and enhance the protein yield. Said WPRE sequence can be of SEQ ID NO:23. It can also contain a Kozak consensus sequence, e.g., the sequence of GCCGCCACCAUGG (SEQ ID NO:89), as disclosed in the exemplary sequences herein proposed (SEQ ID NO: 47, 50, 53, 56, 59, 62, SEQ ID NO:70-75, SEQ ID NO:81, 82, 85 and SEQ ID NO:86). Alternatively, it is possible to use the 5'-(gcc)gccRccAUGG-3' sequence (SEQ ID NO:92), wherein the upper-case letters indicate highly conserved bases and R indicates that a purine (adenine or guanine) is always observed at this position (with adenine being more frequent according to Kozak), and a lower-case letter denotes the most common base at a position where the base can nevertheless vary. Note that the sequence in parentheses (gcc) is of uncertain significance.

The polynucleotide containing the C terminal sequence of the Otoferlin gene contains preferably a DNA sequence which directs polyadenylation of the mRNA encoded by the structural gene. This

polyadenylation DNA sequence can also be included in a vector of the invention. For example, the polyA of the bovine growth hormone (SEQ ID NO:24) can be used with this respect.

Transcription termination regions can typically be obtained from the 3' untranslated region of a eukaryotic or viral gene sequence. Transcription termination sequences can be positioned downstream of a coding sequence to provide for efficient termination. Signal peptide sequence is an amino terminal sequence that encodes information responsible for the relocation of an operably linked polypeptide to a wide range of post-translational cellular destinations, ranging from a specific organelle compartment to sites of protein action and the extracellular environment. Enhancers are cis-acting elements that increase gene transcription and can also be included in a vector of the invention. Enhancer elements are known in the art, and include, but are not limited to, the CaMV 35S enhancer element, cytomegalovirus (CMV) early promoter enhancer element, and the SV40 enhancer element.

Particular vector systems

Particular vector systems of the invention are now described in more details.

In a preferred embodiment of the invention, the vector system of the invention is a trans-splicing vector system comprising at least two different recombinant AAV8 particles, namely:

a) one AAV8 particle comprising:

a first polynucleotide comprising an inverted terminal repeat at each end of said polynucleotide, and, between the said inverted terminal repeats, from 5’ to 3’: the CMV promoter, optionally followed by a kozak sequence of SEQ ID NO:89 or 92, then followed by a partial coding sequence that contains the N-terminal coding part of the Otoferlin gene, and a splice donor site as recombinogenic sequence, and

b) one AAV8 particle comprising a second polynucleotide comprising an inverted terminal repeat at each end of said polynucleotide, and, between the said inverted terminal repeats, from 5’ to 3’: a splice acceptor site as recombinogenic sequence, a partial coding sequence that contains the C-terminal

coding part of the Otoferlin gene, optionally followed by a polyadenylation sequence (for example the polyA of the bovine growth hormone of SEQ ID NO:24).

The CMV promoter, which is preferably of SEQ ID NO:9, is optionally followed by the intronic sequence of SEQ ID NO:10 and/or by a kozak sequence of SEQ ID NO:89 or 92. In this case, the partial coding sequence encoding otoferlin is located downstream of this additional intronic sequence.

The two different types of AAV8 particles can be contained within the same composition or within different compositions and may be administered together or separately.

This vector system can be used in the trans-splicing strategy described above. The enclosed listing provides preferred vectors (SEQ ID NO: 85 - SEQ ID NO: 88) that can be used accordingly. SEQ ID NO:85 and SEQ ID NO:86 encode the N-terminal part (until amino acid 892) of the Otoferlin human isoform 5, respectively under control of the CMV promoter or of the CMV followed by the intronic sequence, whereas SEQ ID NO:87 and SEQ ID NO:88 encode the C-terminal part (as of amino acid 893) of the Otoferlin human isoform 5, with and without the WPRE sequence respectively.

In another embodiment, the vector system of the invention is an overlapping vector system comprising at least two different AAV8 particles, namely:

a) one AAV8 particle comprising a first polynucleotide comprising an inverted terminal repeat at each end of said polynucleotide, and, between the said inverted terminal repeats, from 5' to 3': the CMV promoter, optionally followed by a kozak sequence of SEQ ID NO:89 or 92, then followed by a partial coding sequence that contains the N-terminal coding part of the Otoferlin gene, and

b) one AAV8 particle comprising a second polynucleotide comprising an inverted terminal repeat at each end of said polynucleotide, and, between the said inverted terminal repeats, from 5' to 3': a partial coding sequence that contains the C-terminal coding part of the Otoferlin gene, optionally followed by a polyadenylation sequence (for example the polyA of the bovine growth hormone of SEQ ID NO:24),

wherein the said N and C-terminal coding parts of the Otoferlin gene contain a homologous part which can serve as recombinogenic sequence.

The CMV promoter, which is preferably of SEQ ID NO:9, is optionally followed by the intronic sequence of SEQ ID NO:10 and/or by a kozak sequence of SEQ ID NO:89 or 92. In this case, the partial coding sequence encoding otoferlin is located downstream of this additional intronic sequence.

The two different types of AAV8 particles can be contained within the same composition or within different compositions and may be administered together or separately.

Such a vector system can be used in the overlapping strategy described above. The enclosed listing provides preferred overlapping vectors (SEQ ID NO:81 - SEQ ID NO:84). SEQ ID NO:81 and SEQ ID NO:82 encode the N-terminal part (until amino acid 892) of the Otoferlin human isoform 5, respectively under control of the CMV promoter or of the CMV followed by the intronic, whereas SEQ ID NO:83 and SEQ ID NO:84 encode the C-terminal part (as of amino acid 893) of the Otoferlin human isoform 5, with and without the WPRE sequence respectively.

In another preferred embodiment, the vector system of the invention is an hybrid vector system comprising at least two different recombinant AAV8 particles, namely:

a) one AAV8 particle comprising a first polynucleotide comprising an inverted terminal repeat at each end of said polynucleotide, and, between the said inverted terminal repeats, from 5' to 3': the CMV promoter followed by a partial coding sequence that contains the N-terminal coding part of the Otoferlin gene, and a splice donor site, and

b) one AAV8 particle comprising a second polynucleotide comprising an inverted terminal repeat at each end of said polynucleotide, and, between the said inverted terminal repeats, from 5' to 3': a splice acceptor site, a partial coding sequence that contains the C-terminal coding part of the Otoferlin gene, optionally followed by a polyadenylation sequence (for example the polyA of the bovine growth hormone of SEQ ID NO:24),

wherein the said first and second polynucleotides also contain a second recombinogenic sequence that is located after the splice donor site in said first polynucleotide and before the splice acceptor site in said second polynucleotide.

The CMV promoter, which is preferably of SEQ ID NO:9, is optionally followed by the intronic sequence of SEQ ID NO:10 and/or by a kozak sequence of SEQ ID NO:89 or 92. In this case, the partial coding sequence encoding otoferlin is located downstream of this additional intronic sequence.

The two different types of AAV8 particles can be contained within the same composition or within different compositions and may be administered together or separately.

Such a vector system can be used in the hybrid strategy described above. The enclosed listing provides several exemplary vectors (SEQ ID NO: 47&48 or 47&49, 50&51 or 50&52, 53&54 or 53&55, 56&57 or 56&58, 59&60 or 59&61, and 62&63 or 62&64) corresponding to a cutting site between exons 18-19, 20-21, 21-22, 22-23, 24-25 and 26-27 respectively (the C-terminal vectors containing or not a WPRE sequence) that can be used with this respect. The C-terminal vector of SEQ ID NO:97 can be advantageously used. Also, the N-terminal vectors of SEQ ID NO:70-75 and 90, in which the CMV promoter is followed by the intronic sequence, can be advantageously used.

In any of these vector systems, the first polynucleotide can contain the nucleotides 1-2214, the nucleotides 1-2406, the nucleotides 1-2523, the nucleotides 1-2676, the nucleotides 1-2991, or the nucleotides 1-3126 of the Otoferlin gene of SEQ ID NO:15. And, consequently, the second polynucleotide can contain the nucleotides 2215-5991, the nucleotides 2407-5991, the nucleotides 2524-5991, the nucleotides 2677-5991, the nucleotides 2992-5991, or the nucleotides 3127-5991 of the Otoferlin gene of SEQ ID NO:15.

It is also possible to split the Otoferlin gene into two parts according to any equivalent cutting site corresponding to a natural exon junction in the considered gene.

A particularly preferred vector system of the invention contains:

a) a first polynucleotide comprising an inverted terminal repeat at each end of said polynucleotide, and, between the said inverted terminal repeats, from 5' to 3': the CMV promoter of the invention, optionally a intronic sequence (typically SEQ ID NO:10), optionally a kozak sequence of SEQ ID NO:89 or 92, followed by the nucleotides 1-2214, the nucleotides 1-2406, the nucleotides 1-2523, the nucleotides 1-2676, the nucleotides 1-2991 or the nucleotides 1-3126 of the Otoferlin gene of SEQ ID NO:15, and a splice donor site, and

b) a second polynucleotide comprising an inverted terminal repeat at each end of said polynucleotide, and, between the said inverted terminal repeats, from 5' to 3': a splice acceptor site, the nucleotides 2215-5991, the nucleotides 2407-5991, the nucleotides 2524-5991, the nucleotides 2677-5991, the nucleotides 2992-5991 or the nucleotides 3127-5991 of the Otoferlin gene of SEQ ID NO:15, optionally followed by a polyadenylation sequence (for example the polyA of the bovine growth hormone of SEQ ID NO:24),

each polynucleotide comprising as second recombinogenic sequence of SEQ ID NO:69 derived from the gene encoding alkaline phosphatase.

Preferably, the first polynucleotide in said vector system is chosen among: SEQ ID NO:47, 50, 53, 56, 59, and 62 (without intronic sequence) or among SEQ ID NO:70, 71, 72, 73, 74, and 75 (with a intronic sequence), said polynucleotides containing respectively the nucleotides 1-2214, the nucleotides 1-2406, the nucleotides 1-2523, the nucleotides 1-2676, the nucleotides 1-2991, or the nucleotides 1-3126 of SEQ ID NO:15, thereby encoding N-terminal parts of the isoform 5 of the human Otoferlin.

Preferably, the second polynucleotide in said vector system is chosen among: SEQ ID NO:48, 51, 54, 57, 60 and 63 (with the enhancer WPRE) or among SEQ ID NO:49, 52, 55, 58, 61 and 64 (without the enhancer WPRE), said polynucleotides containing respectively the nucleotides 2215-5991, the nucleotides 2407-5991, the nucleotides 2524-5991, the nucleotides 2677-5991, the nucleotides 2992-5991, or the nucleotides 3127-5991 of SEQ ID NO:15, thereby encoding C-terminal parts of the isoform 5 of the human Otoferlin.

The construction of these polynucleotides is detailed in the example 1 below.

A particularly preferred vector system is a vector system comprising:

a) one AAV8 particle comprising a first polynucleotide comprising an inverted terminal repeat at each end of said polynucleotide, and, between the said inverted terminal repeats, from 5' to 3': the CMV promoter of SEQ ID NO:9 and optionally a intronic sequence of SEQ ID NO:10 and/or a kozak sequence of SEQ ID NO:89 or 92, followed by the nucleotides 1-2214, the nucleotides 1-2676, or the nucleotides 1-2991 of the Otoferlin gene of SEQ ID NO:15, more preferably by the nucleotides 1-2676, and a splice donor site, and

b) one AAV8 particle comprising a second polynucleotide comprising an inverted terminal repeat at each end of said polynucleotide, and, between the said inverted terminal repeats, from 5' to 3': a splice acceptor site, the nucleotides 2215-5991, the nucleotides 2677-5991, or the nucleotides 2992-5991 of the Otoferlin gene of SEQ ID NO:15 (more preferably the nucleotides 2677-5991), optionally followed by a polyadenylation sequence (for example the polyA of the bovine growth hormone of SEQ ID NO:24),

wherein the said first and second polynucleotides also contain the AP recombinogenic sequence of SEQ ID NO:69 that is located after the splice donor site in said first polynucleotide and before the splice acceptor site in said second polynucleotide, and

wherein said second polynucleotide do not contain the WPRE sequence of SEQ ID NO:23.

Another particularly preferred vector system is a vector system comprising:

a) one AAV8 particle comprising a first polynucleotide comprising an inverted terminal repeat at each end of said polynucleotide, and, between the said inverted terminal repeats, from 5' to 3': the CMV promoter of SEQ ID NO:9 and optionally a intronic sequence of SEQ ID NO:10, and optionally a Kozak

sequence of SEQ ID NO : 89 or 92, followed by the nucleotides 1-2214, the nucleotides 1-2676, or the nucleotides 1-2991 of the Otoferlin gene of SEQ ID NO:15, more preferably by the nucleotides 1-2676, and a splice donor site, and

b) one AAV8 particle comprising a second polynucleotide comprising an inverted terminal repeat at each end of said polynucleotide, and, between the said inverted terminal repeats, from 5' to 3': a splice acceptor site, the nucleotides 2215-5991, the nucleotides 2677-5991, or the nucleotides 2992-5991 of the Otoferlin gene of SEQ ID NO:15 (more preferably the nucleotides 2677-5991), optionally followed by a polyadenylation sequence (for example the polyA of the bovine growth hormone of SEQ ID NO:24),

wherein the said first and second polynucleotides also contain the AP recombinogenic sequence of SEQ ID NO:69 that is located after the splice donor site in said first polynucleotide and before the splice acceptor site in said second polynucleotide, and

wherein said second polynucleotide do not contain the WPRE sequence of SEQ ID NO:23.

A particularly preferred vector system according to the invention contains SEQ ID NO: 56 (without intronic sequence) encoding the N-terminal part of the isoform 5 of the human Otoferlin, and SEQ ID NO:58 (without the enhancer WPRE) encoding the C-terminal part of the isoform 5 of the human Otoferlin.

Pharmaceutical compositions of the invention

In another aspect, the present invention targets a pharmaceutical composition comprising the vector system of the invention, as described above (i.e., the virions containing above-described polynucleotides), and a pharmaceutically acceptable carrier.

The present invention also targets pharmaceutical compositions comprising only one population of the viruses of the invention, said viruses containing either the "first" or the "second" polynucleotides which have been described thoroughly above.

Said pharmaceutical compositions typically contain any of the trans-splicing, hybrid or overlapping vectors disclosed above.

In particular, and as an example, the composition of the invention contains the particles comprising the hybrid vector of the invention, namely:

- either AAV8 particles comprising a polynucleotide comprising an inverted terminal repeat at each end of said polynucleotide, and, between the said inverted terminal repeats, from 5' to 3': the CMV promoter, optionally a kozak sequence, followed by a partial coding sequence that contains the N-terminal coding part of the Otoferlin gene, and a splice donor site as recombinogenic sequence and a pharmaceutically acceptable carrier;
- or AAV8 particles comprising a polynucleotide comprising an inverted terminal repeat at each end of said polynucleotide, and, between the said inverted terminal repeats, from 5' to 3': a splice acceptor site as recombinogenic sequence, a partial coding sequence that contains the C-terminal coding part of the Otoferlin gene, optionally followed by a polyadenylation sequence (for example the polyA of the bovine growth hormone of SEQ ID NO:24) and a pharmaceutically acceptable carrier.

In another embodiment, said composition comprises:

a) one AAV8 particle comprising a first polynucleotide comprising an inverted terminal repeat at each end of said polynucleotide, and, between the said inverted terminal repeats, from 5' to 3': the CMV promoter, optionally a kozak sequence, followed by a partial coding sequence that contains the N-terminal coding part of the Otoferlin gene, and a splice donor site, and

b) one AAV8 particle comprising a second polynucleotide comprising an inverted terminal repeat at each end of said polynucleotide, and, between the said inverted terminal repeats, from 5' to 3': a splice acceptor site, a partial coding sequence that contains the C-terminal coding part of the Otoferlin gene, optionally followed by a polyadenylation sequence (for example the polyA of the bovine growth hormone of SEQ ID NO:24),

wherein the said first and second polynucleotides also contain a second recombinogenic sequence that is located after the splice donor site in said first polynucleotide and before the splice acceptor site in said second polynucleotide.

In a preferred embodiment, the present invention also targets a pharmaceutical composition comprising, apart from the pharmaceutically acceptable carrier, AAV8 particles comprising polynucleotides comprising an inverted terminal repeat at each end of said polynucleotides, and, between the said inverted terminal repeats, from 5' to 3': the CMV promoter followed by a partial coding sequence that contains the N-terminal coding part of the Otoferlin gene, and a splice donor site, wherein said polynucleotide also contain a second recombinogenic sequence that is located after the splice donor site in said polynucleotides.

In another preferred embodiment, the present invention also targets a pharmaceutical composition comprising, apart from the pharmaceutically acceptable carrier, AAV8 particles comprising polynucleotides comprising an inverted terminal repeat at each end of said polynucleotides, and, between the said inverted terminal repeats, from 5' to 3': a splice acceptor site, a partial coding sequence that contains the C-terminal coding part of the Otoferlin gene, optionally followed by a polyadenylation sequence (for example the polyA of the bovine growth hormone of SEQ ID NO:24), wherein the polynucleotides also contain a second recombinogenic sequence that is located before the splice acceptor site in said polynucleotides.

All the constitutive parts of such polynucleotides (promoter sequences, enhancer of the translation, recombinogenic sequences, partial coding parts, etc) have been detailed above and do not need to be repeated here. All the embodiments disclosed for the vector systems of the invention (trans-splicing, hybrid and overlapping) apply here, *mutatis mutandis.*

As used herein, "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like that are physiologically compatible. Examples of pharmaceutically acceptable carriers include one or more of water, saline, phosphate buffered saline, dextrose, glycerol, ethanol and the like, as well as combinations thereof. In many cases, it can be preferable to include isotonic agents, for example, sugars, polyalcohol such as mannitol, sorbitol, or sodium chloride in the composition. Pharmaceutically acceptable carriers can further comprise minor amounts of auxiliary substances such as wetting or emulsifying agents, preservatives or buffers, which enhance the shelf life or effectiveness of the the vector system or of the pharmaceutical compositions containing same.

The pharmaceutical compositions of the invention may be in a variety of forms. These include, for example, liquid, semi-solid and solid dosage forms, such as liquid solutions (e.g., injectable and infusible solutions), dispersions or suspensions, tablets, pills, powders, liposomes and suppositories. The form used depends on the intended mode of administration and therapeutic application. Typical compositions are in the form of injectable or infusible solutions.

