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# Comprendre et traiter les surdités génétiques : étude de l'otoferline, et stratégies thérapeutiques utilisant des vecteurs synthétiques

Jean-Christophe Leclère

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# THESE DE DOCTORAT DE

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Par

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**Comprendre et traiter les surdités génétiques : étude de l'otoferline,  
et stratégies thérapeutiques utilisant des vecteurs synthétiques**

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## Abréviations

AAV : virus adéno-associé

ABR : auditory brainstem response (Potentiels Évoqués Auditifs)

ADN : acide désoxyribonucléique

AMPA :  $\alpha$ -amino-3-hydroxy-5-méthyl-4-isoxazolepropionique acid

ANSD : neuropathie auditive

ARN: acide ribonucléique

BSV163 : Brest Synthetic Vector

Ca<sub>v</sub>1.3 : canaux calciques voltage-dépendants de type L

CCE : cellule ciliée externe

CCI : cellule ciliée interne

CMV : cytomégalovirus

CRISPR-Cas9 : Clustered Regularly Interspaced Short Palindromic Repeats – associated protein : outil moléculaire permettant de couper l'ADN génomique au niveau d'une séquence spécifique (endonucléase)

DAPI : 4',6-diamidino-2-phénylindole

dB: décibel

DFNB9 : autosomal recessive deafness 9

DOPE : dioleoylphosphatidylethanolamine

DPOAE : produits de distorsion des oto-émission acoustiques, évaluant le fonctionnement des cellules ciliées externes

DSB : cassure double brin

GFP : green fluorescent protein

gRNA : guide ARN

HEPES : acide 4-(2-hydroxyéthyl)-1-pipérazine éthane sulfonique

HNSCC : cancer de la tête et du cou

IHC : cellule ciliée interne

KLN47 : Karine Le Ny

LBNP : nanoparticule lipidique

NHEJ : jonction d'extrémités non homologues

OCM : oncomoduline

OHC : cellule ciliée externe

OTOF : gène codant pour l'otoferline

PAM : motif adjacent à la séquence intercalaire, séquence nécessaire à l'action de l'endonucléase

PBS : phosphate-buffered saline

PCR : amplification en chaîne par polymérase

PEA : Potentiels Évoqués Auditifs

PEI : polyéthylèneimine

PLGA : poly(lactide-co-glycolide)

RT-PCR : transcription inverse suivie d'une réaction en chaîne de la polymérase

RWM : membrane de la fenêtre ronde de la cochlée

TMD : domaine transmembranaire

# Introduction

## I Système auditif

### 1. Anatomie de l'oreille

L'oreille humaine est constituée de trois parties : l'oreille externe, l'oreille moyenne et l'oreille interne (Fig 1A). L'oreille externe comprend le pavillon et le conduit auditif externe. Le pavillon, constitué de cartilage, présente des reliefs uniques à chaque individu, il diffuse les ondes sonores vers le conduit auditif et participe à la localisation spatiale. L'oreille moyenne est composée de la membrane tympanique, reliée à trois osselets qui forment une chaîne : le marteau, l'enclume et l'étrier, qui est en contact avec la fenêtre ovale de la cochlée. Elle est tapissée d'un épithélium respiratoire. Lorsque des ondes sonores atteignent le conduit auditif externe, la membrane tympanique transmet les vibrations à la chaîne ossiculaire, qui amplifie et transmet ces vibrations à la fenêtre ovale de la cochlée, l'organe de l'audition situé dans l'oreille interne. L'oreille moyenne joue donc un rôle important dans l'adaptation fine de l'impédance des ondes acoustiques entre le milieu aérien externe et le milieu liquide de la cochlée. Une explication sur son fonctionnement en vidéo est disponible à cette adresse :



Figure 1 : Schéma d'une coupe frontale de l'os temporal (A), de la cochlée (B) et de l'un de ses tour de spire, avec de dedans en dehors, le ganglion spiral, la bandelette sillonnée, les neurites, et les cellules ciliées (C). Schéma de l'organe de Corti (D), d'une cellule ciliée interne (E), et de la zone active au contact d'une fibre nerveuse (F).

La cochlée est la partie auditive principale de l'oreille interne, une structure creuse enroulée autour d'une zone centrale, qui contient le nerf auditif, appelé modiolus (Fig 1 B). Elle mesure environ

32 mm de long et fait 2 tours et demi de spires chez l'homme, et sa largeur diminue de la base à l'apex. Elle est divisée en trois compartiments : le canal cochléaire, qui contient de l'endolymphe, et les rampes vestibulaire et tympanique, qui contiennent de la périlymphe (Fig 1 C). Ces rampes communiquent à l'apex par l'hélicotrème. Le canal cochléaire est séparé en haut de la rampe vestibulaire par la membrane de Reissner, et en bas de la rampe tympanique par la membrane basilaire. L'organe de Corti est situé à la partie inféro-médiane du canal cochléaire (Fig 1 D). La cochlée a une caractéristique particulière : une fréquence spécifique provoque un pic de vibration dans une zone spécifique de la membrane basilaire, c'est ce qu'on appelle la tonotopie cochléaire (Fig 2). Chaque segment de la membrane basilaire correspond à une fréquence spécifique. Les hautes fréquences stimulent la base de la cochlée alors que les fréquences graves stimulent l'apex.

En complément de la cochlée, l'oreille interne contient le vestibule, l'organe de l'équilibre, situé à l'arrière de la cochlée. Le vestibule permet le maintien de la posture et la détection de la position et l'accélération de la tête dans l'espace.

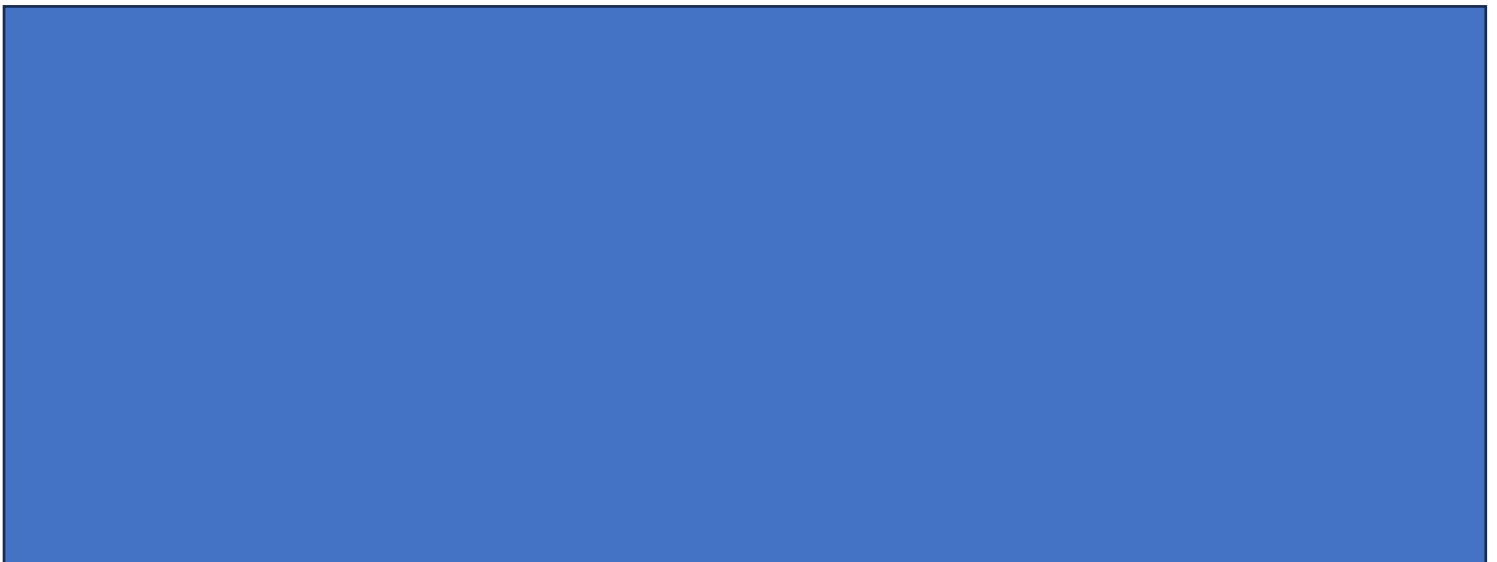


Figure 2. Schéma de la tonotopie : distribution des fréquences le long de la membrane basilaire d'une cochlée humaine, montrant également la variation de la largeur de la membrane basilaire, de la base à l'apex. B Position du maximum de l'onde sonore qui se propage le long de la cochlée en fonction de la fréquence du son.

## 2. Transduction du son

La transduction mécano-électrique est le processus par lequel les cellules ciliées de l'oreille interne convertissent les ondes sonores en signaux électriques. Les mouvements des liquides de l'oreille interne, provoqués par les ondes sonores, induisent la déformation de la membrane basilaire, laquelle entraîne l'activation des cellules ciliées internes (CCI) située dans l'organe de Corti. Les CCI transforment la stimulation mécanique en signaux électriques, qui sont ensuite transmis par les cellules ganglionnaires dans le modiolus, au centre de la cochlée.



Les cellules sensorielles auditives (CCI et cellules ciliées externes (CCE)) possèdent à leur pôle apical des stéréocils (Fig 1 E). Ils sont constitués de filaments d'actine organisés en rangées décroissantes. Ces stéréocils sont ancrés dans une plaque cuticulaire rigide et sont composés de diverses protéines, notamment des myosines, des cadhérines et des protéines de type harmonine (Koffler et al., 2015).

A l'extrémité de ces stéréocils, il existe des canaux de mécanotransduction qui sont des pores cationiques non sélectifs, principalement perméables au potassium ( $K^+$ ) et au calcium ( $Ca^{2+}$ ). La déflexion des stéréocils, entraînée par l'onde sonore, entraîne l'ouverture ou la fermeture de ces canaux de mécanotransduction (Ohmori, 1985).

### 3. Cellules ciliées internes

Les CCI mesurent 30  $\mu m$  de haut, et 10  $\mu m$  de large en moyenne. Elles sont plus larges à la base qu'à l'apex. Elles possèdent plusieurs zones actives présynaptiques à leur pôle basal, qui permettent l'exocytose du neurotransmetteur, le glutamate (Fig 1F). Les zones actives sont constituées de structures denses appelées rubans synaptiques (Schmitz et al., 2000) (Fig 3), auxquels sont liées les vésicules synaptiques contenant le neurotransmetteur, le glutamate. Les rubans permettraient un relargage rapide du contenu des vésicules qui y sont attachées (appelé RRP - Readily Releasable Pool) (Khimich et al., 2005). Ils serviraient aussi de barrière de diffusion du calcium ( $Ca^{2+}$ ) pour maintenir une concentration localement importante pour la libération vésiculaire synchrone (Graydon et al., 2011), et faciliteraient le recrutement des vésicules vers la zone active, située au pôle basal des cellules (Jing et al., 2013).



Figure 3. Microscopie électronique d'une synapse à ruban d'une CCI de souris. Le ruban (rib) est ancré à la membrane plasmique présynaptique, en regard de la densité postsynaptique (PSD) des fibres nerveuses auditives. La flèche et l'étoile indiquent respectivement une vésicule synaptique associée au ruban et une vésicule endocytosomiale libre. D'après Nouvian 2006.

L'activation de l'exocytose des vésicules synaptiques se produit en réponse à la déflexion mécanique de la touffe ciliaire, provoquant l'ouverture des canaux de mécanotransduction et une dépolarisation des CCI. Cette dépolarisation, ou potentiel récepteur, est proportionnelle au courant de mécanotransduction, et déclenche l'ouverture des canaux calciques voltage-dépendants de type L ( $Ca_v1.3$ ) à la base synaptique. A proximité se situe une protéine composée d'un domaine transmembranaire ayant la capacité de fixer le  $Ca^{2+}$ , l'otoferline. L'augmentation du  $Ca^{2+}$  intracellulaire modifie la structure de l'otoferline, ce qui permet la fusion des vésicules, et induit la libération de glutamate dans la fente synaptique, qui active les récepteurs glutamatergiques AMPA des fibres afférentes du nerf auditif (Liberman et al., 2011). L'état des lieux des connaissances actuelles concernant l'otoferline sont détaillées dans l'article n°1 (p.12). En particulier, l'étude du rôle de son domaine trans membranaire, grâce à un nouveau modèle murin d'étude des protéines des cellules ciliées internes est expliqué dans l'article n°2 (p.33).

#### 4. Cellules ciliées externes

Les CCE sont des cellules cylindriques dont la hauteur varie le long de la cochlée en fonction de la tonotopie (Fig 4). Elles régulent la tension mécanique membranaire via un cytosquelette actine/myosine  $Ca^{2+}$ -dépendant (Dulon et al., 1990). De plus, la présence de prestine, une protéine piézoélectrique, confère aux CCE des propriétés d'électromotilité qui permet l'amplification locale des vibrations de la membrane basilaire, contribuant ainsi à l'amplification auditive et à la différenciation des fréquences proches (Zheng et al., 2000).

L'électromotilité des CCE est régulée par le système efférent médian cholinergique, qui agit par le biais de récepteurs nicotiques et de canaux calciques, modulant ainsi l'amplification des CCE et la sensibilité auditive, notamment par le biais de boucles réflexes (Glowatzki and Fuchs, 2000). Ces mécanismes sont indispensables pour la fonction auditive. La fonctionnalité des CCE est évaluée cliniquement par la mesure des otoémissions acoustiques : en plaçant un récepteur dans le conduit auditif externe, il est possible d'enregistrer une réponse acoustique à la suite d'une stimulation sonore, provenant des vibrations générées par les mouvements des CCE.



Figure 4. Schéma d'une cellule ciliée externe, possédant des stéréocils à son pôle apical. Ces stéréocils, situés au niveau de la plaque cuticulaire, sont en contact avec la membrane tectoriale pour les plus longs. Le noyau est situé à la base. Il existe également à proximité une connexion afférente neuronale de type II, en vert ; et une fibre efférente médiane, en rouge. © cochlea.eu

## 5. Innervation

Les CCI sont innervées par des fibres afférentes du nerf auditif, principalement de type I, qui forment des synapses glutamatergiques en face des rubans présynaptiques (Delacroix and Malgrange, 2015). Chaque CCI contient entre 15 et 20 synapses afférentes. Les fibres afférentes de type I transmettent le signal auditif des CCI vers les centres auditifs via les neurones des noyaux cochléaires, tandis que les fibres afférentes de type II, en plus petit nombre, innervent plusieurs CCE et pourraient jouer un rôle dans la détection de fortes intensités sonores (Weisz et al., 2012).

## 6. La voie auditive centrale – Potentiels Évoqués Auditifs – PEA

La voie auditive centrale est constituée de quatre relais après la première synapse entre les CCI et les fibres afférentes, à savoir les noyaux cochléaires, le complexe olivaire supérieur, le colliculus inférieur et le thalamus (Fig 5A). Ces relais permettent un transfert rapide (<10 ms) du signal auditif jusqu'au cortex auditif. En enregistrant les signaux électriques par une électrode cutanée située sur le crâne en réponse à des stimulations sonores répétées, les potentiels évoqués auditifs - PEA (ou Auditory Brainstem Response - ABR), permettent d'évaluer la fonction de cette voie et d'analyser la qualité de la transmission des stimuli auditifs de la cochlée jusqu'au cortex auditif (Fig 5B).

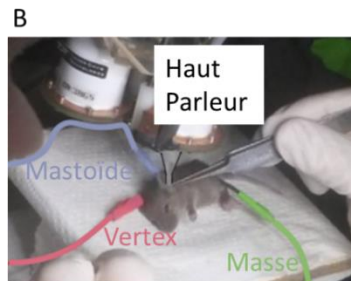


Figure 5 : Schéma de la voie auditive centrale, avec un exemple d'enregistrement de potentiels évoqués auditifs (PEA), avec la correspondance entre les ondes enregistrées et chaque relais de la voie auditive (A). Exemple de positionnement des électrodes pour l'enregistrement de PEA chez la souris (B).

## 7. Surdités et traitements

La surdité est la déficience sensorielle la plus fréquente dans les pays dits développés. En France, le dépistage auditif est proposé systématiquement à la naissance depuis 2012. La prévalence des surdités bilatérales, de niveau modéré à profond, chez les nouveau-nés est estimée à 1,5 à 2,7 cas pour mille enfants dépistés (Hilgert et al., 2009). Plus de 80 % des cas de surdité congénitale ont une origine génétique. Une large majorité (90 %) de ces surdités génétiques sont non-syndromiques, c'est-à-dire isolées, sans autres symptômes (Tanaka-Ouyang et al., 2017). Elles sont de transmission autosomique récessive dans plus de 85 % des cas, autosomique dominante dans 10 à 15 % des cas, liée à l'X ou mitochondrial dans 1 % des cas. Par exemple, la mutation du gène OTOF qui code pour l'otoferline provoque une surdité sévère à profonde, de transmission autosomique récessive, appelée autosomal recessive deafness 9 - DFNB9 (Yasunaga et al., 1999).

Les traitements dépendent du degré de perte auditive. Actuellement, il n'existe aucun traitement étiologique pour les surdités d'origine génétique. Les deux principales options sont les prothèses auditives et les implants cochléaires. Pour les surdités légères (20-40 dB) à modérées (41-70 dB), les prothèses auditives sont privilégiées en première intention. Elles sont portées à l'extérieur de l'oreille, et permettent une amplification du son. Pour les surdités sévères (71-90 dB) à profondes (> 90 dB) bilatérales, les implants cochléaires sont indiqués. Ils encodent le son sous forme d'impulsions électriques transmises au nerf auditif via un réseau d'électrodes (Purcell et al., 2021). Bien que ces dispositifs permettent généralement le développement de la parole et une communication correcte,

ils ont certaines limites en termes d'intelligibilité, justifiant les intérêts actuels pour le développement de traitements étiologiques afin d'améliorer la compréhension et la qualité de vie des patients (Géléoc and Holt, 2014).