Pharmaceutical compositions typically must be sterile and stable under the conditions of manufacture and storage. The pharmaceutical composition of the invention is preferably formulated as a solution, microemulsion, dispersion, liposome, or other ordered structure suitable to high drug concentration. Sterile injectable solutions can be prepared by incorporating the vectors of the invention in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the vectors or viruses of the invention into a sterile vehicle that contains a basic

dispersion medium and the required other ingredients from those enumerated above. In the case of sterile lyophilized powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and spray-drying that yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof. The proper fluidity of a solution can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prolonged absorption of injectable compositions can be achieved by including an agent in the composition that delays absorption, for example, monostearate salts and gelatin.

The pharmaceutical compositions of the invention typically include a "therapeutically effective amount" or a "prophylactically effective amount" of the vectors or viruses of the invention. A "therapeutically effective amount" refers to the amount of the vectors or viruses of the invention that is effective, at dosages and for periods of time necessary, to achieve the desired therapeutic result, in this case for both prophylaxis and treatment of hearing loss without unacceptable toxicity or undesirable side effects.

In a particular embodiment, the pharmaceutical composition of the invention contains a therapeutically effective amount of viruses containing SEQ ID NO: 56 encoding the N-terminal part of the isoform 5 of the human Otoferlin (without intronic sequence) and SEQ ID NO:58 encoding the C-terminal part of the isoform 5 of the human Otoferlin (without the enhancer WPRE).

In a particular embodiment, the pharmaceutical composition of the invention contains a therapeutically effective amount of viruses containing SEQ ID NO: 56 encoding the N-terminal part of the isoform 5 of the human Otoferlin (without intronic sequence) and SEQ ID NO:57 encoding the C-terminal part of the isoform 5 of the human Otoferlin (with the enhancer WPRE).

In a particular embodiment, the pharmaceutical composition of the invention contains a therapeutically effective amount of viruses containing SEQ ID NO: 75 encoding the N-terminal part of

the isoform 5 of the human Otoferlin (with intronic sequence) and SEQ ID NO:57 encoding the C-terminal part of the isoform 5 of the human Otoferlin (with the enhancer WPRE).

In a particular embodiment, the pharmaceutical composition of the invention contains a therapeutically effective amount of viruses containing SEQ ID NO: 75 encoding the N-terminal part of the isoform 5 of the human Otoferlin (with intronic sequence) and SEQ ID NO:58 encoding the C-terminal part of the isoform 5 of the human Otoferlin (without the enhancer WPRE).

In a particularly preferred embodiment, the pharmaceutical composition of the invention contains a therapeutically effective amount of viruses containing :

- AAV8 particles comprising a polynucleotide comprising an inverted terminal repeat at each end of said polynucleotide, and, between the said inverted terminal repeats, from 5' to 3': the CMV promoter followed by the intronic sequence of SEQ ID NO:10, the kozak sequence of SEQ ID NO:89, the N-terminal coding part of the human Otoferlin gene (isoform 5, exons 1-22), a splice donor site and the recombinogenic sequence of SEQ ID NO:69, said polypeptide having for example the sequence displayed in SEQ ID NO:73 or SEQ ID NO:90, together with a pharmaceutically acceptable carrier;

  And/or

- AAV8 particles comprising a polynucleotide comprising an inverted terminal repeat at each end of said polynucleotide, and, between the said inverted terminal repeats, from 5' to 3': the recombinogenic sequence of SEQ ID NO:69, a splice acceptor site, the C-terminal coding part of the human Otoferlin gene (isoform 5, as of exon 23), followed by a polyadenylation sequence (for example the polyA of the bovine growth hormone of SEQ ID NO:24), said polypeptide having for example the sequence displayed in SEQ ID NO:97, together with a pharmaceutically acceptable carrier.

In a most particularly preferred embodiment, the pharmaceutical composition of the invention contains a therapeutically effective amount of viruses containing :

- AAV8 particles comprising a polynucleotide whose sequence is displayed in SEQ ID NO:90, together with a pharmaceutically acceptable carrier;

  And/or

- AAV8 particles comprising a polynucleotide whose sequence is displayed in SEQ ID NO:97, together with a pharmaceutically acceptable carrier.

A therapeutically effective amount of the vectors or viruses of the invention can vary according to factors such as the disease state, age, sex, and weight of the subject, and the ability of said compound to elicit a desired response in same. A therapeutically effective amount can also be one in which any toxic or detrimental effects of the claimed compounds are outweighed by the therapeutically beneficial effects. A "prophylactically effective amount" refers to an amount of the viruses or vectors of the invention that is effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result. Typically, since a prophylactic dose can be used in subjects prior to or at an earlier stage of disease, the prophylactically effective amount is usually less than the therapeutically effective amount.

Dosage regimens can be adjusted to provide the optimum desired response (e.g., a therapeutic or prophylactic response). For example, a single bolus can be administered, several divided doses can be administered over time or the dose can be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of the vector or virus of the invention calculated to produce the desired therapeutic or prophylactic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms can be dictated by and directly dependent on (a) the unique characteristics of the vector or virus and the particular therapeutic or prophylactic effect to be achieved, and (b) the limitations inherent in the art of formulating such vector or virus for treating or preventing hearing loss in a subject.

In some embodiments, where first and second AAV polynucleotides / particles are to be used, the first and second AAV polynucleotides / particles may be contained within the same composition or within different compositions and may be administered together or separately.

In some embodiments, the composition of the invention contains from 10^6 to 10^{14} particles/mL or from 10^{10} to 10^{15} particles/mL, or any values there between for either range, such as for example, about 10^6, 10^7, 10^8, 10^9, 10^{10}, 10^{11}, 10^{12}, 10^{13}, or 10^{14} particles/mL. In one embodiment, the composition of the invention contains more than 10^{13} of AAV particles/mL.

In some embodiments, when a first AAV particle comprising a first polynucleotide and a second AAV particle comprising a second polynucleotide are administered, the amount administered is the same for both particles.

Therapeutical uses and methods of treatment

In another aspect, the present invention also relates to the viruses or the vector system of the invention or the pharmaceutical compositions as defined above, for use for treating patients suffering from DFNB9 deafness or preventing DFNB9 deafness in patients having DFNB9 mutations.

The invention also relates to a treating or preventing method involving the administration of the viruses or the vector system of the invention, or pharmaceutical compositions containing same, to patients suffering from DFNB9 deafness or patients having DFNB9 mutations, respectively.

More generally, these viruses or the vector system of the invention or the pharmaceutical compositions can be administered to human subjects suffering from congenital hearing loss due to altered DFNB59 gene expression or deficiency. Said deficiency can be observed for example when Otoferlin is expressed at normal levels but is not functional.

In other words, the present invention relates to the use of the viruses or vector system of the invention, as described above, for manufacturing pharmaceutical compositions intended to prevent and / or treat patients suffering from disorders linked to altered DFNB9 gene expression or deficiency.

As used herein, the term "treating" is intended to mean the administration of a therapeutically effective amount of the viruses or vector system of the invention to a patient suffering from DFNB9 deafness, in order to restore partially or completely the hearing in said patient. Said recovery can be assessed by testing the auditory brain stem responses (ABRs) with electrophysiologic devices. “Treatment of the DFNB9 deafness” is in particular intended to designate the complete restoration of hearing function regardless of the cellular mechanisms involved.

For patients carrying thermo-sensitive mutations, the viruses or the vector system or the composition of the invention can also be administered to prevent the loss of hearing induced from the body temperature modulation. In the context of the present invention, the term "preventing" designates impairing or delaying the loss of hearing within audible frequency range.

In these and other DFNB9 patients, the viruses or the vector system or the composition of the invention can be administered both for preventing the loss of hearing before it occurs, and for restoring at least partially the hearing capacity when hearing loss has already occurred.

In this aspect of the invention, the viruses or the vector system or the composition of the invention are administered to patients suffering from DFNB9 deafness. By “patients suffering from DFNB9 deafness”, it is herein meant a patient, especially a human patient, that is thought to have (or has been diagnosed to have) a mutation in the constitutive Otoferlin gene, said mutation triggering an abnormal expression, function or both, of the Otoferlin protein. In a particular embodiment, said mutation can be thermo-sensitive.

To date, more than 75 pathogenic mutations have been reported in otoferlin. Among these are at least 7 thermosensitive mutations, identified in patients affected by episodic deafness conditioned by fever

(PQ994VfsX6, P.I515T, p.G541S, PR1607W, pE1804del, c.2975_2978delAG/c.4819C>T, c.4819C>T (c.R1607W)).

These patients can be identified by the skilled physician, e.g., using a combination of electrophysiologic testing of auditory brain stem responses (ABRs) and/or genetic testing to identify mutations in the OTOF gene. In some embodiments, the patient has one or more of the following nonsense or missense mutations in the OTOF gene: TYR730TER, GLN829TER, PR01825ALA, PRO50ARG, LEU1011PRO, ILE515THR, ARG1939GLN, or GLY541SER. In some embodiments, the patient has an A-to-G transition at the intron 8/exon 9 junction (IVS8-2A-G) or a G-to-A transition at position +1, the first intronic nucleotide in the splice donor site of exon 5 or a G-C transversion in the donor splice site of intron 39. In some embodiments, the patient has a one base pair deletion (1778G) in exon 16, leading to a stop codon, and a 6141G-A change, resulting in an ARG-to-GLN substitution in exon 48.

The time of administration of the viruses, or virus system or composition of the invention will be within the purview of the skilled artisan having benefit of the present teachings. It is possible to administer the composition of the invention up to 12 years of age and over, but it can be also carried out as soon as the disease or the mutation is detected, e.g., in utero in embryos or foetuses, or soon after birth, for example before three months after birth, preferably before one month after birth.

The patients to which the viruses or the vector system or the composition of the invention is administered are preferably patients, especially human patients, in which the auditory system, especially the cochlea, is already developed and mature. In this case, these patients, especially human patients, would therefore not be human embryos or foetuses. Accordingly, the patients targeted by the present invention would be preferably new born human babies, typically younger than 6 months old, or even younger than 3 months old, if DFNB9 deafness is diagnosed that young. These human babies would be more preferably between 3 months and 1 year.

Of note the human cochlea as a whole attains an adult size between 17 and 19 weeks' gestation and is fully morphologically mature at 30–36 weeks (corresponding to 12 days after birth in the mouse).

The functional maturation of the inner hair cell ribbon synapse can be evaluated by monitoring the wave I of the ABR recording, that can be recorded at about the 28th week of gestation in humans. Recordings and analyses of the ABR wave I (reflecting the function of the inner hair cell synapses with the primary auditory neurons) have shown a complete functional maturation in human babies at birth (corresponding to 20 days after birth in the mouse). This is well known in the art (see for example Pujol et Lavigne-Rebillard, Acta oto-laryngologica. Supplementum · February 1991).

The present inventors have shown previously that gene therapy with otoferlin is efficient even when achieved on mature auditory system (Akil el al 2019, PNAS; Hardelin et al., médecine / sciences 2019; 35:1213-25).

Accordingly, it is therefore also possible to administer the vector system of the invention to older human patients, such as toddlers (2-6 year old), infants (6-12 year old), teenagers (12-18 year old) or adult humans (18 years and over).

The patients of the invention can be in particular human infants diagnosed as being affected by DFNB9 deafness after language acquisition.

In another particular embodiment, the patients of the invention are human beings that are 6 years and older, i.e., the administration of the treatment occurs when their Central Nervous System is completely mature.

In particular embodiment, the viruses or the vector system or the composition of the invention are administered to human patients suffering from DFNB9 deafness induced by thermosensitive mutations, preferably chosen from: PQ994VfsX6, P.I515T, p.G541S, PR1607W, pE1804del, c.2975_2978delAG/c.4819C>T, c.4819C>T (c.R1607W) more preferably to teenagers or adult humans carrying at least one of the Otoferline thermosensitive mutations mentioned above.

In the context of the invention, the typical mode of administration of the pharmaceutical composition of the invention is intratympanic (in the middle ear), intracochlear, or parenteral (e.g., intravenous,

subcutaneous, intraperitoneal, intramuscular, intrathecal). In one example, the pharmaceutical composition of the invention is administered by intravenous infusion or injection. In another example, the pharmaceutical composition of the invention is delivered to a specific location using stereostatic delivery, particularly through the tympanic membrane or mastoid into the middle ear.

More precisely, the viruses, the vector system or the pharmaceutical composition of the invention can be administered by using a micro-catheter that will be carried out either through the oval window using laser stapedotomy (trans-stapes) or transmastoid / trans-round window (Dai C. et al, JARO, 18:601-617, 2017).

In a preferred embodiment of the invention, the viruses, the vector system or the pharmaceutical composition of the invention is administered in human ear via intra-cochlear administration, more precisely by targeting endolymphatic spaces in the vestibular system or by the semi-circular approach mentioned above.

Multiple routes of delivery to the inner ear have been explored. These include injection into the perilymphatic spaces via the round window membrane (RWM) and via the oval window and injection into the scala tympani or scala vestibule via cochleostomy. Distribution throughout the perilymphatic spaces has been demonstrated for all these routes of delivery. Furthermore, it has been demonstrated that advection flow through the cochlea and vestibular organs can facilitate distribution of therapeutic agents from the site of injection to more distant regions of the inner ear. Delivery into the endolymphatic spaces has also been explored via cochleostomy into the scala media, via canalostomy and by injection into the endolymphatic sac. These approaches have also yielded broad distribution but face the added challenge of breaching the barrier between high potassium endolymph and perilymph. Disruption of the barrier poses two potential problems. First, leakage of high potassium into the perilymphatic spaces that bathe the basolateral surface of hair cells and neurons can chronically depolarize these cells and lead to cell death. Second, breach of the tight junctions between endolymph and perilymph can lead to rundown of the endocochlear potential which typically ranges

between +80 and +120 mV. Rundown of the endocochlear potential reduces the driving force for sensory transduction in hair cells and therefore leads to reduced cochlear sensitivity and elevated auditory thresholds. Avoiding these complications is particularly challenging in the adult cochlea. However, by targeting endolymphatic spaces in the vestibular system, which does not have an endolymphatic potential, but are continuous with cochlear endolymph spaces, these confounding issues may be minimized while still providing sufficient distribution within the cochlea (Ahmed et al, JARO 18:649-670 (2017)).

The cochlea is highly compartmentalized and separated from the rest of the body by the blood-cochlear barrier (BCB), which minimizes the therapeutic injection volume and leakage into the body's general circulation system, to protect cochlear immune privilege and reduce the chance of systemic adverse immune responses. As the hair cells and supporting cells in the cochlea normally do not divide, the cells in the cochlea remain stable, therefore making it possible to use nonintegrating viral vectors (e.g., AAV) for sustained transgene expression.

The semi-circular approach has been suggested as a promising injection route for future cochlear gene therapy in human trials since the posterior semi-circular canal also appears to be accessible in humans (Suzuki et al., Sci. Rep. 7:45524 (2017); Yoshimura et al., Sci. Rep. 8:2980 (2018)).

In a preferred embodiment of the invention, the viruses or the vector system or composition of the invention are administered in human ear via one of the two common and well-established techniques that are routinely used in clinical otologic surgical practice. More precisely, these approaches will be adopted to target the perilymphatic spaces. To this end, the injections using a micro-catheter will be carried out either through the oval window using laser stapedotomy (trans-stapes) or transmastoid / trans-round window (Dai C. et al, JARO, 18:601-617, 2017). Systemic administration by intravenous injections or infusions are also possible.

The skilled person would easily determine if it is required, prior to the administration of the viruses or the vector(s) or composition of the invention, to enhance the permeability of the round window membrane as proposed in WO 2011/075838, depending on the target cell.

New isoforms of the invention

In another aspect, the present invention relates to the three particular homologous proteins of the variant 5 identified by the present inventors (see the Example 2 below).

These three alternative OTOF isoforms have the amino acid sequences of SEQ ID NO:6, SEQ ID NO:7 or SEQ ID NO:8. They can be encoded by the cDNA sequences of SEQ ID NO:16, SEQ ID NO:17 and SEQ ID NO:18, respectively.

Each of them may have a potential to restore hearing in humans. They can therefore be used in gene therapy instead of the usual OTOF isoform proteins 1-5 disclosed in the art.

The present invention also relates to homologous polypeptides thereof, whose amino acid sequence shares at least 70%, at least 75%, and even more preferably at least 80%, at least 85%, or at least 90% identity and/or similarity with SEQ ID NO:6, SEQ ID NO:7 or SEQ ID NO:8. When the homologous polypeptide is much shorter than SEQ ID NO:6, SEQ ID NO:7 or SEQ ID NO:8, then local alignment can be considered.

The present invention also relates to any vector or vector system encoding SEQ ID NO:6, SEQ ID NO:7 or SEQ ID NO:8, or an homologous polypeptide thereof, as defined above.

In particular, it relates to any vector comprising the cDNA sequences of SEQ ID NO:16, SEQ ID NO:17 or SEQ ID NO:18.

These vectors are preferably useful for gene therapy. They include, yet are not limited to, DNA plasmid vectors as well as DNA and RNA viral vectors. In the present invention, such vectors may be used to

express the new isoforms of OTOF in cells of the auditory pathway such as cochlear hair cells. These vectors are well-known in the art. They are for example viral vectors such as lentiviruses, adenoviruses and Adeno-associated viruses (AAV).

FIGURE LEGENDS

Figure 1:

A & B. **Transduction profile of AAV8-CMV-GFP after cochlear delivery in a wild-type mouse at P12.** A. Low and high magnification micrographs of a P30 organ of Corti immuno-labelled for GFP after RWM injection. Note that AAV8-CMV-GFP transduced all IHCs. B. Confocal images of the mid-apex wild-type cochlea injected through the RWM at P20 with AAV8-GFP, Anc80-GFP, and AAV2-GFP. Scale bars: 100µm; 10µm for insets.

C & D. ***In vivo*** **transduction of cochlear cells with AAV8 and AAV2 vectors in NHP**. Confocal representative images of cochlea that underwent AAV8- (C) or AAV2-CMV-GFP (D) injection through the RWM combined to oval window fenestration delivery approach. GFP expressing cells (green) along the length of the cochlea. Cell nuclei were stained with DAPI (blue). Red, phalloidin-stained actin. Scale bars 100µm; 10µm for insets.