## II Thérapie génique

La thérapie génique consiste à introduire du matériel génétique dans des cellules ou à modifier les gènes des cellules pour traiter une maladie. Il existe différentes stratégies pour y parvenir :

### 1. La supplémentation génique

L'objectif est d'introduire *in vivo* une copie fonctionnelle d'un gène dans des cellules cibles, permettant ainsi son expression et la production de la protéine déficiente. L'introduction de la copie du gène est réalisée grâce à l'utilisation de vecteurs. Cette approche est privilégiée dans le traitement des maladies monogéniques.

### 2. L'édition du génome

L'édition du génome permet la correction ciblée de mutations génétiques. Cette approche nécessite l'introduction d'enzymes spécifiques (nucléases) qui coupent le génome à un ou des emplacements précis, ainsi qu'un segment d'ADN pour la réparation du génome, restaurant ainsi un gène fonctionnel. Parmi les nucléases, il existe le système CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) qui permet de couper l'ADN du génome grâce à un guide ARN (gRNA) spécifique (Charpentier and Doudna, 2013) (Fig 6).

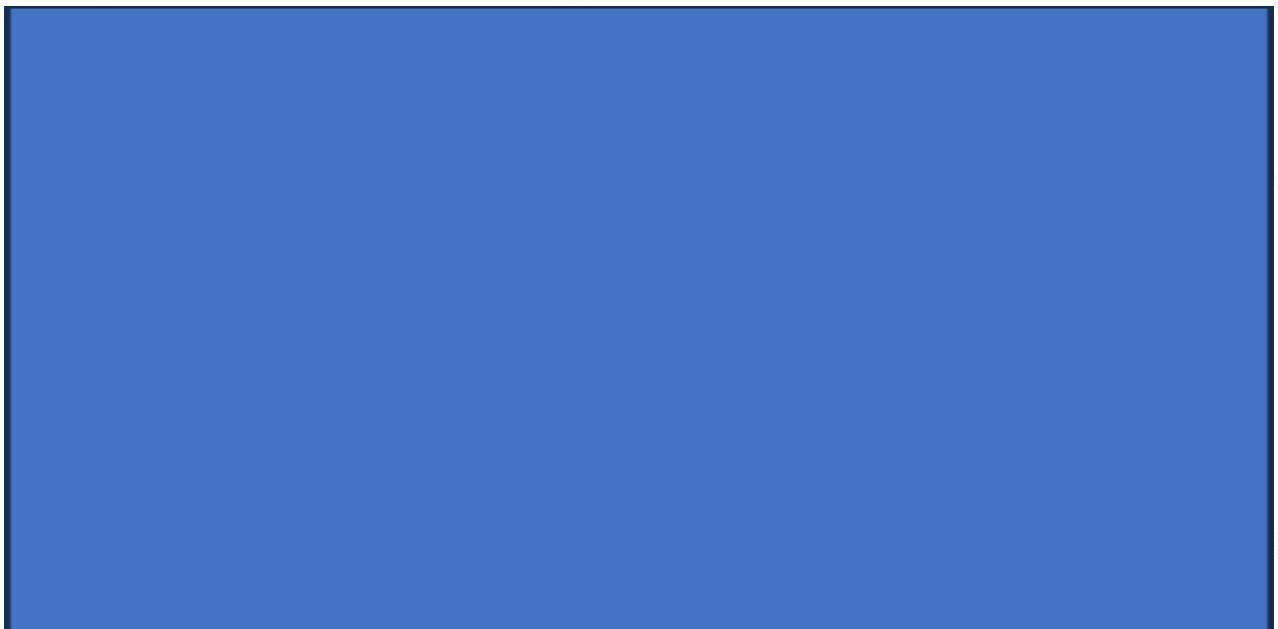


Figure 6. Schéma explicatif de l'action de la CRISPR-Cas9. Pour obtenir une coupure de l'ADN double brin, plusieurs éléments sont nécessaires. Il faut un ARN guide (ARNg), en vert, spécifique de la séquence d'ADN que l'on souhaite cibler. Cet ADN cible doit par ailleurs contenir une séquence PAM (Protospacer Adjacent Motif) de 2 à 6 paires de base, en jaune. Ensuite, l'ARNg est combiné avec une

enzyme Cas9. La Cas9 est une endonucléase capable de couper l'ADN à un endroit spécifique déterminés par l'ARNg. Lorsque l'ARNg se lie à sa séquence cible, la Cas9 induit une coupure double brin dans l'ADN. Après cette coupure, la cellule active des mécanismes de réparation de l'ADN. Ces processus peuvent entraîner des modifications génétiques, telles que des insertions, des délétions, voire des remplacements de séquences d'ADN. ©Gunilla Elam / Science Photo Library

### 3. Modification de l'ARN

Cette approche consiste à inciter les cellules à produire des versions modifiées de la protéine déficiente. Elle implique l'injection d'oligonucléotides antisens qui se fixent à l'ARN messager transcrit à partir du gène muté, modifiant son épissage, une étape indispensable avant sa traduction en protéine fonctionnelle. Des essais cliniques ont démontré avec succès l'efficacité de la modulation de l'épissage, notamment pour le traitement de l'amyotrophie spinale (Hammond et al., 2016).

### 4. Transfert de gènes

Une des difficultés de la thérapie génique est d'introduire un acide nucléique à visée thérapeutique et/ou une endonucléase dans les cellules cibles. Actuellement, environ 20% des essais de thérapie génique ont été réalisés par injection directe d'ADN, modifié et protégé des nucléases grâce à des modifications chimiques, ou intégré dans un plasmide. Cette approche est possible pour la peau et le muscle, mais inefficace pour l'oreille interne (Wolff et al., 1990). Les vecteurs viraux sont actuellement les plus utilisés, dans 75% des essais cliniques de thérapie génique. Il existe deux types de vecteurs viraux, intégratifs : l'ADN du vecteur viral s'intègre dans l'ADN de l'hôte (lentiviraux), et non intégratifs : le gène thérapeutique demeure dans la cellule sans s'intégrer au génome (virus adéno associés, AAV). Cependant, leur utilisation peut se trouver limitée en raison de l'exposition naturelle au virus AAV sauvage : un certain nombre de patients ont des anticorps dirigés contre ces virus (Lahlou et al., 2023). En outre, le vecteur déclenchant une réaction immunitaire, son utilisation est souvent restreinte à une injection unique. En parallèle, la mise au point de vecteurs et de techniques non viraux pour la délivrance de gènes et de complexes permettant une édition génomique se développe. Bien que ces approches soient moins efficaces, elles ont deux principaux avantages, les réactions immunitaires sont plus limitées et leur production est plus aisée. Les différentes approches non virales de thérapie génique de l'oreille interne sont détaillées dans l'article n°3 (p.53). Plusieurs nouveaux vecteurs non viraux ont été testés au cours de cette thèse, notamment des lipides cationiques. Ce sont des molécules amphiphiles constituées de trois parties distinctes : une tête polaire constituée de fonctions amines protonées, qui permet les interactions électrostatiques avec l'ADN, un bras espaceur, impliqué dans la stabilité et la biodégradabilité du vecteur, et un domaine hydrophobe constitué de chaînes lipophiles, permettant l'interaction avec les membranes cellulaires et l'assemblage supramoléculaire du complexe. Les résultats de l'utilisation de nouveaux vecteurs de type liposomes cationiques pour la thérapie génique de l'oreille interne sont détaillés dans l'article 4 (p.75).

### III Problématiques et objectifs de la thèse

#### 1. Première partie : étude de l'otoferline

Le rôle de l'otoferline n'est que partiellement connu. Il s'agit d'une protéine agissant comme un senseur calcique de haute affinité, qui est impliquée dans le processus de recrutement des vésicules vers la zone active. De plus, elle régule la vitesse de fusion des vésicules synaptiques. L'objectif initial était de réaliser un état des lieux des connaissances actuelles sur l'otoferline. Bien qu'impliqué en pathologie, avec de nombreuses mutations entraînant des surdités, le rôle du domaine transmembranaire est mal connu. Il nous a paru opportun de nous focaliser sur cette partie afin de mieux appréhender son rôle précis dans la transmission du signal. La création d'un modèle d'étude in vivo avec une expression mosaïque de l'otoferline tronquée, permettait d'évaluer son rôle en fonction du niveau d'expression de l'otoferline. L'hypothèse principale était que ce domaine transmembranaire avait un rôle majeur dans les processus de fusion des vésicules synaptiques. En conséquence, l'absence ou la modification de ce domaine pouvait provoquer des conséquences analogues à l'absence totale d'otoferline, conduisant potentiellement à un phénotype similaire à celui observé chez les souris KO-OTOF.