Figure 2: Dual-AAV8 CMV promoter drives murine otoferlin expression in *Otof*$^{-/-}$ mouse cochlea

(**A**) Maximum-intensity projections of confocal z-sections of P64 organs of Corti in Otof$^{-/-}$ mice injected at P10 and immunostained for otoferlin. Almost all the IHCs expressed murine otoferlin. (**B**) ABR recording at forty-two days in Otof$^{-/-}$ uninjected mice (black dashed line), wild-type uninjected mice (grey dashed line) and Otof$^{-/-}$ mice injected at p10 with a dual AAV of the invention encoding murine otoferlin (solid line) in response to tone-bursts at 8, 10, 15 and 20 kHz. Note that the hearing of the Otof$^{-/-}$ AAV otoferlin treated mice is restored to near normal thresholds. (**C**) The hearing longevity for

15 kHz frequency (for which the best hearing threshold was observed) was recorded for at least 47 weeks after injection of the dual vector of the invention encoding murine otoferlin in p10 old Otof $^{-/-}$ treated mice. Individual ABR recordings over time in response to tone-bursts at 15 kHz in non-injected Otof$^{-/-}$ mice (dashdot line), and Otof$^{-/-}$ mice treated at p10 are presented (n=6). (**D**) ABR recordings between 28 and 319 days after intracochlear injection of the dual otoferlin vector of the invention encoding murine otoferlin in 10 days old Otof$^{-/-}$ mice (n=8, solid lines, mean ± SEM), non-injected wild-type mice (dotted line, mean ± SEM, n=3) and non-injected Otof$^{-/-}$ mice (dashdot line, mean ± SEM, n=3) in response to tone-bursts at 5, 10, 15, 20, 32, and 40 kHz. (**E**) ABR recordings 18 weeks post-treatment in Otof$^{-/-}$ 21/22 days old mice injected with PBS (dashdot line, mean ± SEM, n=5), PBS-injected wild-type 21/22 days old mice (dashed line, mean ± SEM, n=6), non-injected wild-type mice (densely dashed line, mean ± SEM, n=3), Otof$^{-/-}$ mice injected at p21/p22 with the dual AAV vector of the invention encoding murine otoferlin (dotted line, mean ± SEM, n=9), and the Otof$^{-/-}$ mouse #8 (solid line, n=1) injected at p21/p22 with a dual vector of the invention encoding murine otoferlin, said mouse showing the best recovery in response to tone-bursts at 5, 10, 15, 20, 32, and 40 kHz. (**F**) ABR recording 18 weeks post-injection in PBS-injected Otof$^{-/-}$ 18 - 25 days old mice (dashdot line, mean ± SEM, n=5), PBS-injected wild-type 18 - 25 days old mice (dashed line, mean ± SEM, n=7), non-injected wild-type 18 - 25 days old mice (densely dashed line, mean ± SEM, n=3), and Otof$^{-/-}$ mice injected at p18-p25 with the dual AAV of the invention encoding human otoferlin (dotted line, mean ± SEM, n=4), among which the Otof$^{-/-}$ mouse #3 (solid line, n=1) that has been injected with the dual vector of the invention at p18-p25, said mouse showing the best recovery in response to tone-bursts at 5, 10, 15, 20, 32, and 40 kHz. (**G**) Longevity of hearing recovery at 15 kHz in mice treated with either dual AAV8-CMV-muOTOF (n=8) or with AAV8-smCBAmuOTOF (n= 15). Mean +/- SEM at 15kHz is represented. dB=decibel; SPL=sound pressure level; kHz=kilohertz. **(H)** Maximum-intensity projections of confocal z-sections of the apex, MT, and basal turn of the injected left cochlea immunostained for murine otoferlin (blue) from one the best responder mice (#8). IHC and their nuclei are stained with Ribeye (green). Scale bars: 10 µm. (I) Maximum-intensity projections of confocal z-sections of the apex, MT,

and basal turn of the injected left cochlea immunostained for human otoferlin (blue) from one the best responder mice (#3). IHC and their nuclei are stained with Ribeye (green). Scale bars: 10 µm.

Figure 3: Schematic representation of the recombination, transcription, splicing, and translation processes producing the full-length human protein Otoferlin in transduced cells. pA=polyadenylation site, SD= splice donor element, SA= splice acceptor element, AP= alkaline phosphatase recombinogenic region, ITR= Inverted terminal repeats.

Figure 4: Schematic diagram of dual AAV OTOF vector strategies. pA=polyadenylation site, SD= splice donor element, SA= splice acceptor element, AP= alkaline phosphatase recombinogenic region, ITR= Inverted terminal repeats.

Figure 5: **A. Scheme of the *in vitro* ubiquitous CMV promoter-driven expression of human otoferlin protein after dual vector delivery using lipofectamine-based transfection. B. Efficacy evaluation of 3 different human otoferlin cutting sites in dual plasmid configurations.** HEK293 cells were transfected with the Dual OTOF Nter-Cter 738-739 (black), 892-893 (dark grey), and 997-998 (light grey) plasmids and their respective controls (Nter or Cter alone). Experiments were performed 3 times with at least 2 independent wells per condition. For each condition, quantification of cells positive (Nter+Cter) for otoferlin was performed on at least 3 fields of 200-300 DAPI positive cells per well.

Figure 6: **Diagram summarizing the exons present in the mouse cochlea**, highlighting the exons present on the murine transcript (A1-B2-C2) and the exons present on the human isoform 5 transcript (A2-B1-C2).

Figure 7: A. RT-PCR analysis of the otoferlin transcript produced by the reconstituted full-length human cDNA in co-transfected HEK293 cells with the pair of recombinant plasmids. The RNA extracts were reverse-transcribed, and subjected to PCR amplification with primers designed to amplify a 898 or 946 bp fragment of the otoferlin cDNA encompassing the junction between the Otof Nter and Otof Cter cDNA. A negative (non-transfected HEK293 cells) and positive (pcDNA3 containing the

HuOTOFcDNA under the control of the CMV promoter) control are shown. M: DNA molecular weight marker. The position of the 0.5, and 1.5 kb molecular mass markers of the DNA ladder is indicated on the left side of the electrophoresis gel.

B. Full-length otoferlin expression following HEK293 cells transfection with dual otof plasmids. M: For each of the dual plasmids, the corresponding Nter and Cter or only Nter or Cter parts were used for transfection. Otoferlin specific antibodies were used to identify the full-length otoferlin protein. HEK293 cells transfected with the HuOTOF (Hu cDNA) plasmid were used as positive control. M: Pre-stained protein marker. TOT: Input (soluble protein extracts). IP: Extracts from HEK293 cells subjected to immunoprecipitation with the FP2 otoferlin antibody. NT: Cell lysates from non-transfected cells. ACTB: An anti human beta-actin monoclonal antibody was used as an internal-loading control between the different lanes. The position of the different molecular mass markers of the protein ladder is indicated on the left side of the electrophoresis gel. Stars indicated non-specific antibody detection (bands also observed in the non-transfected cells).

EXAMPLES

EXAMPLE 1: activity of the AAV8 vector of the invention

1. Material and methods

Generation of AAV vector plasmids

All the AAV otoferlin recombinant vector were synthetized (Genscript).

To generate dual otoferlin vector constructs, the human otofelin coding sequence (OTOF transcript variant 5; NM_001287489.2) was split at different exon-exon natural junctions: exon 18-19 (nt 1-2214/2215-5991, aa 738-739), exon 20-21 (nt 1-2406/2407-5991), exon 21-22 (nt 1-2523/2524-5991, aa 841-842), exon 22-23 (nt 1-2676/2677-5991, aa 892-893), exon 24-25 (nt 1-2991/2992-5991, aa

997-998), and exon 25-26 (nt 1-3126/3127-5991, aa 1042-1043).

To generate the 5' vector p0101-CMV-NterhuOTOF892 (SEQ ID NO:56), synthetic fragments were synthetized (Genscript) and cloned into p0101_CMV_eGFP plasmid (SEQ ID NO:77).. To generate p0101-CMV-NterhuOTOF1042, a 450 bp insert (nucleotides 3617-4066) was synthesized and cloned into the plasmid p0101-CMV-NterhuOTOF892-AP. Then, plasmids P0101_CMV-huOTOF738, 841 and 997 were generated by mutagenesis from p0101-CMV-NterhuOTOF1042 Genscript.

The 5' vectors p0101-CMV-intron-NterhuOTOF738, 802, 841, 892, 997, and 1042 were generated by mutagenesis from p0101-CMV-NterhuOTOF738, 802, 841, 892, 997, and 1042 constructs described above (Genscript).

To generate the 3' vectors (human OTOF transcript variant 5; NM_001287489.2), synthetic fragments were synthetized (Genscript) and cloned into p0101_CMV_eGFP plasmid (SEQ ID NO:77), which was digested by NheI[205]-HindIII[1702] (fragment of 4007 bp) or NheI[205]-BglII[2256] (fragment of 3453 bp).

The full-length coding sequence of the murine otoferlin cDNA sequence (Otof1 isoform1; NM_001100395.1) was divided into a 5' fragment (nucleotides 1-2448) and a 3' fragment (nucleotides 2449-5979), and these fragments were synthetized (Genscript). The 5' and 3' fragments have been cloned into p0101_CMV_eGFP plasmid (SEQ ID NO:77). The 5' vector p0101-CMV-Nter murine OTOF816_AP (SEQ ID NO:79) and 3' vector Cter OTOF murine817_AP (SEQ ID NO:80) were generated (see Akil et al. 2019).

The 5' fragment has been also cloned into p0101_smCBA_eGFP plasmid (SEQ ID NO:96). The 5' vector p0101-smCBA-Nter murine OTOF816_AP (SEQ ID NO:95) was generated (see Akil et al. 2019).

AAV production

AAV vectors were produced by either University of Pennsylvania Vector Core (UPenn) or ETH (Zurich) Vector Core facility. AAV titers were given in viral genomes per ml (vg/ml) as determined by ddPCR-based method (UPenn) or fluorometric assay (ETH). Final concentrated AAV vector stocks were stored in PBS with Pluronic-F68 (0,001%) (UPenn) or in PBS with MgCl2 (1mM) and KCl (2.5mM) (ETH).

Transgene expression in transfected HEK 293 cells

HEK293 cells were cultured at 37°C in a humidified chamber containing 5% CO_2. HEK293 cells were grown in six-well plates on polylysine-coated coverslips in DMEM/F-12 Thermofisher) supplemented with 1x non-essential amino acids and 10% FBS (Gibco), and penicillin/streptomycin (Pen/Strep; Invitrogen). For immunocytochemistry analysis, cells were grown on polylysine-coated coverslips. On the next day, cells were transfected at 70-80% of confluency with Lipofectamine 3000® (Thermofisher). Briefly, Lipofectamine® 3000 Reagent in was diluted in Opti-MEM® Medium– Mix. A master mix of DNA was prepared by diluting DNA (0.25 to 10 μg) in OptiMEM® Medium, then added to P3000™ Reagent. Diluted DNA was added to each tube of diluted Lipofectamine® 3000 Reagent (1:1 ratio). After an incubation for 5 minutes at room temperature the DNA-lipid complex was added to cells. The next day of transfection medium was replaced. Between 24-48 hours post-transfection, cells were collected for immunocytochemistry and RT-PCR analysis.

OTOF expression by RT-PCR

Transfected HEK293 cells were scratched and total RNA were extracted using the Nucleospin RNA kit (Macherey Nagel, 740955) according to manufacturer's instruction. RNA dosage was then assessed using a Nanovue Plus spectrophotometer. Retro-transcription PCR for the Otoferlin gene was performed on extracted RNA with the SuperScript™ III One-Step RT-PCR System (Thermofisher, 12574018) with different couple of primers designed to amplify specific junction fragments (forward 4F TGGAGGCCTCAATGATCGAC, SEQ ID NO:45 and reverse 4R AGCCACAGGGCAGGCCGCAC SEQ ID

NO:46 for exon 18-19 junction, and forward 5F GAGCTGAGCTGTGGCTGCTG SEQ ID NO:93 and reverse 5R AGTACGCCTCGTCTGCCATC SEQ ID NO:94 for exon22-23 and 24-25 junction). For each RT-PCR reaction, 1µg of RNA extract was used as template. PCR products was then migrated on a 0,8% agarose gel with Ethidium Bromide. Sanger sequencing of PCR products was performed by Transnetyx automated sequencing services.

OTOF expression analysis by Immunoblot

Proteins were extracted by mechanical homogenization in RIPA buffer supplemented with protease inhibitor cocktail. Samples were incubated on ice for 30 minutes, with vortex shaking, then centrifugated at 12,000 x g for 30 minutes at 4°C. Supernatant was collected, frozen in liquid nitrogen and kept at -80°C. Protein concentration of each lysate was assessed using a colorimetric BCA protein assay (commercial kit). Immunoprecipitation (IP) was carried out using 14CC antibodies, preincubated with protein A-agarose (Pharmacia). Immunoprecipitates were resuspended in 50µl at a ratio 1:1 with NuPAGE™ LDS Sample Buffer (4X) (Invitrogen) and NuPage Sample Reducing Agent (10X) (Invitrogen) then incubated at 70°C for 10 minutes. Samples were mixed at a ratio 1:1 with NuPAGE™ LDS Sample Buffer (4X) (Invitrogen) and NuPage Sample Reducing Agent (10X) (Invitrogen) then incubated at 70°C for 10 minutes. These denaturated proteins were loaded in a 3-8% NuPAGE™ Tris-Acetate Novex Mini Gels (Invitrogen) (15µL per wells), and run in NuPAGE™ Tris-Acetate SDS Running Buffer (1X) at 150V for 1 hour. Proteins were transferred to a nitrocellulose membrane with the Power Blotter-Semi Dry Transfer System (Thermofisher) according to the commercial protocol during 15 minutes. After an incubation of one hour in a blocking buffer (PBS-Tween (0.1%) and milk solution (1%)), the membrane was probed overnight at 4°C with primary antibodies (Rabbit polyclonal antibody FP2, directed against the N-ter part of the otoferlin protein, 1:100 dilution) loaded in blocking buffer. After several washes with PBS-Tween (0.1%), the membrane was probed with secondary HRP -antibodies (Goat anti-Rabbit IgG (H+L) HRP, 1:5000 dilution, Invitrogen) in blocking buffer. Chemiluminescence was revealed with the Clarity™ Western ECL Substrate (Biorad) for 5 minutes at RT and detected with the ChemiDoc

Imaging Systems (Biorad). The transfected otoferline is expected at ~230 kDa. Membrane was then incubated in a stripping buffer (Thermo Scientific), washed with PBS-Tween (0.1%) and blocked 1 hour in blocking buffer. Housekeeping protein assessment was performed by a blotting with a monoclonal antibody to beta-actin. The membrane was probed with a primary antibody (mouse anti-beta-actin antibody, 1:5000 dilution, Sigma) in blocking buffer during 1 hour, then in anti-mouse HRP-antibodies (1:5000 dilution, Jackson Immunoresearch) in the same blocking buffer. Chemiluminescence was revealed with ECL substrate for 5 minutes at RT and detected with the ChemiDoc Imaging Systems (Biorad).

Vector delivery to the cochlea of mice

All surgical procedures and viral injections were carried in a Biosafety level 2 Laboratory. C57BL/6 wild-type or Otof$^{-/-}$ mice at different postnatal stages (P10 to P25) were anesthetized with isoflurane (4% for induction and 2% for maintenance). In order to reduce pain, mice received, at the beginning of the surgery, a subcutaneous injection of an analgesic, meloxicam (Metacam®, 0.2 mg/kg/day), and a subcutaneous injection of a local anesthesia in the retro-auricular region (Laocaïne ®, 5mg/kg). The anesthetized animal was placed on a thermopad throughout the procedure until the mouse was totally awake. Intracochlear injection was carried out as described by Akil et al. (2019). The left ear was approached via a retro-auricular incision. After dissecting the cervical muscles, the otic bulla was exposed and punctured with a 25G needle. The opening was expanded as necessary with forceps to visualize the stapedial artery and the round window membrane (RWM). The RWM was gently punctured in the center with a glass pipette, then a fixed volume (2 microliters) of a PBS or viral solution containing either the AAV8-CMV_GFP of SEQ ID NO:77 ($5.6x10^{13}$ vg/ml), or the Anc80L65-CMV-GFP of SEQ ID NO:77 ($5.5x10^{12}$ vg/ml), or the AAV2-CMV-GFP of SEQ ID NO:77 ($1.2x10^{13}$ vg/ml) or the AAV8-CMV-NterOTOFmu816_AP of SEQ ID NO:79 ($1.3x10^{13}$ vg/ml) and AAV8-CterOTOFmu817_AP of SEQ ID NO:80 ($1.5x10^{13}$ vg/ml) vector pair, or AAV8-smCBA NterOTOFmu816_AP of SEQ ID NO:95

($1.5x10^{13}$ vg/ml) and AAV8-CterOTOFmu817_AP of SEQ ID NO:80 ($1.5x10^{13}$ vg/ml) vector pair, or the AAV8-CMVNterOTOFhu892_AP of SEQ ID NO: 56 ($1.1x10^{13}$ vg/ml) and AAV8-CterOTOFhu893_AP of SEQ ID NO:58 ($0.85x10^{13}$ vg/ml) vector pair was injected through the RWM using a pump system coupled to a glass micropipette. The RW niche was sealed quickly after pulling out the pipette with a small plug of muscle secured with a small drop of biological glue (Vetbond® 3M) placed on the muscle, to avoid leakage from the round window and with a small plug of fat to close the opening of the bulla. GFP or OTOF expression in cochlea was assessed by immunofluorescence.

Vector delivery to the cochlea of non human primate

Following an overnight food deprivation, the animals were anesthetized by intramuscular injection of a mixture of Ketamine (10mg/kg) and Propofol (5-10 mg/kg) and intubated, and maintained with oxygen and isoflurane during surgery. Animals received a prophylactic injection of an antibiotic (Duphamox 15mg/kg, IM) and an anti-inflammatory (tolfedine 4mg/kg, IM). The round window niche (RW) of the left or the right ear was exposed using a trans-canal approach. Access to the canal was achieved through preauricular incision. The skin of the canal and the eardrum were uplifted. A canaloplasty was performed by drilling the posterior and inferior part of the external ear canal to access to the middle ear and expose the RW niche and the stapes. The niche of the round window was drilled to expose its membrane without opening it, than a platinotomy was performed using a diode laser and a 300 micrometer external diameter fiber. The opening of the platinum was checked with a trephine, with visualization of a perilymph leak from the oval window. Incision of the RW membrane to access the cochlea using the tip of a 25-gauge needle under binocular operating microscope. Insertion of a thin catheter (Medel catheter) through the RW on a 1 mm length. The total duration of surgery for one ear was 1 hour on average. After injection, sealing of the oval and round window opening using small muscular graft was performed. Injection of the viral preparation (30 µL) expressing GFP under the control of the CMV promoter (AAV8-CMV-GFP $2.8x10^{13}$ vg/ml, AAV2-CMV-GFP $1.4x10^{13}$ vg/ml) was performed using a volume-controlled syringe pump. To avoid overpressure effects within

the inner ear the injection rate and volume set to 1µl/5s. The animals were observed for postural imbalance, nystagmus and other signs of vestibular dysfunction the following 8 hours. Except during the 3-4 days following surgery where the animals were housed in the infirmary for close monitoring, they were housed at least in pairs. In addition, an adapted enrichment program was in place to limit stress and group monitoring is carried out to identify and manage conflicts that may arise in a social group. The animals were anesthetized by injection of Ketamine (10 mg/kg; IM) then put in deep anesthesia maintained with Propofol following the placement of a venous catheter (1ml/kg h, IV). Under deep anesthesia (without awakening), chest cage was open then animals received intracardiac perfusion first with PBS (200 mL) followed by 4% paraformaldehyde (pH 7.4) (500 mL) while deeply anesthetized.