#### 2. Deuxième partie : stratégies thérapeutiques utilisant des vecteurs synthétiques

Les possibilités thérapeutiques actuelles pour les patients atteints de surdité d'origine génétique sont uniquement fonctionnelles et reposent principalement sur les aides auditives et les implants cochléaires. Toutefois, ces dispositifs ont des limites en termes de gain, en particulier dans les environnements bruyants. Ceci explique l'intérêt croissant pour le développement de traitements étiologiques afin d'améliorer non seulement la compréhension, mais aussi la qualité de vie des patients. La thérapie génique pourrait être un traitement étiologique prometteur pour restaurer au mieux la fonction auditive ; et dans cette optique, se sont les vecteurs viraux qui sont les plus utilisés. Cependant ils ont un certain nombre d'inconvénients dont leur risque d'effets secondaires notamment hépatotoxiques et/ou immunogènes. La présence d'une possibilité thérapeutique pour les surdités y compris sévères à profonde, avec peu d'effets secondaires (implant cochléaire) rend indispensable le développement d'un traitement étiologique avec un profil de tolérance optimal. Après réalisation d'un état des lieux des différentes options de transfert de gène sans utiliser de vecteur viral afin d'éviter ces effets secondaires, les lipides cationiques semblaient être des candidats idéaux, avec un bon profil de tolérance mais avec une efficacité moindre a priori. Actuellement, de nouveaux vecteurs de ce type sont développés dans le laboratoire UMR1078, permettant leur évaluation.

L'objectif principal était d'évaluer l'efficacité de l'incorporation génique par ces nouveaux vecteurs non viraux dans la cochlée *in vitro*. Après sélection, des tests *in vivo* étaient réalisés. A terme, l'objectif était d'envisager un traitement étiologique de la surdité. Les hypothèses étaient d'une part l'amélioration de l'efficacité de transfection par rapport aux composés actuellement disponibles, et d'autre part l'élargissement des types cellulaires ciblés, ce qui permettait d'envisager un traitement étiologique sans utiliser de vecteurs viraux. En cas d'efficacité insuffisante, l'évaluation des types cellulaires ciblés était nécessaire. En effet, en fonction du type de surdité, les types cellulaires cochléaires atteints sont différents. Ceci pourrait ainsi ouvrir la voie à un traitement étiologique en utilisant ces nouveaux vecteurs non viraux.



Partie I  
Comprendre les surdités génétiques : étude de  
l'otoferline

## Article n°1 L'otoferline, un senseur calcique avec de multiples fonctions : des synapses de l'oreille interne à la cancérogénèse.

### Résumé

Chez l'homme, la famille des ferline est composée de 6 protéines : la dysferline, la myoferline, l'otoferline, fer1L4, fer1L5 et fer1L6. Ces protéines partagent des caractéristiques communes : de multiples domaines C2 de liaison au  $\text{Ca}^{2+}$ , des domaines FerA et un unique domaine transmembranaire en C-terminal. Ces protéines interviennent dans la fusion membranaire médiée par le calcium et dans le trafic des vésicules. Une de ces protéines a un rôle majeur dans l'audition et la fonction vestibulaire : l'otoferline. Dans cette revue, nous expliquerons comment l'otoferline fonctionne en tant que senseur calcique, qui régule la fusion des vésicules synaptiques au niveau des synapses à ruban des cellules ciliées. Bien qu'elle soit également présente dans le système nerveux central, en particulier dans le cortex et l'amygdale, son rôle dans les tissus cérébraux reste inconnu. Des mutations du gène OTOF sont à l'origine de l'une des formes génétiques les plus fréquentes de surdité congénitale, nommée DFNB9. Ces mutations entraînent une surdité sévère à profonde due à un défaut de la transmission synaptique excitatrice glutamatergique entre les cellules ciliées internes et les fibres nerveuses du nerf auditif. Des protocoles de thérapie génique permettant l'expression normale de l'otoferline dans les cellules ciliées viennent d'être lancés et sont actuellement en phase préclinique. Parallèlement, des études ont établi un lien entre les ferlines et le cancer en raison de leur effet sur le développement et la signalisation cellulaire, ce qui permet le développement tumoral, et l'adaptation des cellules cancéreuses à un environnement hostile. Les processus métastatiques sont modulés par deux éléments importants que sont les forces mécaniques locales notamment membranaires et la signalisation calcique. Bien que le rôle des ferlines dans le cancer soit peu étudié, des données montrent que l'expression de l'otoferline est significativement associée à la survie dans plusieurs types de cancer, incluant le carcinome rénal à cellules claires et à cellules papillaires, et le cancer urothélial de la vessie. Ces résultats indiquent que l'otoferline joue un rôle dans la carcinogénèse de ces tumeurs, ce qui nécessite des recherches supplémentaires pour confirmer et comprendre son rôle exact, en particulier parce qu'il semble varier en fonction du cancer primitif. Le ciblage de cette protéine pourrait à l'avenir potentiellement permettre le développement de nouvelles thérapies anticancéreuses.

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# Otoferlin as a multirole $\text{Ca}^{2+}$ signaling protein: from inner ear synapses to cancer pathways

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Humans have six members of the ferlin protein family: dysferlin, myoferlin, otoferlin, fer1L4, fer1L5, and fer1L6. These proteins share common features such as multiple  $\text{Ca}^{2+}$ -binding C2 domains, FerA domains, and membrane anchoring through their single C-terminal transmembrane domain, and are believed to play a key role in calcium-triggered membrane fusion and vesicle trafficking. Otoferlin plays a crucial role in hearing and vestibular function. In this review, we will discuss how we see otoferlin working as a  $\text{Ca}^{2+}$ -dependent mechanical sensor regulating synaptic vesicle fusion at the hair cell ribbon synapses. Although otoferlin is also present in the central nervous system, particularly in the cortex and amygdala, its role in brain tissues remains unknown. Mutations in the OTOF gene cause one of the most frequent genetic forms of congenital deafness, DFNB9. These mutations produce severe to profound hearing loss due to a defect in synaptic excitatory glutamatergic transmission between the inner hair cells and the nerve fibers of the auditory nerve. Gene therapy protocols that allow normal rescue expression of otoferlin in hair cells have just started and are currently in pre-clinical phase. In parallel, studies have linked ferlins to cancer through their effect on cell signaling and development, allowing tumors to form and cancer cells to adapt to a hostile environment. Modulation by mechanical forces and  $\text{Ca}^{2+}$  signaling are key determinants of the metastatic process. Although ferlins importance in cancer has not been extensively studied, data show that otoferlin expression is significantly associated with survival in specific cancer types, including clear cell and papillary cell renal carcinoma, and urothelial bladder cancer. These findings indicate a role for otoferlin in the carcinogenesis of these tumors, which requires further investigation to confirm and understand its exact role, particularly as it varies by tumor site. Targeting this protein may lead to new cancer therapies.

## KEYWORDS

ferlins,  $\text{Ca}^{2+}$  sensor, membrane fusion, auditory synaptopathy, deafness, brain synapses, dysferlinopathy, cancer

## 1. Introduction

Interest in otoferlin began with the discovery of mutations in the OTOF gene that were identified as responsible for recessive profound deafness in humans (Yasunaga et al., 1999, 2000). This deafness is called DFNB9 (for autosomal recessive deafness 9). It represents about 2% of non-syndromic prelingual deafness, i.e., without any other associated health problem,

and is the most frequent cause of auditory neuropathy spectrum disorder (ANSD) (Iwasa et al., 2019). To date, about 220 mutations leading to DFNB9 deafness have been identified (Azaiez et al., 2021). Remarkably, some otoferlin mutations, notably in the C2F domain, can lead to exacerbated deafness when body temperature rises during a fever (Varga et al., 2006; Marlin et al., 2010; Matsunaga et al., 2012; Strenzke et al., 2016). This peculiar property underlines the importance of a precise functional 3D architecture of the multi-C2 protein, which is known to interact at the presynaptic active zone of the sensory hair cells with SNARE (soluble N-ethylmaleimide-sensitive-factor attachment protein receptor) proteins such as SNAP25 (Roux et al., 2006; Ramakrishnan et al., 2014; Hams et al., 2017; Calvet et al., 2022) and with  $\text{Ca}_v1.3$   $\text{Ca}^{2+}$  channels (Ramakrishnan et al., 2009; Vincent et al., 2017, 2018; Table 1 and Figures 1, 2).

DFNB9 is caused by a defect in synaptic neurotransmission from auditory inner hair cells (IHCs) to their contacting spiral ganglion afferent fibers (Roux et al., 2006; Beurg et al., 2010; Michalski et al., 2017). The sound-induced electrical analog signal of the hair cells, also called cochlear microphonic, is incorrectly transduced into nerve impulses at the auditory nerve fibers. We recall that one of the morphological characteristics of the afferent IHC synapses is the presence of a presynaptic ribbon, an electron-dense presynaptic structure 300–350 nm wide (Figures 1, 3). Each of the 15–20 presynaptic ribbons per IHC are facing a single postsynaptic auditory nerve structure bearing AMPA (glutamate) ionotropic receptors composed of GluA2, GluA3, and GluA4 subunits (Rutherford et al., 2023). This presynaptic ribbon, composed of RIBEYE proteins arranged in staircase pattern, allows the attachment of numerous synaptic vesicles at the membrane active zone of release. This ribbon structure is essential for spike timing precision of the auditory nerve fibers (Wittig and Parsons, 2008; Becker et al., 2018; Jean et al., 2018).