The dissected cochleae were perfused with the same fixative and then transferred to an EDTA solution for decalcification for 10 days (Kos micro-wave). EDTA was refreshed two times and decalcified bone was trimmed at each change. The cochlear sensory epithelium (organ of Corti) was micro dissected and then processed for GFP immunodetection.

The organ of Corti was preincubated in PBS with 20% horse serum, 0.3% Triton X-100, and 0.3% Saponin in PBS (blocking buffer) at RT for one hour, then incubated with the primary antibody (chicken anti-GFP, Invitrogen) in diluted blocking buffer (dilution 1:20) at RT overnight. The samples were rinsed three times with PBS, and incubated with the appropriate secondary antibody (Alexa Fluor 488 Goat anti-chicken IgG, Invitrogen) in PBS at room temperature for one hour. The samples were then stained with phalloidin-Atto 565 (Sigma) and 4',6-diamidino-2-phenylindole (DAPI) to visualize cell nuclei, mounted on a glass slide with a drop of Fluorsave medium, and observed with a Zeiss confocal immunofluorescence microscope.

Auditory testing

Auditory testing was carried out in anesthetized Otof$^{+/+}$, Otof $^{-/-}$, and rescued Otof$^{-/-}$ mice at different time points, in a sound-proof chamber as previously described (Akil O. et al., 2019). Pure-tone stimuli were used at frequencies of 5, 10, 15, 20, 32, and 40 kHz. The hearing threshold was defined as the lowest stimulus level at which ABR peaks for waves I-V were clearly defined and repeatedly present upon visual inspection. ABRs were analyzed with Matlab software.

Fluorescence microscopy

In vitro studies

Transfected cells grown on polylysine-coated coverslips were fixed using 4% paraformaldehyde in phosphate buffered saline (PBS), pH 7.4, at room temperature (RT) for 20 min, rinsed three times with PBS, and incubated with 0.25% Triton X-100 at RT for 15 minutes. Cells were rinsed two times with PBS and blocked with horse serum (20%) in PBS at RT for one hour. The cells were then incubated with a mixture of the rabbit polyclonal antibody FP2 (Institut Pasteur, dilution 1:200) directed against the C-terminal part of otoferlin and of the mouse monoclonal antibody (Institut Pasteur, dilution 1:100), directed against the N-terminal part of otoferlin at RT for 1 hour. The samples were washed twice with PBS, and incubated with secondary antibodies (AlexaFluor goat anti-rabbit 488, goat anti-mouse 555, Life Technologies, dilution 1:500) in PBS at room temperature for one hour. The samples were then rinsed twice in PBS, stained with 4',6-diamidino-2-phenylindole (DAPI) to visualize cell nuclei, mounted on a glass slide with a drop of Fluorsave medium (EMB Millipore), and observed with an Olympus confocal immunofluorescence microscope.

In vivo studies

Mouse cochleas were perfused with 4% paraformaldehyde in 0.1 M PBS (pH 7.4) and incubated in the same fixative at 4 °C for 45 minutes at RT. The cochleas were rinsed three times with PBS and decalcified by incubation with ethylenediamine tetraacetic acid (EDTA) 0.5 M at 4 °C overnight. After several rinses (three times in PBS) the cochlear sensory epithelium (organ of Corti) was microdissected into a surface preparation, preincubated in 0.03% Triton X100 and 20% horse serum in PBS (blocking buffer) at room temperature for 1 h, and incubated with the primary antibody at 4 °C overnight. The following antibodies were used: Chicken-polyclonal GFP (1:400 dilution, Abcam), or rabbit anti-otoferlin (1:100 dilution, Institut Pasteur), mouse (IgG1) anti-CtBP2 (1:200 dilution, Millipore) and anti-glutamate receptor subunit A2 (1:2000 dilution; Millipore). The samples were rinsed three times in PBS and incubated with the appropriate secondary antibody: Fluor 488-conjugated anti-chicken IgY (1:500 dilution; Life Technologies) or Atto Fluor 647-conjugated anti-rabbit IgG (1:200 dilution; Sigma), Fluor 488-conjugated anti-mouse IgG1 and Alexa Fluor 568-conjugated anti-mouse IgG2a (1:500 dilution; Life Technologies). The samples were rinsed three times in PBS and incubated with the appropriate secondary antibody: The samples were washed three times in PBS, and mounted on a glass slide in one drop of Fluorsave, with DAPI (1:7500 dilution) to stain cell nuclei. Fluorescence confocal z stacks of the organ of Corti were obtained with an LSM 700 confocal microscope (Zeiss) equipped with a high-resolution objective (63× oil-immersion objective). The images were then analyzed using the FIJI software.

Transfection rates

The proportion of cells expressing the otoferlin protein was calculated as follows: Number of cells with detectable green fluorescent signal/Total number of cells (DAPI-stained cell nuclei). This count was carried out on the whole coverslip or slide by using the NIS Elements 3.1 Imaging software (Nikon).

2. Results

2.1. ***In vivo investigation of the tropism of the AAV8 vector with the CMV promoter (WT animals)***

2.1.1.First, the ability of AAV8-CMV-GFP to transduce inner ear cells was analysed at a mature stage (P12) after a single intracochlear injection in C57BL/6 mice. It was found that AAV8-CMV-GFP targeted largely the IHCs, where otoferlin is expressed and that the transduction rate was 89 and 100% at the base and apex, respectively (figure 1A). These results demonstrate the suitability of this AAV capsid/promoter combination to deliver therapeutic genes to IHCs.

2.1.2. ***Inner ear tropism of several AAV serotypes in mice***

The ability of several adeno-associated virus (AAV) serotypes (AAV2, AAV8, and Anc80) to transduce mature murine hair cells was assessed. The IHC transduction rate achieved after a single viral intracochlear injection of each recombinant vector, wherein the CMV promoter drives the expression of the GFP as the reporter gene, was examined.

The AAV recombinant vectors were injected (2 microliters) through the RWM in C57BL/6 wild-type mice at a mature stage (P20). More precisely, the left ear was approached via a dorsal incision and the virus was delivered to the cochlea as previously described Akil et al. (2019). Anesthetized C57BL/6 wild-type or Otof$^{-/-}$ mice received an injection of 2 microliters of a viral solution containing either the AAV8-CMV-GFP containing SEQ ID NO:77 ($5.6x10^{13}$ vg/ml), the Anc80L65-CMV-GFP containing SEQ ID NO:77 ($5.5x10^{12}$ vg/ml), or the AAV2-CMV-GFP containing SEQ ID NO:77 ($1.2x10^{13}$ vg/ml) through the round window membrane of the cochlea. GFP expression in cochlea was assessed by immunofluorescence (figure 1B).

The analysis shows that AAV8-CMV-GFP largely targeted IHCs (IHC transduction 94%) throughout the entire cochlear spiral, and not the outer hair cells (OHCs) (<1%). Anc80L65 mainly transduced IHCs

(97%) and in a lesser extent OHCs (13%) throughout the cochlear spiral. AAV2 transduced not only IHCs (95%) but also OHCs (60-80%) (see exemplary images on figure 1B).

AAV8 combined with the ubiquitous CMV promoter is thus a highly efficient recombinant vector to target mature IHCs *in vivo.*

2.1.3. ***AAVs tropism in the Inner ear of non-human primate*** *(NHP)*

The tropism of several AAVs tested in mice were further investigated in non-human primates. The viral preparations were injected (injection volume: 30 microliters) into the cochlea of non-human primates through the round window (trans-canalar hypotympanotomy approach) combined with a tiny fenestration in the oval window. All the injected cochleae were fixed 3 weeks after the transgene delivery and processed for immunolabelling for the GFP reporter gene.

Importantly, the results (see figures 1C and 1D for exemplary images) show that the transduction rate and pattern profile of the AAV8-CMV-GFP was similar to those obtained in mice: the AAV8 vector transduced efficiently and specifically IHCs (up to 95%) and to a lesser extent supporting cells of the injected cochlea. None of the OHCs were transduced by the AAV8 vector. On the contrary, AAV2 was able to transduce OHCs (40%), as well as IHCs (79%) as it was observed in mice (cf. 2.1.1.). This non-specific infectivity could increase the risk of non-specific expression of the therapeutic gene by targeting OHCs as well, although these cells are not defective in the case of DFNB9 deafness.

These results therefore demonstrate that the AAV8 capsid/CMV promoter combination of the invention delivered to NHP cochlea transduces very efficiently and specifically IHCs but not OHCs (contrary to AAV2-CMV-GFP).

2.2. *Efficacy evaluation of different human otoferlin cutting sites in Dual vector configurations*

The Inventors adopted the same strategy used for dual murin otoferlin CMV vectors and engineered the dual AAV vectors expressing the human otoferlin protein (figure 3). As the efficacy of the dual AAV may be affected by the split site within the cDNA, several human otoferlin fragments were generated

using various cutting sites and compared *in-vitro* their capacity to recombine to reconstitute the full-length otoferlin protein.

To generate dual human otoferlin vector constructs, the full-length coding sequence of the cochlear isoforms of the human otoferlin cDNA (transcript variant 5, and the new transcript variant) was divided into different 5' fragments (nt 1-2214, nt 1-2406, nt 1-2523, nt 1-2676, nt 1-2991, and nt 1-3126) and 3' fragments (nt 2215-5991, nt 2407-5991, nt 2524-5991, nt 2677-5991, nt 2992-5991, and nt 3127-5991). The 5' constructs contained the 5' fragment of the hOTOF cDNA (encoding the amino-acids (aa) 1-738, aa 1-802, aa 1-841, aa 1-892, aa 1-997 and aa 1-1042) under the control of the CMV promoter, optionally followed by the intronic sequence, and/or by a kozak sequence, then followed by a splice donor site (SD), and the 3' constructs contained the 3' part of the hOTOF cDNA (encoding the aa 739-1997, aa 803-1997, aa-842-1997, aa 893-1997, aa 998-1997, and aa 1043-1997), and a splice acceptor (SA) site (Figure 3). All the fragments, which contain the alkaline phosphatase recombinogenic bridging sequence (AP), were inserted into the AAV-p0101 plasmid referred to as p0101–CMV-Nter huOTOF (738, 802, 841, 892, 997 and 1042) and p0101-hOTOF Cter huOTOF (739, 803, 842, 893, 998 and 1043) constructs.

The sequences of these constructs are given in the enclosed listing, in SEQ ID NO:47-64 and in SEQ ID NO:70-75, encoding N-terminal or C-terminal part of the isoform 5 of human OTOF.

Cutting site	part	nt	aa	Constructs	
Exons 18-19	N-term	nt 1-2214	aa 1-738	P0101 CMV N ter 738 (SEQ ID NO:47)	P0101 CMV + intron + N ter 738 (SEQ ID NO:70)
	C-term	nt 2215-5991	aa 739-1997	P0101 C ter WPRE 739 (SEQ ID NO :48)	P0101 C ter sine WPRE 739 (SEQ ID NO:49)

Exons 20-21	N-term	nt 1-2406	aa 1-802	P0101 CMV N ter 802 (SEQ ID NO:50)	P0101 CMV + intron + N ter 802 (SEQ ID NO:71)
	C-term	nt 2407-5991	aa 803-1997	P0101 C ter 803 WPRE (SEQ ID NO :51)	P0101 C ter 803 sine WPRE (SEQ ID NO:52)
Exons 21-22	N-term	nt 1-2523	aa 1-841	P0101 CMV N ter 841 (SEQ ID NO:53)	P0101 CMV + intron + N ter 841 (SEQ ID NO:72)
	C-term	nt 2524-5991	aa 842-1997	P0101 C ter 842 WPRE (SEQ ID NO :54)	P0101 C ter 842 sine WPRE (SEQ ID NO:55)
Exons 22-23	N-term	nt 1-2676	aa 1-892	P0101 CMV N ter 892 (SEQ ID NO:56)	P0101 CMV + intron + N ter 892 (SEQ ID NO:73)
	C-term	nt 2677-5991	aa 893-1997	P0101 C ter 893 WPRE (SEQ ID NO :57)	P0101 C ter 893 sine WPRE (SEQ ID NO:58)
Exons 24-25	N-term	nt 1-2991	aa 1-997	P0101 CMV N ter 997 (SEQ ID NO:59)	P0101 CMV + intron + N ter 997 (SEQ ID NO:74)
	C-term	nt 2992-5991	aa 998-1997	P0101 C ter 998 WPRE (SEQ ID NO :60)	P0101 C ter 998 sine

					WPRE (SEQ ID NO:61)
Exons 25-26	N-term	nt 1-3126	aa 1-1042	P0101 CMV N ter 1042 (SEQ ID NO:62)	P0101 CMV + intron + N ter 1042 (SEQ ID NO:75)
	C-term	nt 3127-5991	aa 1043-1997	P0101 C ter 1043 WPRE (SEQ ID NO :63)	P0101 C ter 1043 sine WPRE (SEQ ID NO:64)

HEK293 cells were transfected using lipofectamine with either p0101 CMV-NTerhuOTOF alone (738, 802, 841, 892, 997 and 1042, Left panel), p0101 CTerhuOTOF alone (739, 803, 842, 893, 998 and 1043, Right panel), or both p0101 CMV-NTerhuOTOF and p0101 CTerhuOTOF (738-739, 802-803, 892-893, 997-998 and 1042-1043) (figure 5A).

Cells were stained for otoferlin expression with the previously characterized mouse monoclonal antibody 10H9 (red) and rabbit polyclonal antibody FP2 (green), directed against the N-terminal and the C-terminal part of human Otoferlin, respectively 48 h after transfection (dilution 1:200). The actin filaments were labeled with phalloidin (orange) and cell nuclei were labelled with DAPI (blue) (not shown). Only the results for three cutting sites are shown.

The results showed that all the tested dual plasmid configurations containing junction 738-739, 892-893, and 997-998 were capable of reconstituting the ofoferlin protein. Even though for a given junction site, the ratio of cells expressing otoferlin showed variability, the statistical test indicate that these ratios did not differ significantly, implying that the recombination efficacy involving the 3 different cutting sites are quite similar (Figure 5B).

These results indicated that co-transfection of the dual OTOF plasmids, regardless of the junction site used, led to recombination of the full-length cassette resulting in human otoferlin protein expression.

We conclude that the reconstitution of the otoferlin full-length cDNA occurs as long as the cutting sites give DNA fragments that remain within the limit of the packaging capacity of the AAVs.

2.3. In vitro assessment of the recombination efficacy of different dual AAV8-CMV-huOTOF constructs

To assess the recombination efficacy of various dual AAV OTOF vector pairs (examples thereof are given with the dual human OTOF Nter-Cter 738-739, 892-893, and 997-998 vectors containing the CMV promoter), transfected cells were harvested, and RNA transcript expression was evaluated by RT-PCR with specific primers encompassing the splicing junction.

The RNA extracts were reverse-transcribed, and subjected to PCR amplification with primers designed to amplify a 898 or 946 bp fragment of the otoferlin cDNA encompassing the junction between the Otof Nter and Otof Cter cDNA. A negative (non-transfected HEK293 cells) and positive (pcDNA3 containing the HuOTOFcDNA under the control of the CMV promoter) control are shown. M: DNA molecular weight marker. The position of the 0.5, and 1.5 kb molecular mass markers of the DNA ladder is indicated on the left side of the electrophoresis gel (figure 7A).

The RT-PCR amplified a fragment of the expected size, which was of 946 bp (Nter-CTer 738-739) or 898 pb (Nter-Cter 892-893 and NTer-Cter 997-998) similar to the fragment amplified from the huOTOF cDNA control (Figure 7A). There was no amplificons obtained when only the 5' or 3' portion of huOTOF were used as template. Purification of the specific amplicons followed by Sanger sequencing showed a complete sequence alignment of the 738-739, 892-893, or 997-998 junction portion with the native cDNA sequence of huOTOF, confirming the recombination of the vector pairs (not shown). More precisely, the recombination event of dual AAV OTOF vector pairs in transfected cells correctly allow the excision of splice donor (SD), splice acceptor (SA) and ITR sequences originally contained in 5' and 3' vector sequences. Sanger sequencing of transcripts amplified by RT-PCR shows complete homology of the amplicons with the Otoferlin exonic sequence. These results indicated that accurate homologous recombination and mRNA splicing occurred in HEK293 transfected cells with dual OTOF vector pairs.

The recombination of full-length huOTOF protein was also evaluated by Western blot. The dual 738-739, 892-893, and 997-998 plasmids were transfected into HEK293 cells. Cells were collected 48 hours and subjected to efficient protein extraction and lysis followed by Western immunoblotting using anti-otoferlin antibodies (figure 7B). For each of the dual plasmids, the corresponding Nter and Cter or only Nter or Cter parts were used for transfection. Otoferlin specific antibodies were used to identify the full-length otoferlin protein. HEK293 cells transfected with the HuOTOF (Hu cDNA) plasmid were used as positive control.

The results displayed on figure 7B showed that all dual plasmid configurations resulted in the expression of a protein with an apparent molecular weight of ~230 kDa, which was comparable to that of the human otoferlin protein. As predicted, no bands of the expected size were observed when only the Nter or Cter dual plasmid was used for transfection.

In conclusion, the various dual OTOF plasmid configurations tested herein led to the assembly of the two otoferlin cDNA fragments and to the *in vitro* expression of the full-length human OTOF protein.

2.4. In vivo validation of the dual AAV8 huOTOF vectors

The next step aimed to investigate the efficacy of gene therapy using a dual AAV8 vector with the CMV promoter driving the expression of either human or murine otoferlin protein to rescue hearing when administered before hearing onset (at P10), and reverse deafness phenotype when administered at mature stage (i.e., well after hearing onset, between P18-P25) into cochleae of DFNB9 mice.