Otoferlin-induced synaptopathy is due to a defective  $\text{Ca}^{2+}$ -evoked exocytosis of synaptic vesicles containing glutamate, the main cochlear excitatory neurotransmitter, at the IHC ribbon synapses (Figure 3). Like in most neurosecretory cells, vesicle trafficking and synaptic exocytosis in IHCs are thought to involve interactions of SNARE complex proteins such as SNAP25 (Calvet et al., 2022). Synaptotagmins (Syts), a large family of transmembrane proteins containing tandem  $\text{Ca}^{2+}$ -binding C2-domains, confer  $\text{Ca}^{2+}$  sensitivity to SNARE-dependent vesicle fusion in the CNS (central nervous system) (Chapman, 2008). However, the implication of Syts in hair cell synaptic exocytosis has been shown to be limited to immature cochlear developmental stages when hair cells fire action potentials (Beurg et al., 2010). At post-hearing mature stages, when hair cells display gradual depolarization upon sound stimulation, otoferlin becomes the major  $\text{Ca}^{2+}$  sensor triggering synaptic vesicle exocytosis.

DFNB9 is classified as a neuropathy and, more specifically, as an auditory cochlear synaptopathy (Moser and Starr, 2016). In addition to the cochlea, otoferlin expression has also been shown in the brain with unknown function (Schug et al., 2006) and, in the peripheral vestibular organs where hair cell  $\text{Ca}^{2+}$ -dependent fast synaptic vesicle exocytosis is also impaired (Dulon et al., 2009). In DFNB9 patients, it is important to note that the active electromechanical sound amplification function by the outer hair cells (OHCs) is normal, as indicated by measurements of the distortion products of otoacoustic emissions (DPOAEs). The

DFNB9 auditory cochlear synaptopathy is currently overlooked in clinics when the systematic hearing screening performed at birth relies only on DPOAE measurements. Since delays in the management of sensory pathology reduce the quality of language development, it is becoming essential to screen for deafness with automated auditory brainstem recordings (ABRs). For DFNB9 hearing loss, hearing aids or cochlear implantation are the only effective treatments currently available. Future clinical gene therapies are on the way, as demonstrated by successful rescue viral gene therapies in otoferlin-deficient mouse models (Al-Moyed et al., 2019; Akil, 2020; Rankovic et al., 2020). However, in these studies, for unknown reasons, there was only a partial rescue of ABR wave I amplitude (the neural response associated with the electrical activity of the IHC ribbon synapses), suggesting a non-restored loss of some ribbon synapses. This incomplete rescuing is likely due to the late developmental stage (postnatal days P1 to P3 in mice) at which AAV treatment was performed in these studies. Indeed, otoferlin seems essential for normal early prenatal development and the maintenance of ribbon synapses (Stalman et al., 2021).

## 2. Otoferlin structure and isoforms

Otoferlin is a large 1997 amino acid (aa) protein, that includes a single C-terminal transmembrane domain (TMD) anchoring the protein to the vesicular membrane and six C2 domains (A–F) oriented toward the IHC cytosol (Figures 3, 4). The C2 domains are structures composed of eight antiparallel  $\beta$ -sheets and negatively charged top loops due to the presence of five aspartate residues, constituting the putative binding site for  $\text{Ca}^{2+}$  ions (Sutton et al., 1995; Xue et al., 2008). The C2-A domain is the only C2 domain predicted to be unable to bind  $\text{Ca}^{2+}$  due to a shorter top loop, thus lacking aspartates coordinating calcium binding (Helfmann et al., 2011). The main function of C2 domains is to target membrane surfaces following  $\text{Ca}^{2+}$ -binding. C2 domains bind to their target membranes by using a combination of hydrophobic and electrostatic interactions, preferentially with phosphatidylinositol 4,5-bisphosphate (PIP2) or phosphatidylserine (PS) (Corbalan-García and Gómez-Fernández, 2014). Otoferlin also has a FerA domain, a four-helix bundle fold with its own  $\text{Ca}^{2+}$ -dependent phospholipid-binding activity, suggesting that the interaction of this domain with the membrane is enhanced by the presence of  $\text{Ca}^{2+}$  (Harsini et al., 2018).

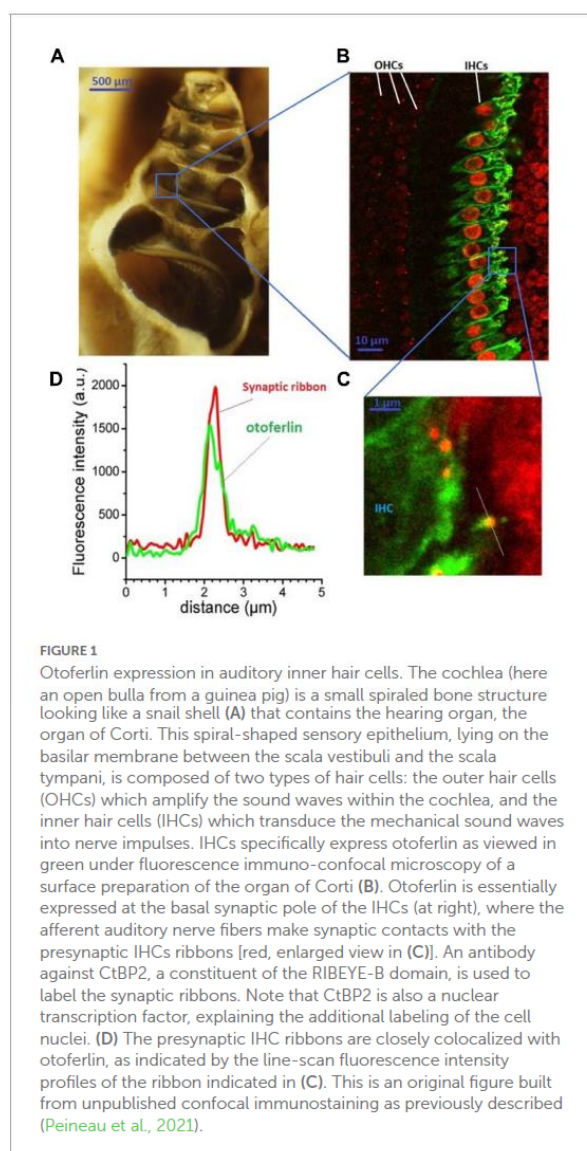
Otoferlin is produced mainly in the brain and inner ear (cochlea and vestibule), and in smaller amounts in other organs: the heart, liver, pancreas, kidney, and skeletal muscle (Yasunaga et al., 2000). Otoferlin appears as a globular protein when using the AlphaFold protein structure prediction software (Jumper et al., 2021; Figure 4). In humans, a long and short isoform of otoferlin have been identified (Yasunaga et al., 1999, 2000), with the long one comprising all six C2 domains and a C-terminal *trans*-membrane domain (TMD) (1997 aa), while the short one expresses only the last three C-terminal C2 domains (C2-DEF) with the TMD (1230 aa). The role of these two isoforms remains unknown. Of note, the short otoferlin isoform is not present in mice, whereas the human long isoform has 98% sequence similarity with murine otoferlin.

The long isoform of otoferlin is encoded by 48 exons, of which exons 6 and 31 can have alternative splicing. Exon 47

TABLE 1 The otoferlin interactome.

Interacting factors	Putative function	Identified interacting domains of otoferlin								References
		Otoferlin	C2A	C2B	C2C	C2D	C2E	C2F	TMD	
Ca <sup>2+</sup>	Bind to Asp residues in C2 domains	✓		✓	✓	✓	✓	✓		Johnson and Chapman, 2010; Michalski et al., 2017
Syntaxin 1A	SNARE complex	✓	✓	✓		✓	✓	✓		Ramakrishnan et al., 2009, 2014
SNAP-25	SNARE complex	✓						✓		Ramakrishnan et al., 2014
Ca <sub>v</sub> 1.3	L-type Ca <sup>2+</sup> channel	✓	✓	✓		✓		✓		Ramakrishnan et al., 2014; Hams et al., 2017
CaMKII $\delta$	CaM kinase	✓						✓		Meese et al., 2017
Endophilin-A1	Endocytic adaptors	✓								Kroll et al., 2019
PIP2	Phospholipid binding	✓			✓			✓		Roux et al., 2006; Ramakrishnan et al., 2009; Padmanarayana et al., 2014
Myosin VI	Motor for vesicular membrane traffic	✓				✓				Heidrych et al., 2009
Golgi marker GM130	Endosome- network dynamics	✓								Heidrych et al., 2008
Rab8b GTPase	Protein transport regulator	✓								Heidrych et al., 2008
NSF	SNARE chaperone membrane fusion	✓	✓	✓		✓		✓		Selvakumar et al., 2017
Ergic2 (ER/Golgi)	Brain specific partner	✓				✓				Zak et al., 2012
Dynamin	Fast endocytosis GTPase	✓				✓	✓	✓		Tertrais et al., 2019
AP2	Clathrin adaptor protein, slow endocytosis	✓	✓	✓		✓		✓		Duncker et al., 2013; Jung et al., 2015; Selvakumar et al., 2017
Calpain	Ca <sup>2+</sup> activated protease cleaving otoferlin	✓								Redpath et al., 2014
Tryptophan-rich basic protein (WRB)	TRC40 complex, transmembrane recognition complex	✓							✓	Vogl et al., 2016