In this aim, dual vector pairs according of the invention, encoding either the murine otoferlin protein or the human otoferlin protein, were engineered as disclosed in the Material and Methods.

2.4.1. Dual-AAV8 expression of mouse Otof in Otof$^{-/-}$ mouse cochlea using CMV promoter

A single unilateral injection of the AAV8 dual murine CMV vector (AAV8-CMV-NterOTOFmu816_AP of SEQ ID NO:79 ($1.3x10^{13}$ vg/ml) and AAV8-CterOTOFmu817_AP of SEQ ID NO:80 ($1.5x10^{13}$ vg/ml) was administered to Otof$^{-/-}$ mice at P10. Fifty-four days after injection of the recombinant vector pair, the

sensory epithelium of the treated cochleas was microdissected and immunolabeled for otoferlin to estimate the transduction rate of IHC. The murine protein was detected in almost all of the IHCs (Figure 2A). This result provides evidence that the murine otoferlin cDNA can effectively be reconstituted in cochlear sensory cells upon *in vivo* co-delivery of two halves of the otoferline cDNA using dual AAV8 capsid/CMV promoter combination.

ABR recordings 52 days after P10 injection demonstrated a substantial restoration of hearing thresholds (up to 40dB) in response to tone-burst stimuli (5, 10, 15, and 20 kHz) in the treated mice (n=6), but no restoration in the uninjected $Otof^{-/-}$ mice (Figure 2B). The long-term efficacy of gene therapy was evaluated by carrying out ABR recordings in response to tone-bursts of 15 kHz at time points between 4 and 47 weeks (Figure 2C). From the 4th to 47th week post-injection, hearing longevity for 15 kHz frequency was comparable between the responding treated mice (6 out of 8) and the uninjected WT mice. The long-term efficacy of gene therapy in the treated mice was also evaluated using ABR recordings in response to tone-burst stimuli at other frequencies (5, 10, 15, 20, 32 and 40 kHz) at different time points between 4 weeks and 47 weeks post-injection (figure 2D). ABR analysis showed that the rescued hearing thresholds were maintained overtime in all responding mice (6 out of 8) and were near wild-type level except for high frequencies (32 and 40 kHz).

The longevity of hearing restoration was also tested at the sound frequency of 15 kHz after intracochlear injection of the dual AAV8-CMV-muOTOF versus AAV8-smCBA-muOTOF vector in $Otof^{-/-}$ mice at P10.

P10 $OTOF^{-/-}$ mice were injected through the round window membrane with 2µL of either AAV8-smCBA-muOTOF (SEQ ID NO:95 and SEQ ID NO:80) (n=15; circle) or AAV8-CMV-muOTOF (SEQ ID NO:79 and SEQ ID NO:80) (n=8; square) at doses of 3.0E+10 and 2.8E+10 total vg respectively (1:1 ratio). Auditory Brainstem Response (ABR) thresholds were regularly measured over a year at different frequencies.

From the 4th (ABR1) to 40th (ABR14) week post-injection, ABR thresholds are higher at the 15 kHz

frequency in mice treated with the dual AAV8-smCBA-muOTOF vector compared to those injected with the dual AAV8-CMV-muOTOF vector (figure 2G).

The vector pair consisting of the AAV8-CMV-NterOTOFmu816_AP of SEQ ID NO:79 ($1.3x10^{13}$ vg/ml) and AAV8-CterOTOFmu817_AP of SEQ ID NO:80 ($1.5x10^{13}$ vg/ml), encoding the murine Otoferlin protein, was also used to treat twenty $Otof^{-/-}$ mice after hearing onset (between p21-p22). More precisely, these dual vectors were delivered to the cochlea of DFNB9 mice after hearing onset in P21- to 22-day-old $Otof^{-/-}$ mice (n=20), as described in the section "Material and Methods".

Eighteen weeks post-injection, ABR analysis showed a robust hearing rescue, which was maintained overtime in all the responding treated mice (9 out of 20), to a threshold of 70 dB on average (figure 2E). Mouse #8 hearing was remarkably rescued to a wild-type level 18 weeks post-injection (figure 2E).

The IHC transduction rate and otoferlin expression were next studied in mice injected treated with the dual AAV8-CMV-muOTOF vector at a mature stage. The mice were euthanized and their cochleae microdissected and immunolabeled for otoferlin and ribeye, a synaptic marker that also stains the IHC nuclei. The injected cochlea of all the responder mice displayed a variable IHC transduction rate. In the cochlea of the best responder mice (ABR threshold 40 dB on average, 15 kHz frequency), between 60 to 81% of IHCs were transduced throughout the cochlear spiral (apex to base, figure 2H). None of the OHCs of the responder mice expressed otoferlin confirming the specificity of our therapeutic vector.

These results demonstrate that the delivery before or after the hearing onset, of a fragmented murine otoferlin cDNA using dual-AAV8 vectors and the CMV promoter to the cochlea of $Otof^{-/-}$ mice, leads to the production of the full-length protein, which is restricted to the IHC. Despite variability of the transduction rate, the AAV gene therapy rescued the hearing of $Otof^{-/-}$ mice that would otherwise remain profoundly deaf. Importantly, the hearing restoration lasted for at least nearly a year.

2.4.2. Dual-AAV8 expression of human Otof in $Otof^{-/-}$ mouse cochlea using CMV promoter

Next, the efficacy of gene therapy was tested using a dual AAV8 vector with the CMV promoter driving the expression of human otoferlin to reverse the deafness phenotype when administered at a mature stage (well after hearing onset, between P18-P25) into cochleae of DFNB9 mice.

One of the best performing dual plasmids (Dual OTOF Nter-Cter 892-893), based on the *in vitro* reconstruction of a full-length human otoferlin protein, was also tested. The vectors consisting of the AAV8-CMVNterOTOFhu892_AP of SEQ ID NO: 56 ($1.1x10^{13}$ vg/ml) and AAV8-CterOTOFhu893_AP of SEQ ID NO:57 ($0.85x10^{13}$ vg/ml), encoding the full-lenth human otoferlin protein, were injected in seventeen $Otof^{-/-}$ mice after hearing onset (between P18-P25).

More precisely, these dual vectors were delivered to the cochlea of DFNB9 mice after hearing onset in P18- to 25-day-old Otof $^{-/-}$ mice, as described in the section "Material and Methods".

Eighteen weeks post-injection, ABR analysis showed that the hearing rescue was maintained in the responding mice (4 out of 14) to a threshold of 90 dB on average (figure 2F). However, the ABR thresholds in the treated mice did not reach the WT level. Mouse (#3) was remarkably rescued to a wild-type level 18 weeks post-injection (Figure 2F).

The IHC transduction rate and otoferlin expression were studied in mice treated with the dual AAV8-CMV-huOTOF vector at a mature stage. The mice were euthanized and their cochleae microdissected and immunolabeled for otoferlin and ribeye, a synaptic marker that also stains the IHC nuclei. The injected cochlea of all the responder mice displayed transduced IHCs with a variable transduction rate. In the cochlea of the best responder mouse (#3, 60 dB on average, 15 kHz frequency), between 60 to 80% of IHCs were transduced throughout the cochlear spiral (apex to base, figure 2I).

Importantly, none of the OHCs of the responder mice expressed human otoferlin, confirming further the efficiency of the therapeutic vector of the invention (achieving a very high transfection rate in IHC, > 60%) and its advantageous specificity.

To conclude, delivering a dual AAV8 vector encoding the human otoferlin therapeutic transgene to a mature Otof $^{-/-}$ cochlea lead to significant hearing restoration in adult Otof $^{-/-}$ mice. These results demonstrate that just like the murine otoferlin, the dual AAV8 /CMV promoter driving the human otoferlin restores the hearing in DFNB9 mouse model and confirm the suitability of this combination to deliver therapeutic genes to IHCs at a mature stage.

EXAMPLE 2: Identification of new isoforms of OTOF in humans

The murine transcript, for which a hearing recovery has been demonstrated in the PNAS publication (Akil et al., PNAS 2019), is different from the human isoform 5 transcript on 2 points:

- The murine transcript has an additional exon (exon 6 in the mouse sequence), and
- Exon 31, in the murine transcript, is shorter than the human exon 30 which is the equivalent in the human isoform 5 transcript (numbering related to the absence of exon 6 reported in human).

Consequently, a similar sequence of exon 6 was searched in the human intron 5 sequence. An almost identical region was identified in the human sequence.

It was then looked for splice donor and acceptor sites flanking that newly discovered sequence and they were present. Finally, the amino acid sequence obtained by the translation of the identified sequence were compared and it was found that the sequence is highly conserved between human and mouse:

mouse_Otof-202_exon6_genomic_seq = SEQ ID NO:65

CAAAGGCAGAGAGAAGACCAAGGGAGGCAGAGATGGCGAGCACAA 45

human_OTOF-205_putative_exon6_genomic_seq = SEQ ID NO:66

CAAAGGCAGAGAGAAGACCAAGGGAGGCAGAGATGACGAGCACAA 45

All the nucleotides are the same except the underlined one.

mouse_Otof-202_exon6_protein_seq = SEQ ID NO:67

KGREKTKGGRDGEH 14

human_OTOF-205_putative_exon6_protein_seq = SEQ ID NO:68

KGREKTKGGRDDEH 14

All the amino acids are the same except the underlined one.

These elements together constitute very strong evidences in favor of the existence of an exon 6 also in the *OTOF* gene in humans.

In addition, the existence of a short form of exon 30 has been observed in other human isoforms of OTOF reported in databases (for instance, in isoform 2 and 3). It is therefore likely that the human therapeutic cDNA could also contain a short form of exon 30 (future exon 31 when taking into account a supplementary exon).

According to previous publication (Yasunaga et al., J Hum Genet 2000), alternative exonic configurations have been observed in mouse cochlea and concern 3 exons (configurations are described in Figure 6). C2 is unique to the cochlea, A1 and A2 exist in the cochlea as well as B1 and B2.

It is possible that the human functional OTOF is in fact encoded by cDNA sequences containing:

- A1-B2-C2 (identical to the murine transcript): including exon 6 and a shorter exon 30 (SEQ ID NO:16), encoding the protein of SEQ ID NO:6

- A1-B1-C2: including exon 6 and the normal size exon 30 (SEQ ID NO:17), encoding the protein of SEQ ID NO:7

- A2-B2-C2: with no exon 6 but with a shorter exon 30 (SEQ ID NO:18), encoding the protein of SEQ ID NO:8.

These new isoforms would encode proteins of SEQ ID NO:6, SEQ ID NO:7 and/or SEQ ID NO:8 having a potential to restore hearing in humans, in addition to the current human isoform 5 transcript.

5

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CLAIMS

1. A vector system comprising at least two different AAV particles, namely:

a) at least one AAV8 particle comprising a first polynucleotide comprising an inverted terminal repeat at each end of said polynucleotide, and, between the said inverted terminal repeats, from 5' to 3': a CMV promoter sequence followed by a partial coding sequence that contains the N-terminal coding part of the Otoferlin gene, and

b) at least one AAV8 particle comprising a second polynucleotide comprising an inverted terminal repeat at each end of said polynucleotide, and, between the said inverted terminal repeats, from 5' to 3': a partial coding sequence that contains the C-terminal coding part of the Otoferlin gene, optionally followed by a polyadenylation sequence,

wherein the first and second polynucleotides comprise a recombinogenic polynucleotide sequence,

and wherein the coding sequences in the first and second polynucleotides when combined encode the isoform5 of the Otoferlin polypeptide, or a functional fragment thereof.

2. The vector system according to claim 1, wherein said Otoferlin gene has the sequence SEQ ID NO:15 or an homologous sequence thereof.

3. The vector system according to any of claim 1 to 2, comprising:

a) at least one AAV8 particle comprising a first polynucleotide comprising an inverted terminal repeat at each end of said polynucleotide, and, between the said inverted terminal repeats, from 5' to 3': the CMV promoter followed by a partial coding sequence that contains the N-terminal coding part of the Otoferlin gene, and a splice donor site, and

b) at least one AAV8 particle comprising a second polynucleotide comprising an inverted terminal repeat at each end of said polynucleotide, and, between the said inverted terminal repeats, from 5' to

3': a splice acceptor site, a partial coding sequence that contains the C-terminal coding part of the Otoferlin gene, optionally followed by a polyadenylation sequence,

wherein the said first and second polynucleotides also contain a second recombinogenic sequence that is located after the splice donor site in said first polynucleotide and before the splice acceptor site in said second polynucleotide.

4. The vector system according to claim 1 to 3, wherein said second recombinogenic sequence is the exogenous sequence of SEQ ID NO:69.

5. The vector system according to claim 1 to 4, wherein said CMV promoter has the sequence of SEQ ID NO:9 or an homologous sequence thereof.

6. The vector system according to claim 1 to 5, wherein said CMV promoter is followed by a intronic sequence, preferably of SEQ ID NO:10, said sequence being located upstream of the N-terminal coding part of the Otoferlin gene.

7. The vector system according to claim 1 to 6, wherein said second polynucleotide also contains a WPRE sequence of SEQ ID NO:23.

8. The vector system according to any of claims 1-7, wherein said N-terminal coding part of the Otoferlin gene consists in: the nucleotides 1-2214, the nucleotides 1-2406, the nucleotides 1-2523, the nucleotides 1-2676, the nucleotides 1-2991 or the nucleotides 1-3126 of the Otoferlin gene of SEQ ID NO:15 or of an homologous sequence thereof.

9. The vector system according to claim 8, comprising at least:

a) one AAV8 particle comprising a first polynucleotide comprising an inverted terminal repeat at each end of said polynucleotide, and, between the said inverted terminal repeats, from 5' to 3': the CMV promoter of SEQ ID NO:9 and optionally an intronic sequence of SEQ ID NO:10, followed by the

nucleotides 1-2214, the nucleotides 1-2406, the nucleotides 1-2523, the nucleotides 1-2676, the nucleotides 1-2991 or the nucleotides 1-3126 of the Otoferlin gene of SEQ ID NO:15, and a splice donor site, and

b) one AAV8 particle comprising a second polynucleotide comprising an inverted terminal repeat at each end of said polynucleotide, and, between the said inverted terminal repeats, from 5' to 3': a splice acceptor site, the nucleotides 2215-5991, the nucleotides 2407-5991, the nucleotides 2524-5991, the nucleotides 2677-5991, the nucleotides 2992-5991 or the nucleotides 3127-5991 of the Otoferlin gene of SEQ ID NO:15, optionally followed by a WPRE sequence and/or a polyadenylation sequence,

wherein the said first and second polynucleotides also contain the AP recombinogenic sequence of SEQ ID NO:69 that is located after the splice donor site in said first polynucleotide and before the splice acceptor site in said second polynucleotide.

10. The vector system according to any of claim 8 or 9, comprising at least:

a) one AAV8 particle comprising a first polynucleotide comprising an inverted terminal repeat at each end of said polynucleotide, and, between the said inverted terminal repeats, from 5' to 3': the CMV promoter of SEQ ID NO:9 and optionally an intronic sequence of SEQ ID NO:10, followed by the nucleotides 1-2214, the nucleotides 1-2676, or the nucleotides 1-2991 of the Otoferlin gene of SEQ ID NO:15, and a splice donor site, and

b) one AAV8 particle comprising a second polynucleotide comprising an inverted terminal repeat at each end of said polynucleotide, and, between the said inverted terminal repeats, from 5' to 3': a splice acceptor site, the nucleotides 2215-5991, the nucleotides 2677-5991, or the nucleotides 2992-5991 of the Otoferlin gene of SEQ ID NO:15, optionally followed by a polyadenylation sequence,

wherein the said first and second polynucleotides also contain the AP recombinogenic sequence of SEQ ID NO:69 that is located after the splice donor site in said first polynucleotide and before the splice acceptor site in said second polynucleotide, and

wherein said second polynucleotide do not contain the WPRE sequence of SEQ ID NO:23.

11. A pharmaceutical composition comprising the vector system of any one of claim 1 to 10 as well as a pharmaceutically acceptable vehicle.

12. The pharmaceutical composition as defined in claim 11, for use for treating patients suffering from DFNB9 deafness or preventing DFNB9 deafness in patients having DFNB9 mutations.

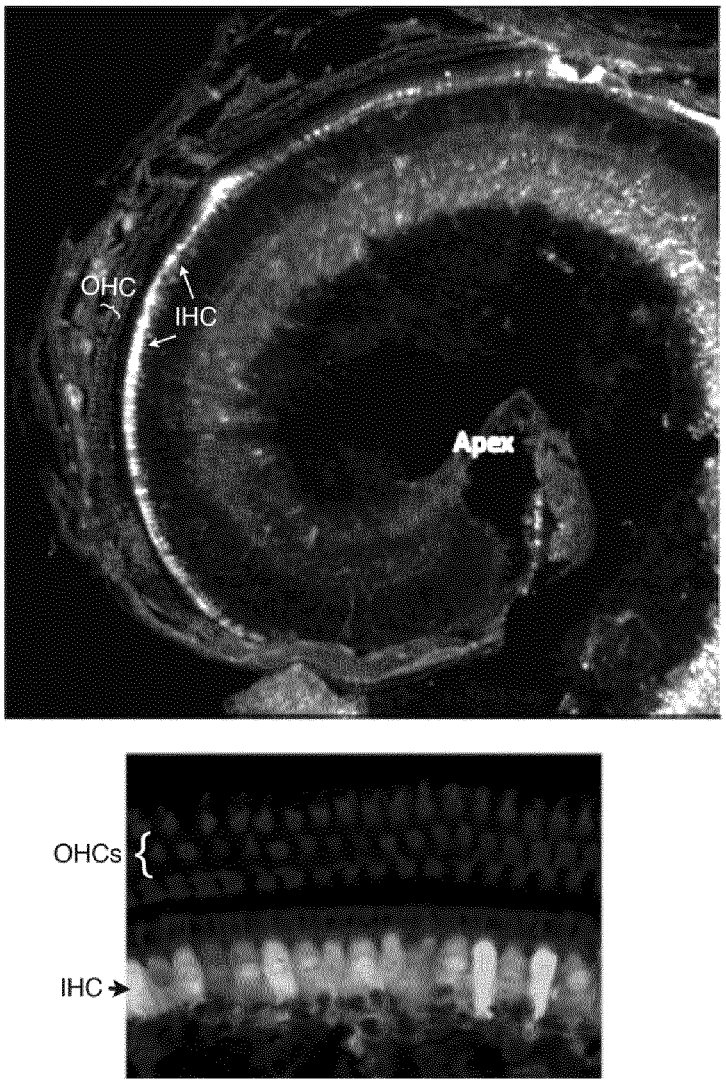
13. The composition for use according to claim 12, wherein said patients are human patients that have been diagnosed from the DFNB9 deafness after language acquisition.

14. The composition for use according to claim 12 or 13, wherein said patients are teenagers or adult humans suffering from DFNB9 deafness induced by thermosensitive mutations.

15. The composition for use according to claim 14, wherein said thermosensitive mutations are chosen from: PQ994VfsX6, P.I515T, p.G541S, PR1607W, pE1804del, c.2975_2978delAG/c.4819C>T, c.4819C>T (c.R1607W).

Figure 1

A


OHC
IHC
Apex
OHCs
IHC

B

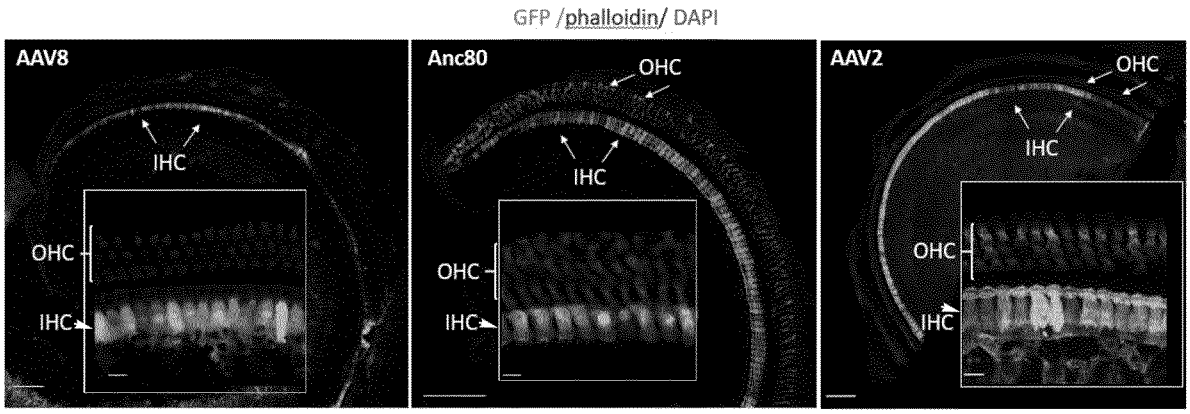
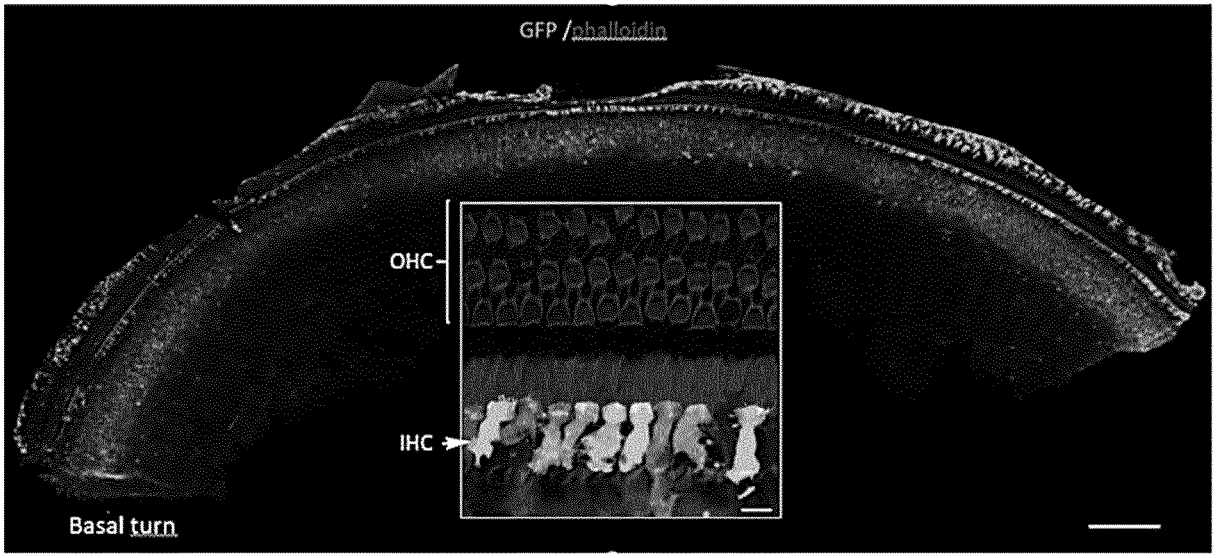

GFP /phalloidin/ DAPI
AAV8
IHC
OHC
IHC
Anc80
OHC
IHC
OHC
IHC
AAV2
OHC
IHC
OHC
IHC

Figure 1 (continuation)

C



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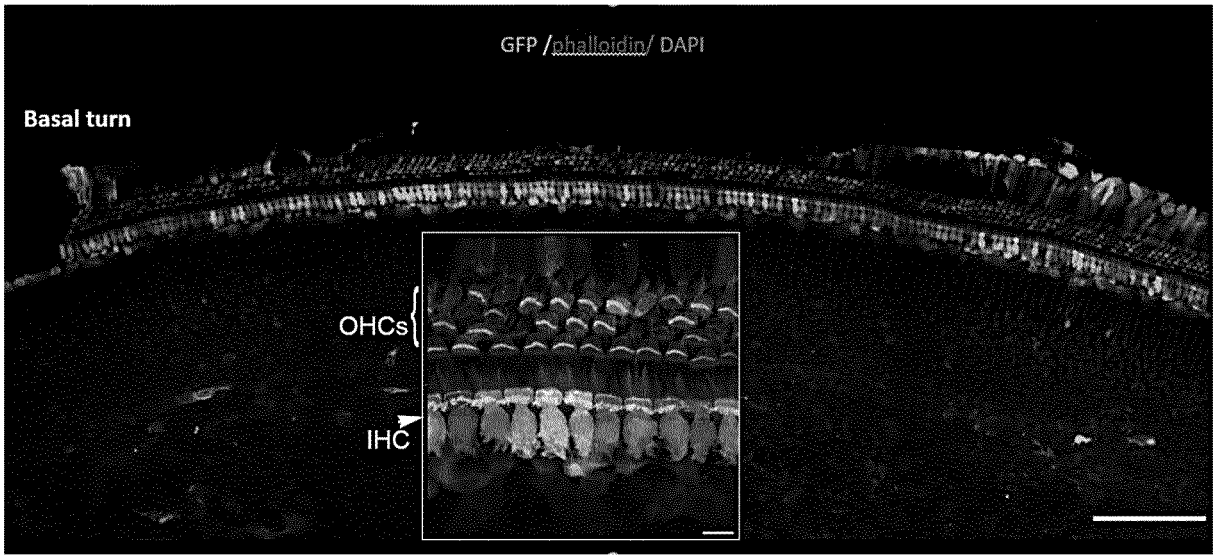
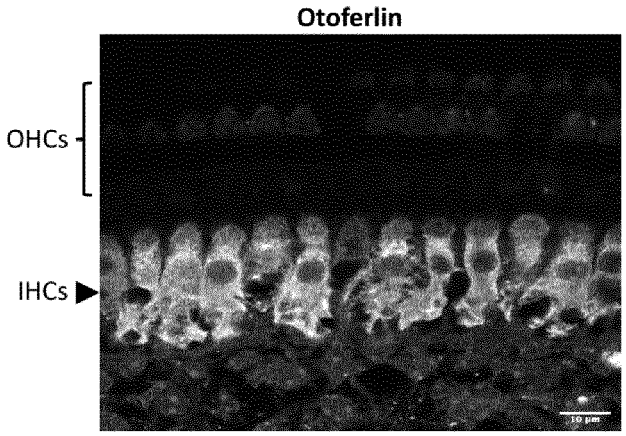


Figure 2

A


Otoferlin
OHCs
IHCs

B

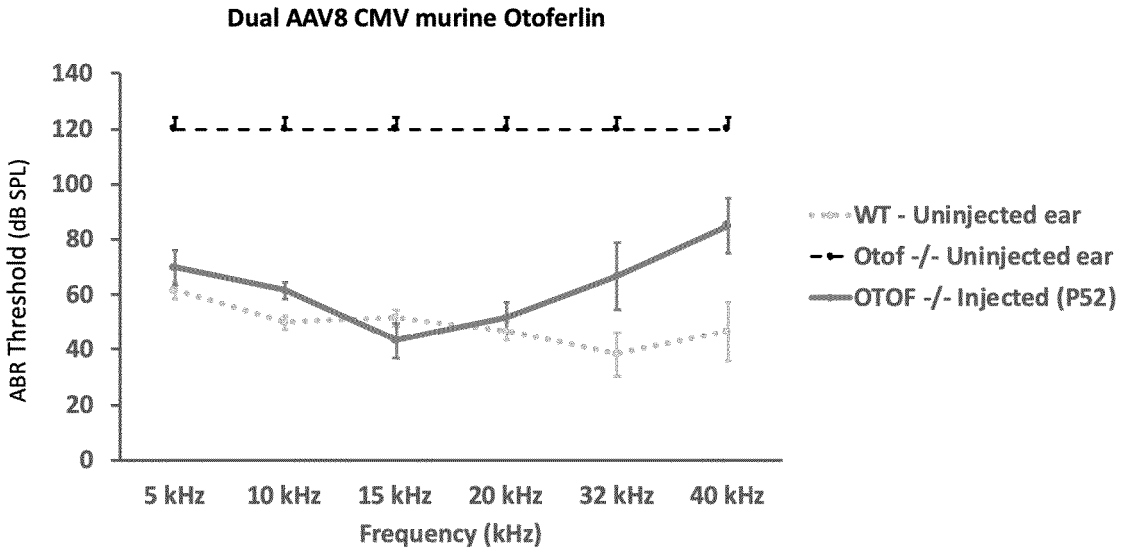
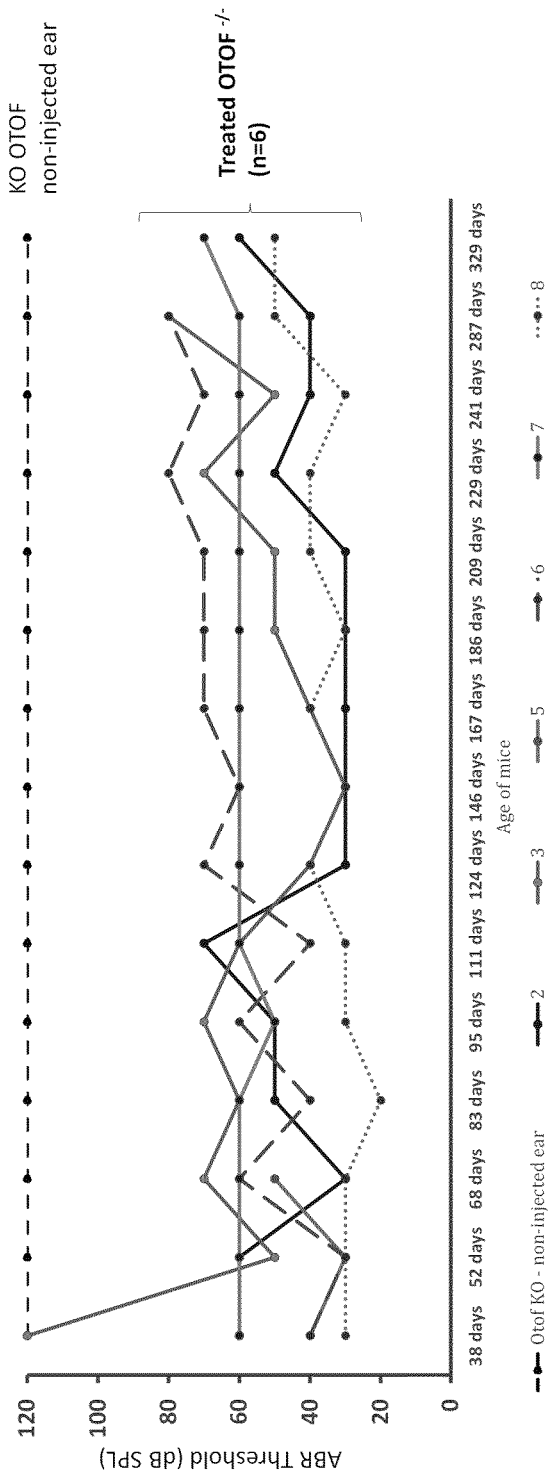

Dual AAV8 CMV murine Otoferlin
ABR Threshold (dB SPL)
140
120
100
80
60
40
20
0
5 kHz
10 kHz
15 kHz
20 kHz
32 kHz
40 kHz
Frequency (kHz)
WT - Uninjected ear
Otof -/- Uninjected ear
OTOF -/- Injected (P52)

Figure 2 (Continuation)

C



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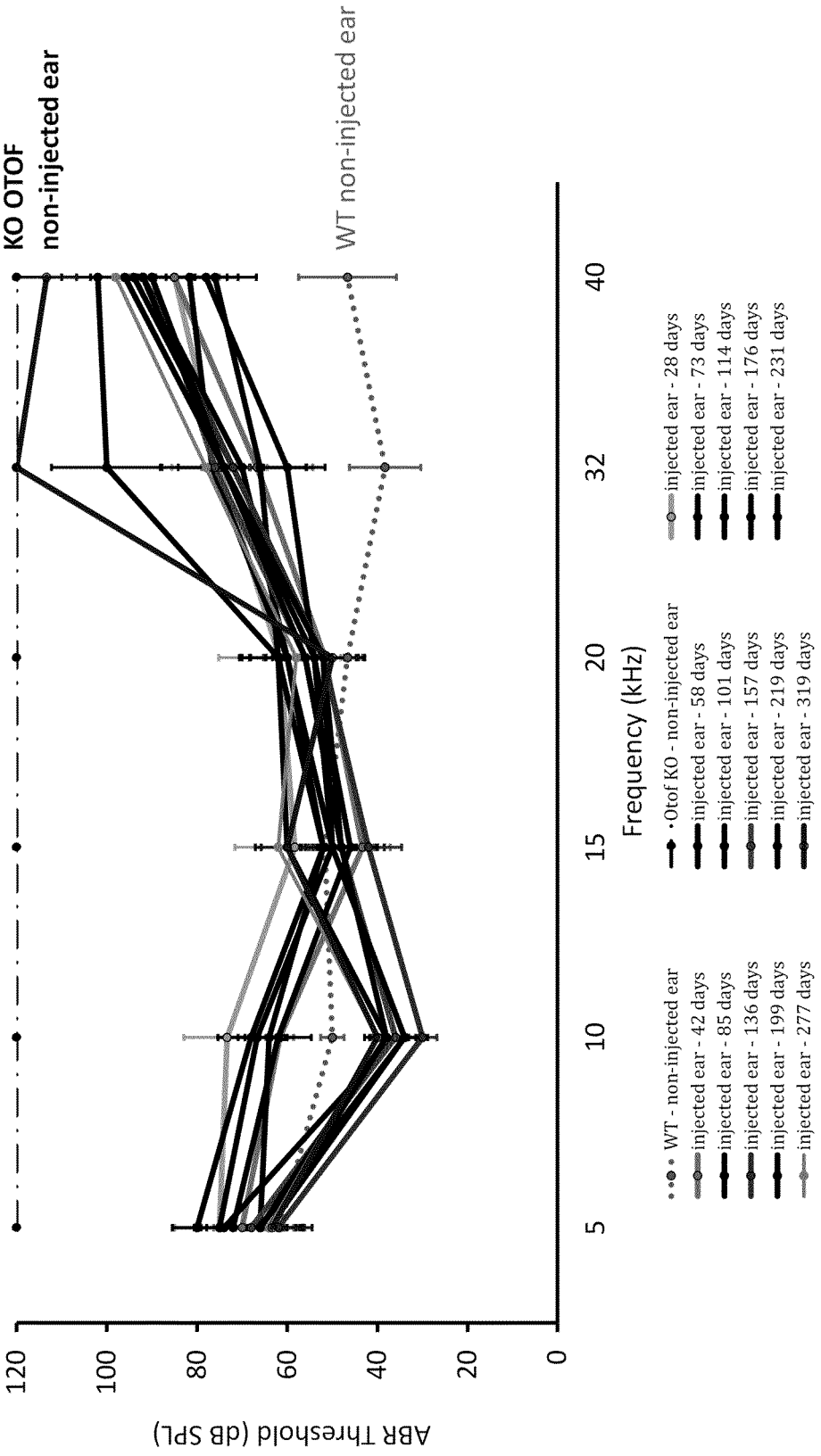

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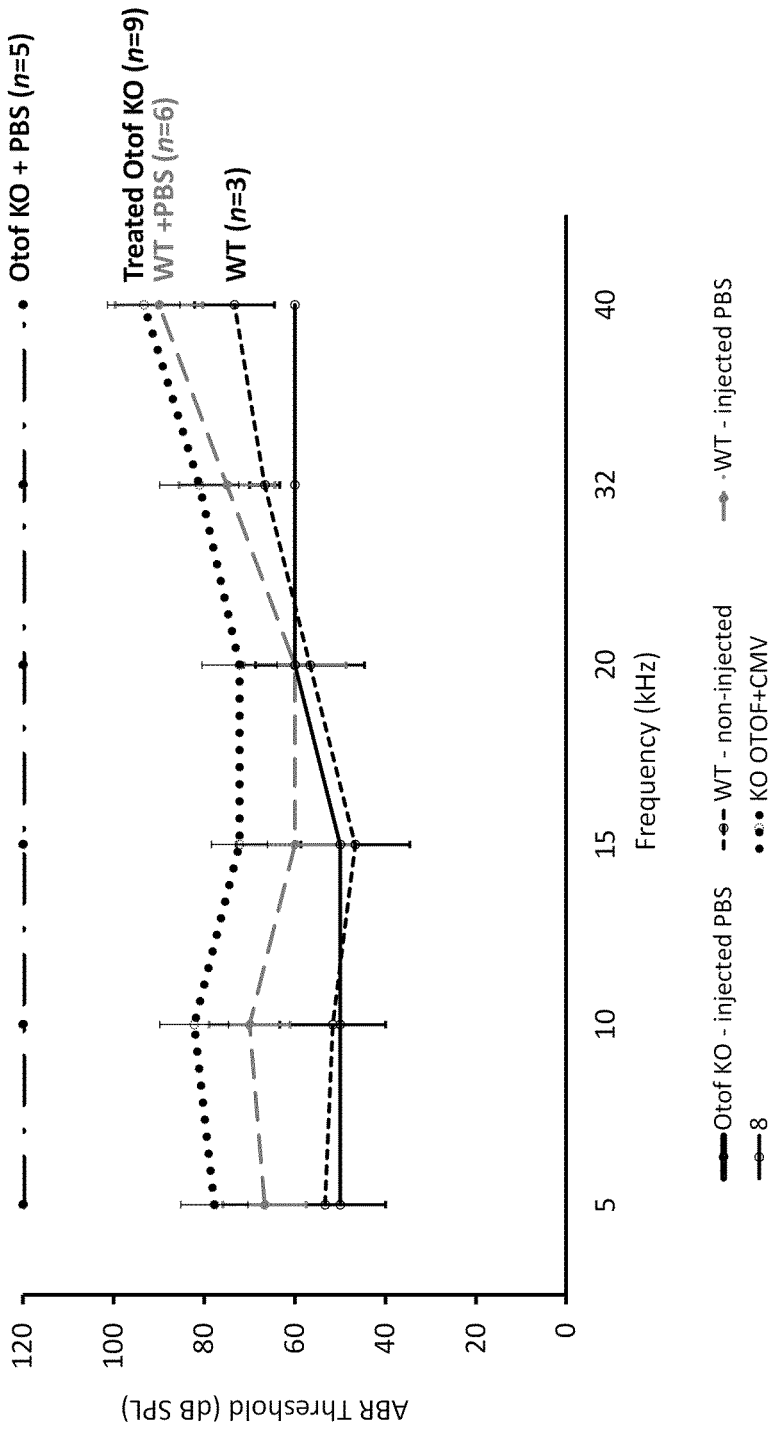