Otoferlin is a multi-C2-domain transmembrane vesicular protein with mutations associated with congenital deafness. Based on the published literature, this table recapitulates the putative factors (left column) interacting with otoferlin, its specific C2 domains, or its transmembrane domain (TMD). Most of these factors interacting with otoferlin share functions with SNARE proteins or have other neurotransmission-related functions.



also carries an alternative splice site, and upon splicing, exon 48 is expressed and encodes a transmembrane domain called TMD1. If exon 47 is not spliced, it bears a stop codon at its end, so the translation of exon 48 is excluded, and otoferlin has a different TMD domain, TMD2 (Figure 4). In humans and mice, the TMD2 otoferlin isoform (exon 47) is preferentially expressed in the brain, while the TMD1 isoform (exon 48) is specifically found in the cochlea (Yasunaga et al., 2000; Schug et al., 2006). Specific mutations in exon 48 cause hearing loss (Choi et al., 2009), underscoring the importance of TMD. According to AlphaFold3D structure predictions, the differences between the two TMD isoforms are partly based on the angulation of the transmembrane domain relative to the rest of the protein (Figure 4E). These two TMDs only share less than 57% amino acid sequence similarity (Figure 4D). Notably, the brain TMD2 isoform contains a large peptidic sequence with numerous contiguous Leu residues (Leu-block of more than 9 Leu) in comparison

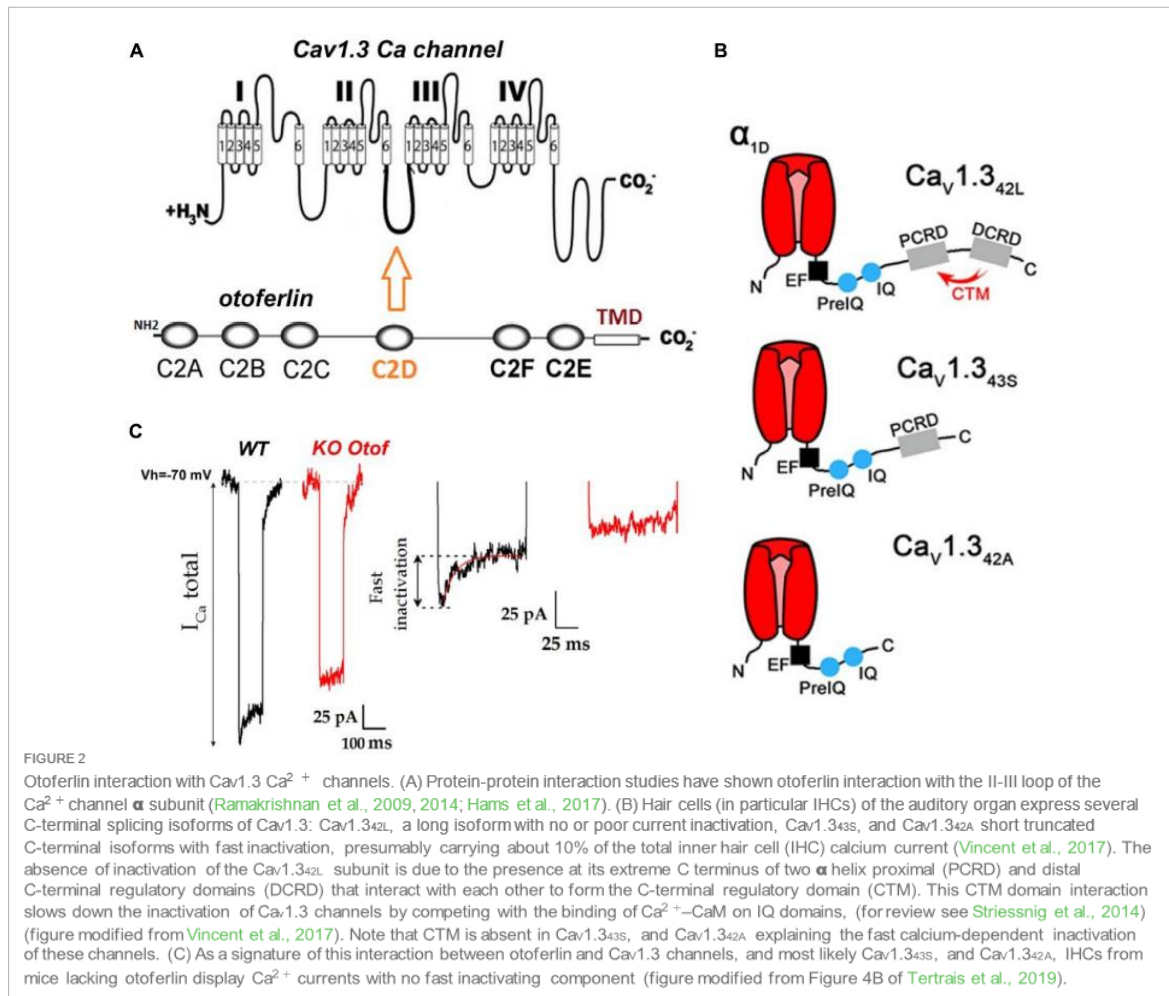
to the cochlear TMD isoform, which contains discontinuous stretches of Leu residues (Figure 4D). Large hydrophobic Leu-blocks in TMD peptides confer greater helicity and circumferential hydrophobicity that facilitate biological membrane insertion (Stone et al., 2015). The use of specific TMD should be important for targeting the protein to specific subcellular locations, such as particular glutamatergic excitatory presynaptic active zones, and specifying the interaction with specific organelles and ligands (Sharpe et al., 2010). Mutations in exon 48, such as the in-frame deletion of a conserved isoleucine in the cochlear TMD1 at position 1967 (p.Ile1967del), lead to hearing impairment and underline the importance of this domain in targeting the protein to the endoplasmic reticulum (ER) membrane in hair cells (Vogl et al., 2016).

Another isoform, with an alternative splice in exon 31 might explain deafness caused by heat sensitivity, but its presence in the human cochlea remains uncertain (Strenzke et al., 2016). A recent study, by Liu et al. (2023) provides new insights into the role of alternative otoferlin isoforms in auditory function and their modulation by environmental factors such as noise and aging. This study discovered a new short transcript of otoferlin derived from an unannotated exon 6b whose expression is increased in IHCs encoding high frequency sounds and varies under noise and aging conditions.

### 3. Genetic mutations and DFNB9

Approximately 220 mutations causing DFNB9-type deafness have been identified, some of which cause deafness during a febrile episode (Vona et al., 2020; Azaiez et al., 2021). Interestingly, there is no described pathological mutation of the C2A domain (Figure 5; Stenson et al., 2003). All other areas of the protein can be affected. According to the Human Gene Mutation Database, 41% of the variants are missense, 22% nonsense, 15% deletions, 14% splicing, 6% insertions, and 2% copy number variants. The missense variants alter the folding, stability, or function of the protein. Truncating variants (nonsense, deletion, splicing, insertion, and copy number variation) result in a missing or truncated non-functional protein. Both types of variants result in severe to profound hearing loss. One of the most common OTOF mutations (p.R1939Q) is located at the junction between the calcium binding C2F domains and the C-terminal TMD (Matsunaga et al., 2012; Kim et al., 2018). As mentioned above, some of the mutations lead to a disorder of the temperature-sensitive auditory neuropathy spectrum. Elevated body temperature causes severe to profound hearing loss, whereas normal body temperature results in normal to moderate hearing. Interestingly, all identified thermosensitive mutations are located between the C2C and C2F domains, underscoring the functional importance of these regions (Zhu et al., 2021).

The mechanisms underlying the temperature sensitive hearing loss remain essentially unknown, and they may vary with their site of location. These mutations probably alter the folding or unfolding properties of the long multi-C2-domain protein, impairing specific  $\text{Ca}^{2+}$ -dependent C2-domain interactions and synaptic vesicle fusion, as temperature rises. Another hypothesis is that there is increased degradation and loss of the protein. This is caused by the heat sensitivity of otoferlin, especially in the presence of variants



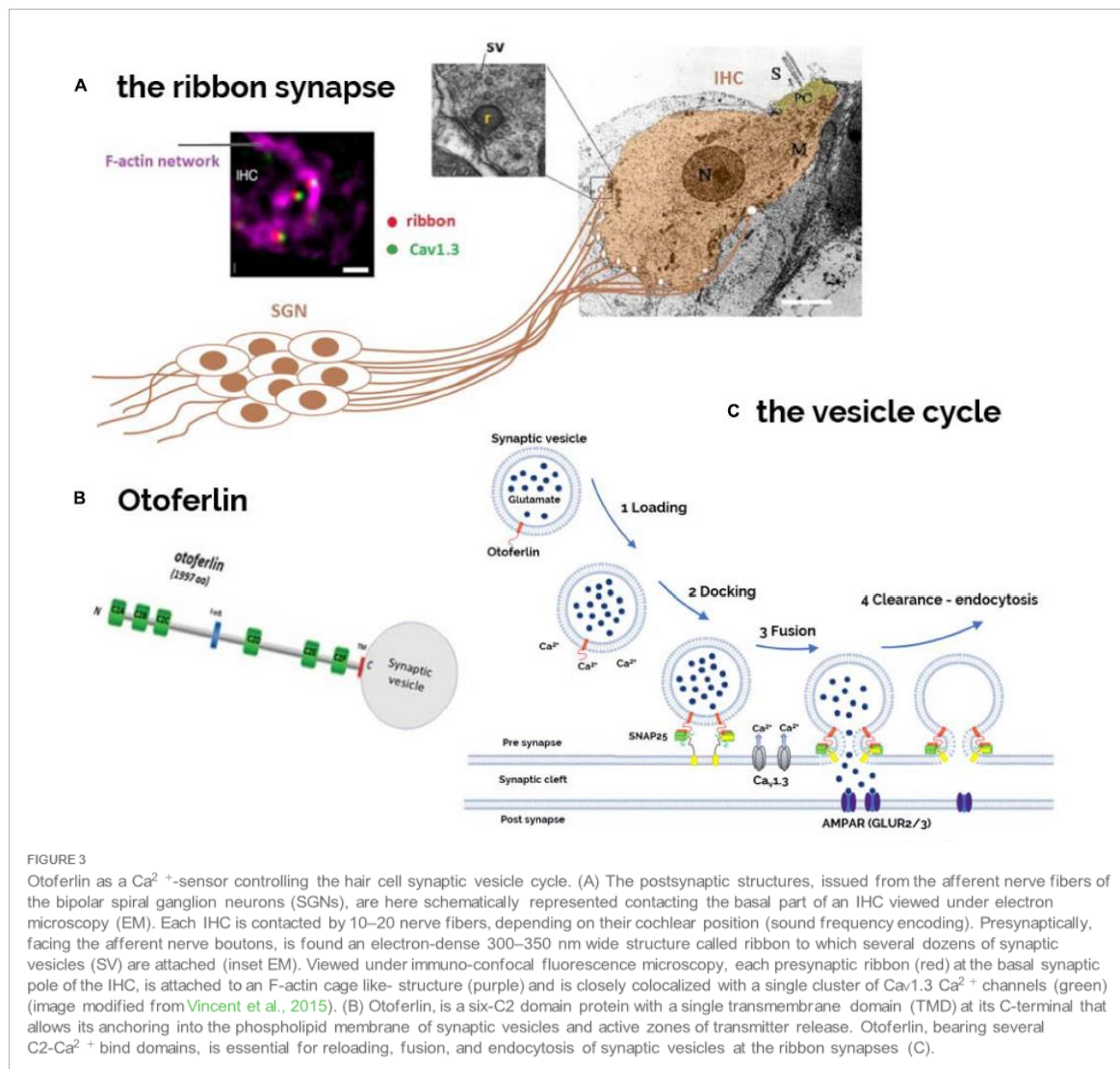
such as Ile515Thr (Strenzke et al., 2016). The protein is then degraded more quickly.

#### 4. Otofferin tissue expression

The tissue distribution of otofferin is primarily observed in the brain and inner ear (Schug et al., 2006). In the brain it is mainly found in the amygdala and cerebral cortex (Figure 6). We recall that the two TMD otofferin isoforms have different distributions: one is almost exclusively found in the cochlea (TMD1, exon 48), and the other is mainly found in the brain (TMD2, exon 47) (Yasunaga et al., 2000). However, this TMD variant distribution in the brain and inner ear, only demonstrated by RT-PCR and Northern blot analysis at the tissue level, needs to be confirmed at the protein and cellular levels.

Otofferin expression increases in hair cells as they mature, remaining permanently expressed in IHCs and only transiently expressed in immature OHCs (largely decreasing after P4-P6 in mice) (Roux et al., 2006; Schug et al., 2006). This transient expression in OHCs is essential for spontaneous synaptic exocytosis

by immature OHCs, a process likely involved in the establishment of frequency tonotopy of the central auditory nuclei during development (Beurg et al., 2008). In IHCs and vestibular cells, otofferin was localized in synaptic vesicles by immunogold labeling (Roux et al., 2006; Dulon et al., 2009) or in the Golgi apparatus and presynaptic zone by immunohistochemistry (Schug et al., 2006; Heidrych et al., 2008). In HEK cell expression, otofferin is found at the plasma membrane and colocalizes with some of the *trans*-Golgi markers (GM130 or TGLON2) (Redpath et al., 2016). In IHCs, otofferin is distributed throughout the cytoplasm and plasma membrane, except for the apical portion that forms the cuticular plate and tight junctions with neighboring cells. It is also interesting to note that the expression of the protein differs according to its two isoforms, TMD1 and TMD2, when expressed in heterologous cell lines. In HEK293 cell cultures, TMD1 showed a dispersed pattern within the cell, while TMD2 was predominantly found at the plasma membrane (Redpath et al., 2016). Otofferin is also expressed by type I and type II vestibular utricular hair cells (Dulon et al., 2009). Note that truncation of the TMD of otofferin alters the development of hair cells and reduces membrane docking (Manchanda et al., 2021). This tail-anchored protein can be inserted



into the endoplasmic reticulum (ER) of hair cells via the TRC40 receptor tryptophan-rich basic protein (Wrb) (Vogl et al., 2016).

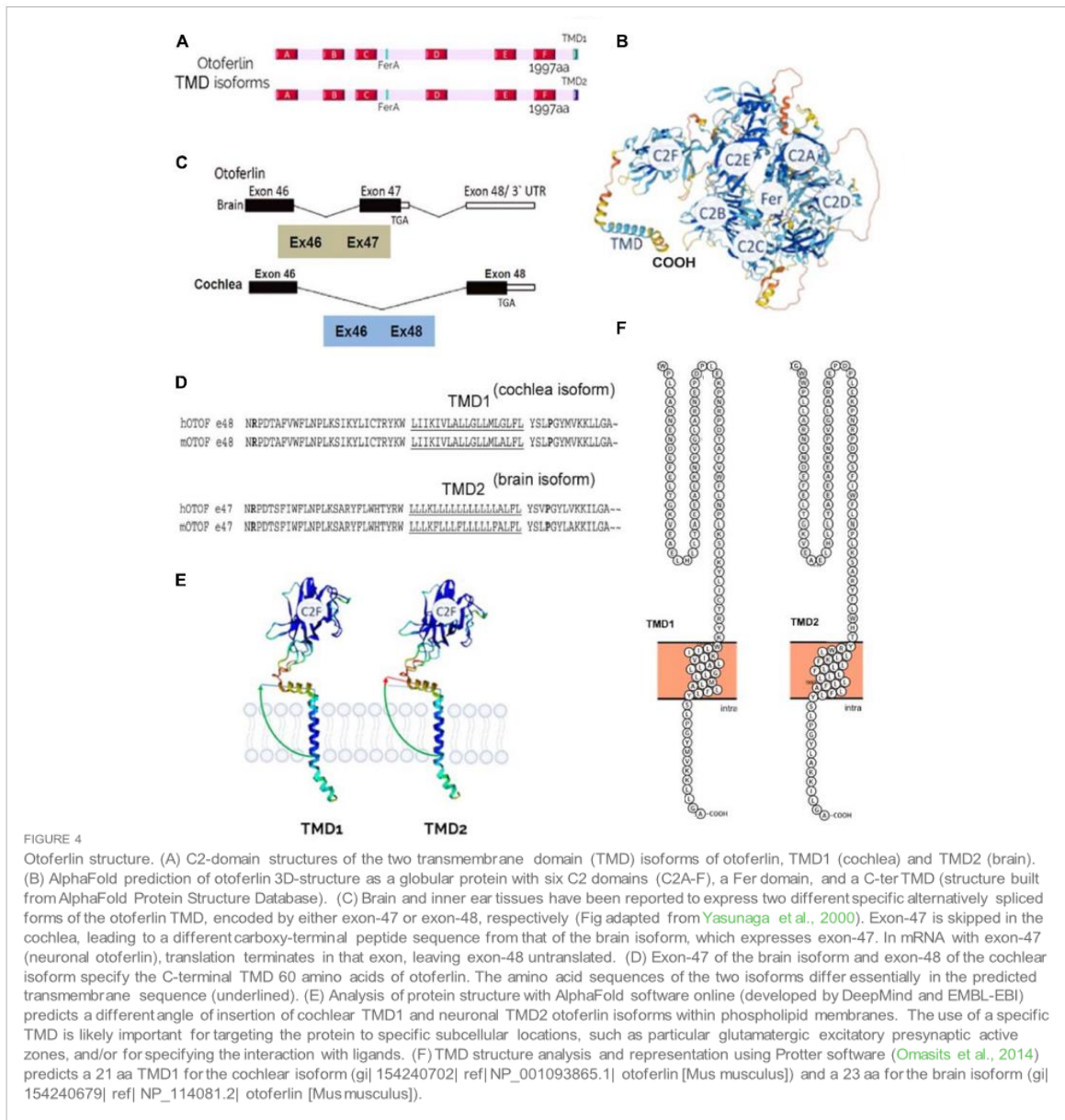
## 5. Otoferlin functions and mechanisms of action