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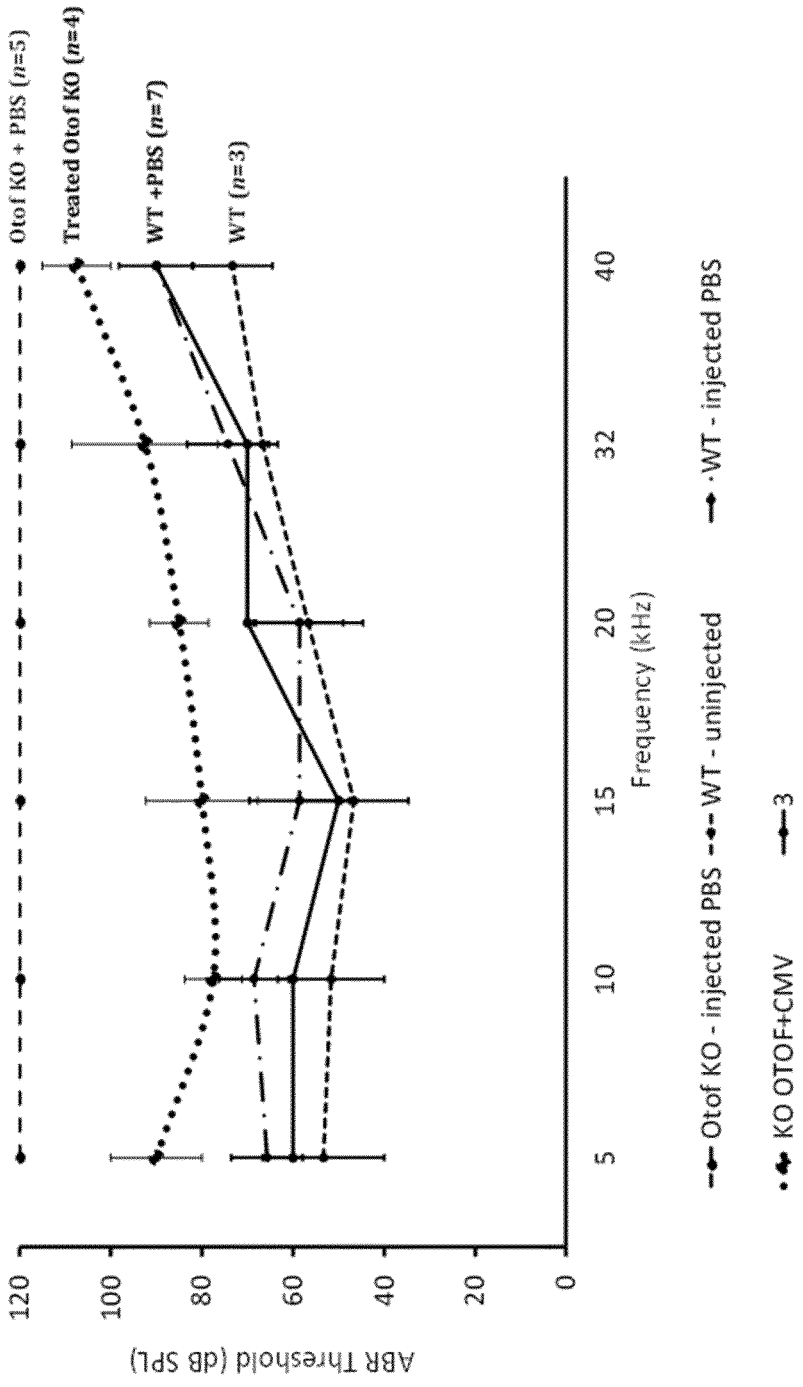
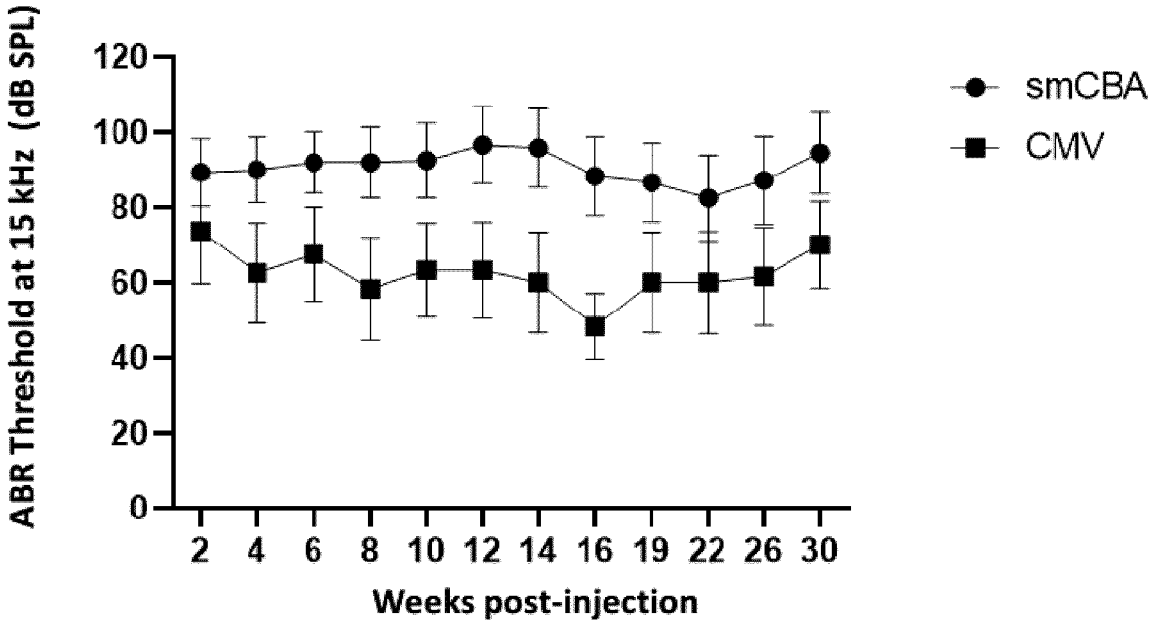


Figure 2 (continuation)

G



H

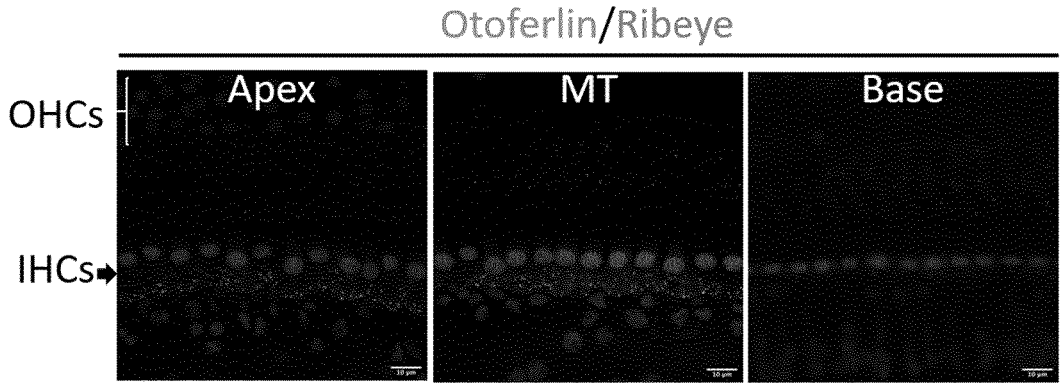


Figure 2 (continuation)

I

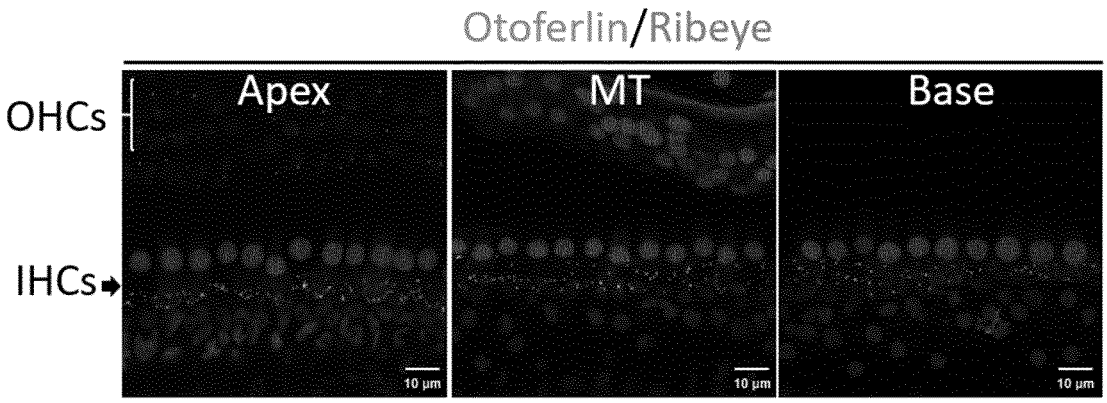


Figure 3

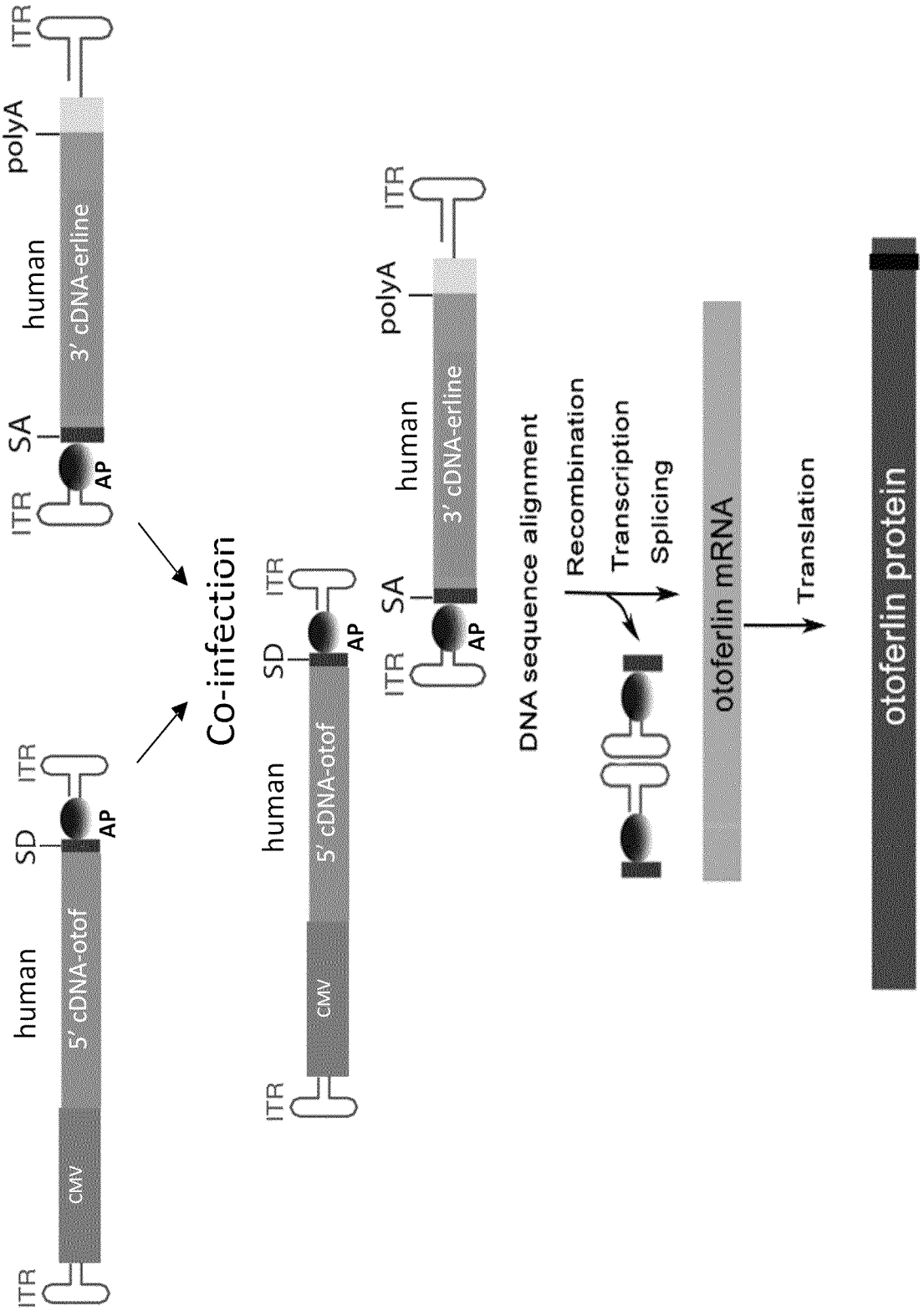

ITR
polyA
human
3′ cDNA-erline
SA
AP
ITR
ITR
SD
AP
human
5′ cDNA-otof
CMV
ITR
Co-infection
ITR
SD
AP
human
5′ cDNA-otof
CMV
ITR
ITR
polyA
human
3′ cDNA-erline
SA
AP
ITR
DNA sequence alignment
Recombination
Transcription
Splicing
otoferlin mRNA
Translation
otoferlin protein

Figure 4

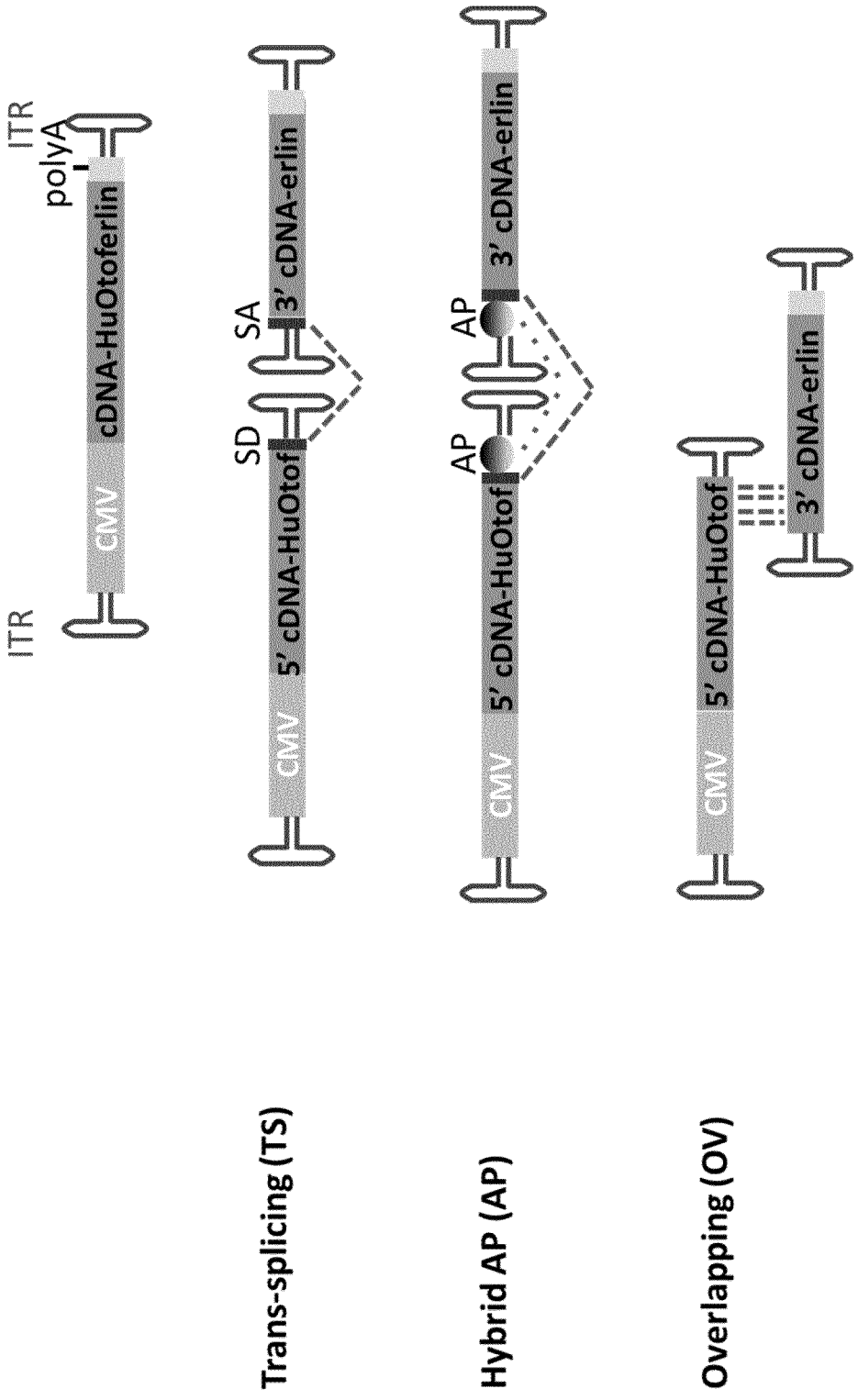

ITR
polyA
cDNA-HuOtoferlin
CMV
ITR
Trans-splicing (TS)
SA
3' cDNA-erlin
SD
5' cDNA-HuOtof
CMV
Hybrid AP (AP)
AP
3' cDNA-erlin
AP
5' cDNA-HuOtof
CMV
Overlapping (OV)
3' cDNA-erlin
5' cDNA-HuOtof
CMV

Figure 5

A

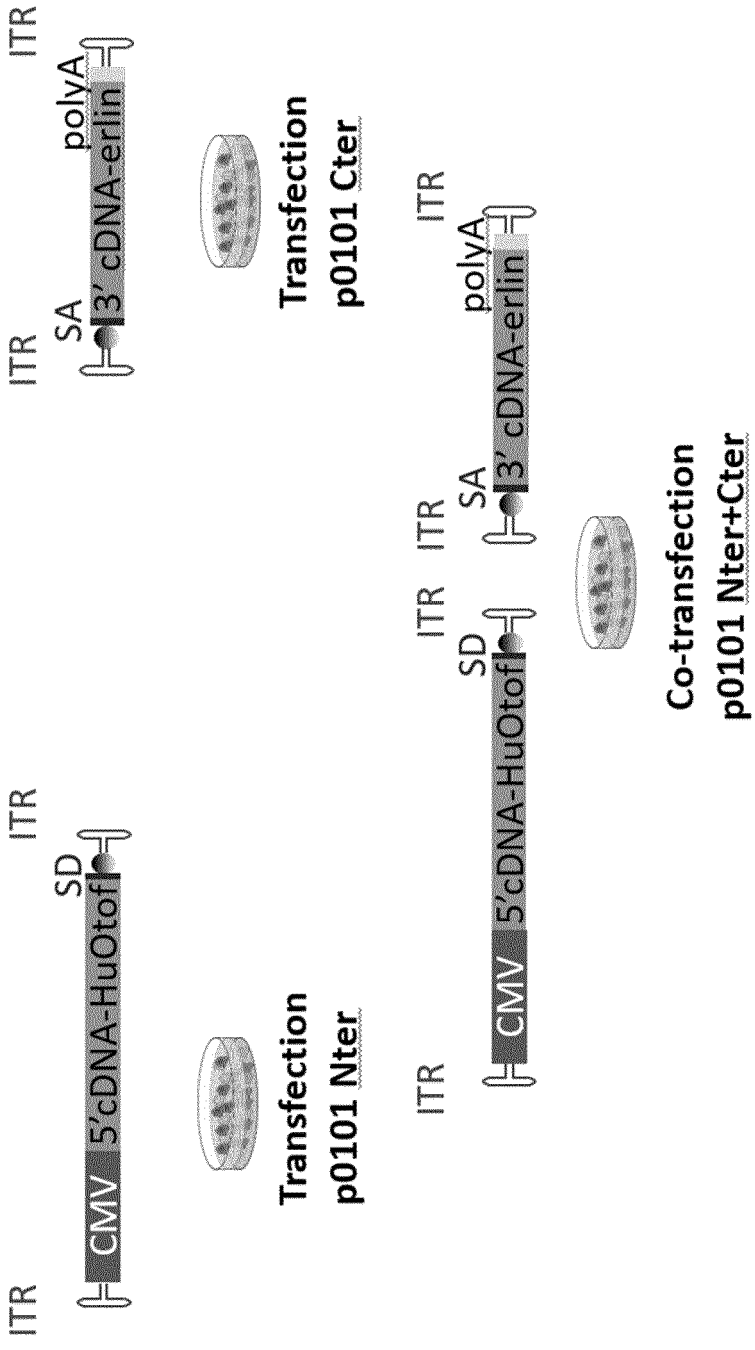

ITR
CMV
5'cDNA-HuOtofl
SD
ITR
Transfection
p0101 Nter
ITR
SA
3' cDNA-erlin
polyA
ITR
Transfection
p0101 Cter
ITR
CMV
5'cDNA-HuOtofl
SD
ITR
ITR
SA
3' cDNA-erlin
polyA
ITR
Co-transfection
p0101 Nter+Cter

Figure 5 (continuation)

B

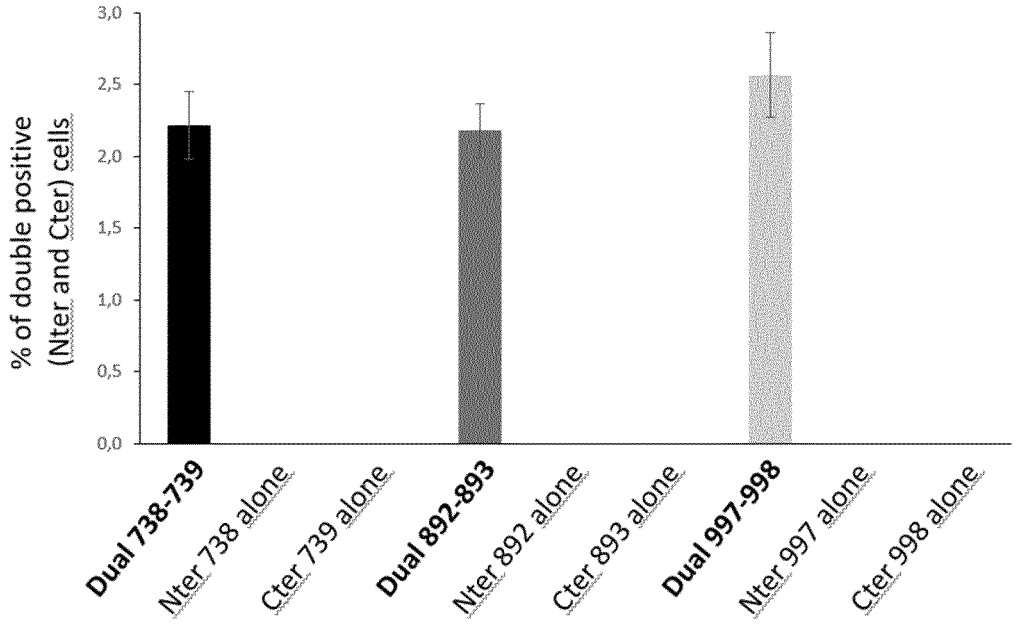


Figure 6

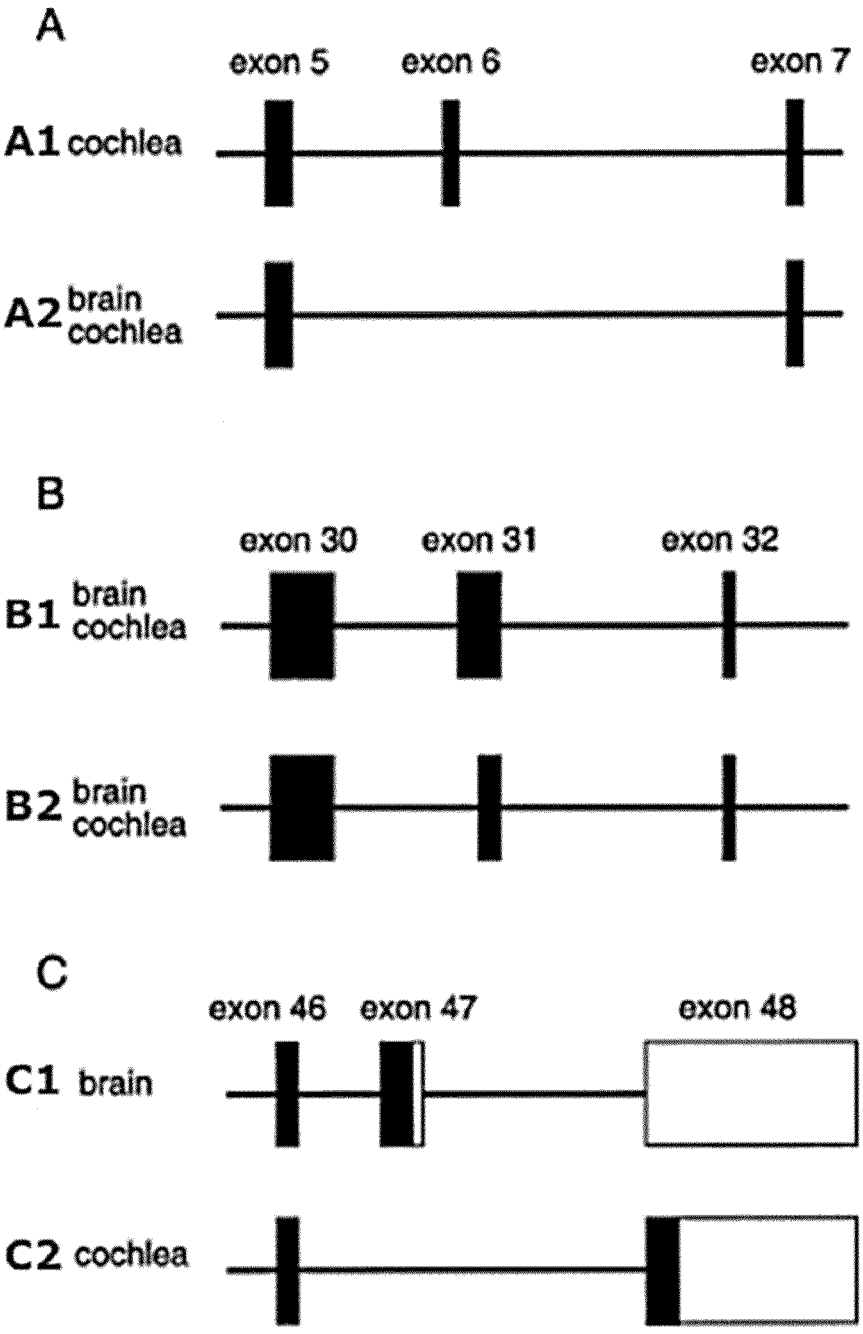

A
exon 5
exon 6
exon 7
A1 cochlea
A2 brain cochlea
B
exon 30
exon 31
exon 32
B1 brain cochlea
B2 brain cochlea
C
exon 46
exon 47
exon 48
C1 brain
C2 cochlea

Figure 7

A

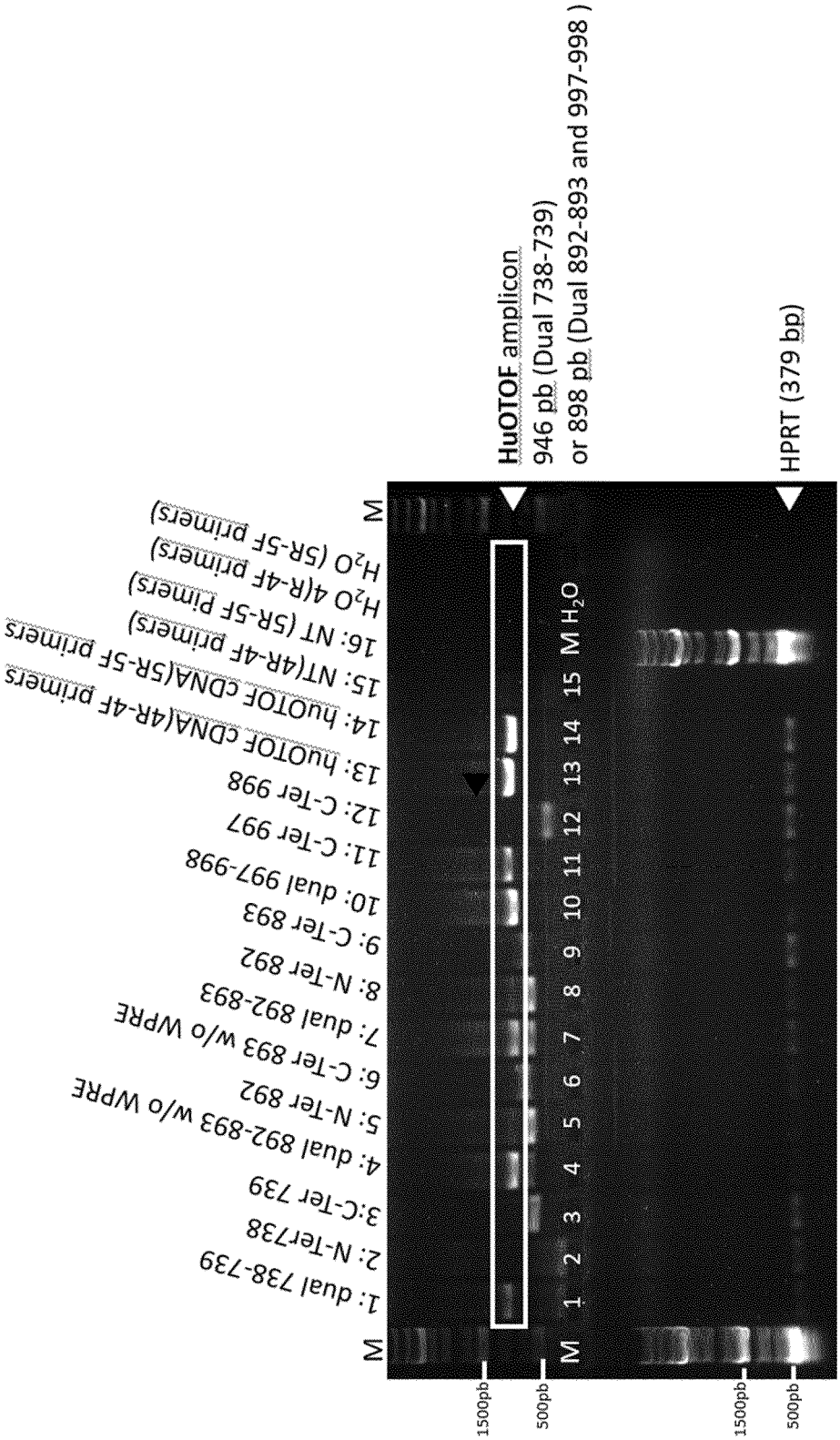

HuOTOF amplicon
946 pb (Dual 738-739)
or 898 pb (Dual 892-893 and 997-998)
HPRT (379 bp)
M
1: dual 738-739
2: N-Ter738
3:C-Ter 739
4: dual 892-893 w/o WPRE
5: N-Ter 892
6: C-Ter 893 w/o WPRE
7: dual 892-893
8: N-Ter 892
9: C-Ter 893
10: dual 997-998
11: C-Ter 997
12: C-Ter 998
13: huOTOF cDNA(4R-4F primers
14: huOTOF cDNA(5R-5F primers
15: NT(4R-4F primers)
16: NT (5R-5F primers)
H2O 4(R-4F primers)
H2O (5R-5F primers)
M
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 M H2O
1500pb
500pb
1500pb
500pb

Figure 7 (continuation)

B

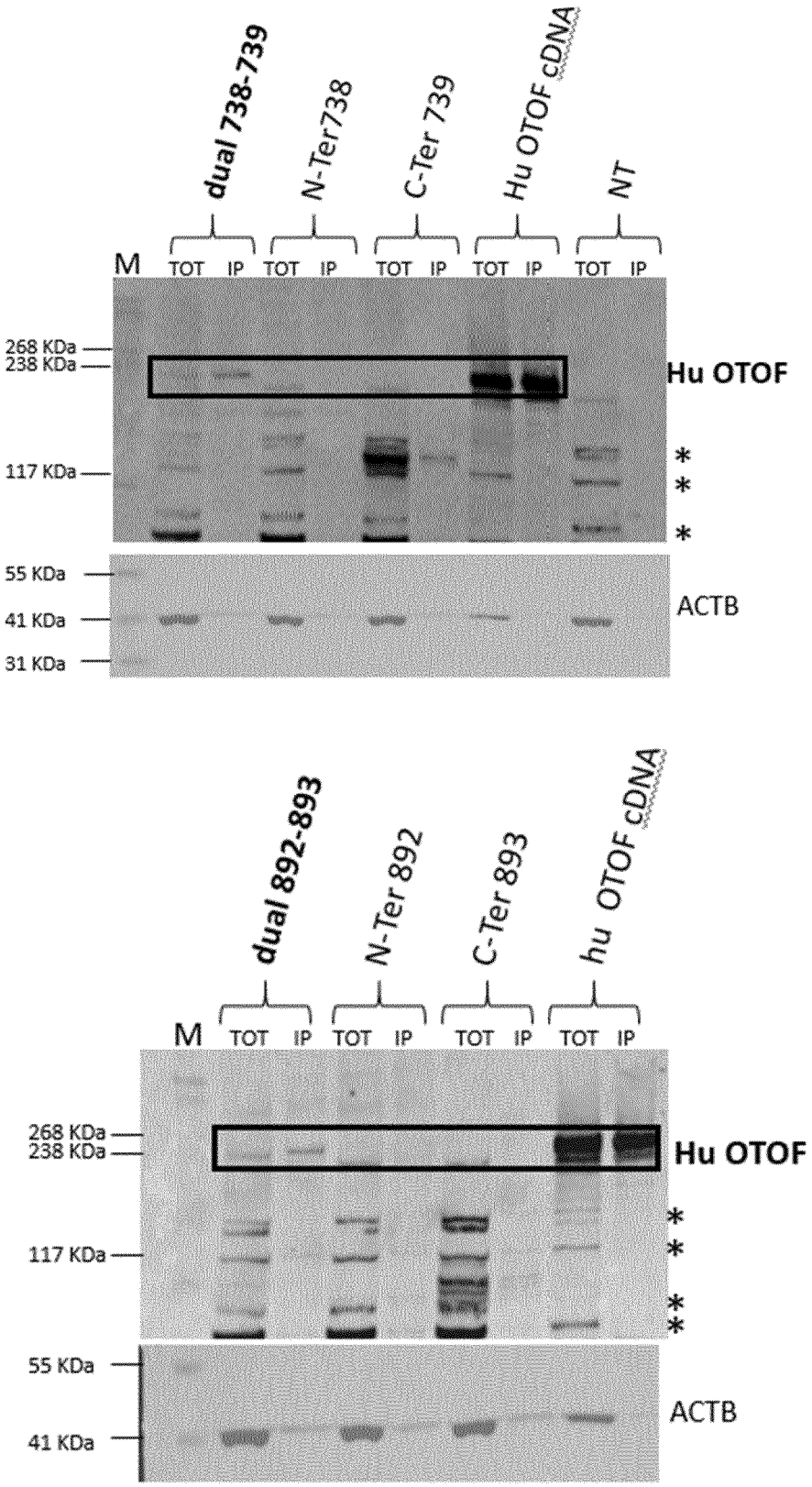


Figure 7 (continuation)

B