### 5.1. Otoferlin multirole at the IHC synaptic vesicle cycle

Remarkably, mature inner hair cells (IHCs) lack the  $\text{Ca}^{2+}$ -sensors synaptotagmin (I and II) and Munc13, which are known to play a crucial role in vesicle exocytosis at conventional central neuronal synapses (Beurg et al., 2010; Reisinger et al., 2011; Vogl et al., 2015). It is worth recalling that mature IHCs, contrary to central neurons, do not fire action potentials but are

capable responding to a large dynamic range of sound intensity (> 100 dB) by graded depolarization that is encoded into an increasing discharge rate at the postsynaptic nerve fibers. It is now established that otoferlin plays a role at several essential stages of the vesicle cycle in IHCs, including functional docking, priming, fusion, endocytosis, and possibly transport and maturation of vesicles (Figure 3; Michalski et al., 2017; Tertrais et al., 2019). In otoferlin knockout mice, the average tether length between vesicles and the active zone is increased, suggesting a possible defect in vesicle attachment and priming (Vogl et al., 2015). The downstream calcium-triggered exocytosis of vesicles is almost completely blocked in otoferlin knockout mice. Otoferlin is also involved in active zone clearance, including the transport of exocytic material, through interactions with adaptor protein 2  $\mu$ , a motor protein, and a GTPase (Jung et al., 2015). Vesicle size may indicate a role for otoferlin in vesicle reformation and maturation, and the functions of otoferlin may be regulated by phosphorylation.





## 5.2. Otoferlin and membrane fusion

Structural analysis and prediction using Protter (Omasits et al., 2014) and AlphaFold (Jumper et al., 2021) display otoferlin as a globular protein anchored to the vesicle and/or plasma membrane through its C-terminal domain, while its N-ter  $Ca^{2+}$ -sensing C2-domains are in the cytosol (Figure 4). The structural features of its C2-domains, through their  $Ca^{2+}$ -binding and phospholipid-binding activities, allow otoferlin to play a role in membrane fusion and trafficking, in endocytic, secretory, and lysosomal pathways. By deleting exons 14 and 15 of the OTOF gene, an OTOF KO mouse model was created (Roux et al., 2006). These otoferlin-deficient mice (*Otof*<sup>-/-</sup>) are profoundly deaf. Patch-clamp recordings for

time-resolved change of membrane capacitance in IHCs from *Otof*<sup>-/-</sup> mice show a significant impairment of fast and sustained exocytotic activity (Beurg et al., 2010; Michalski et al., 2017). Another mouse model, Pachanga, carrying a missense mutation in the C2-F domain of otoferlin, was obtained by ENU (N-ethyl-N-nitrosourea) mutagenesis (Schwander et al., 2007). Pachanga mice have profound hearing loss, but unlike *Otof*<sup>-/-</sup> mice, IHCs still weakly express otoferlin and maintain rapid fusion of the pool of fusogenic vesicles, forming the RRP (for “readily releasable pool of vesicles”) (Pangrsic et al., 2010). However, IHC exocytosis cannot be sustained upon repeated stimulations in these Pachanga mutant mice, indicating impaired recruitment of vesicles to IHC fusion sites. These results suggested that



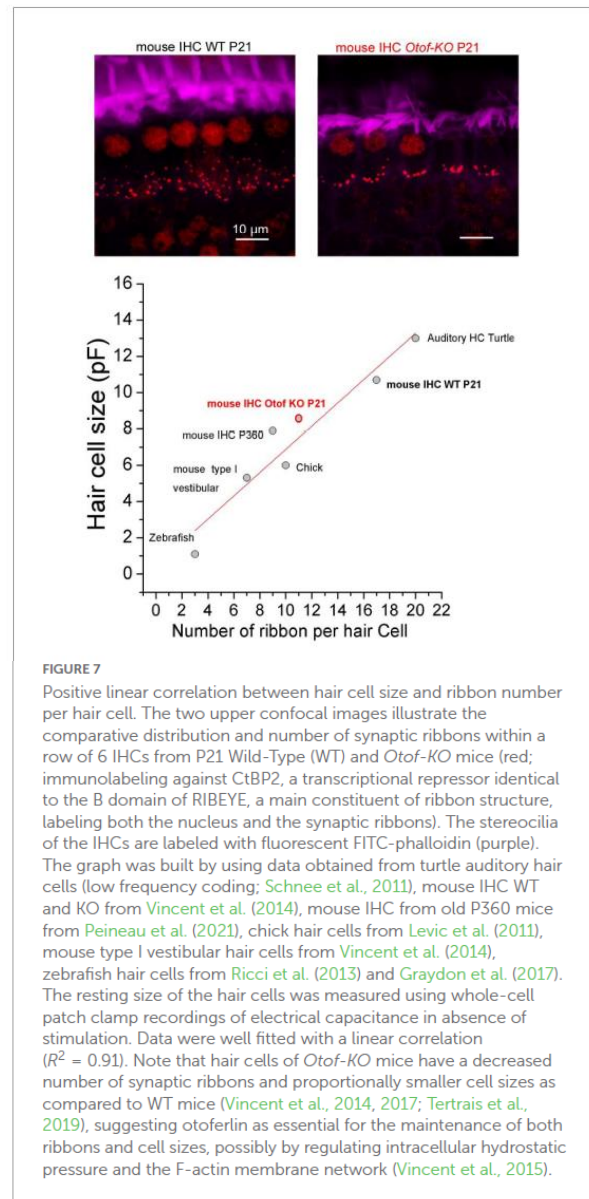
contrast to ferlin proteins, the TMD of synaptotagmins is located at the N-terminal region of the protein for unknown reasons. The homolog of otoferlin, dysferlin, has also been shown to dimerize in living cells, at its transmembrane domain and at its multiple C2 domains (except C2A), as probed by fluorescence resonance energy transfer (FRET) (Xu et al., 2011).

The C2-A domain is the only one of the six otoferlin domains that cannot bind  $\text{Ca}^{2+}$ , which probably explains the absence of pathogenic mutations in this domain (Johnson and Chapman, 2010; Helfmann et al., 2011; Padmanarayana et al., 2014). The idea of a simple repeat between each C2 domain is rejected. An *Otof* KI mouse model carrying two mutations in C2C (substitution of 2 aspartic acid by alanine, affecting  $\text{Ca}^{2+}$  binding), demonstrated that otoferlin is essential in both rapid fusion and vesicle recruitment (Michalski et al., 2017). Interestingly, the viral expression of truncated forms of otoferlin (C2-EF, C2-DEF, and C2-ACEF) can only partially rescue the fast and transient release component of exocytosis in mouse hair cells lacking otoferlin (*Otof*<sup>-/-</sup>), yet cannot sustain exocytosis after long, repeated stimulations (Tertrais et al., 2019). To note, a C2-EF otoferlin truncated form can also be produced by intracellular calpain-1 digestion in cell lines (Redpath et al., 2014) but it is unknown whether such truncation occurs in auditory IHCs.

Note that otoferlin is also essential for the fast exocytosis of type I vestibular cells. *Otof*<sup>-/-</sup> mice show altered vestibular compound action potentials, suggesting impaired vestibular hair cell function (Dulon et al., 2009). In intact utricles *ex vivo*, otoferlin was found to be critical for a highly sensitive and linear calcium-dependent exocytosis, facilitating the linear encoding of low-intensity stimuli at the vestibular hair cell synapse. Surprisingly, mice and humans lacking otoferlin do not have apparent vestibular symptoms. The lack of vestibular phenotype may be explained by some compensatory mechanisms, in particular by stimulus-evoked acidification of the synaptic cleft of vestibular hair cells (protons acting as neurotransmitters) (Highstein et al., 2014) or by the use of another type of neurotransmission via potassium accumulation in the calyx, which depolarizes it (Holt et al., 2007). Although these compensatory modes of transmission are rather slow and non-linear, they may explain the absence of a vestibular phenotype in OTOF patients and animal models.

### 5.3. A specific mechanical tuning between $\text{Ca}^{2+}$ , otoferlin, F-actin, and the ribbon could determine the specific firing frequency of each auditory nerve fiber

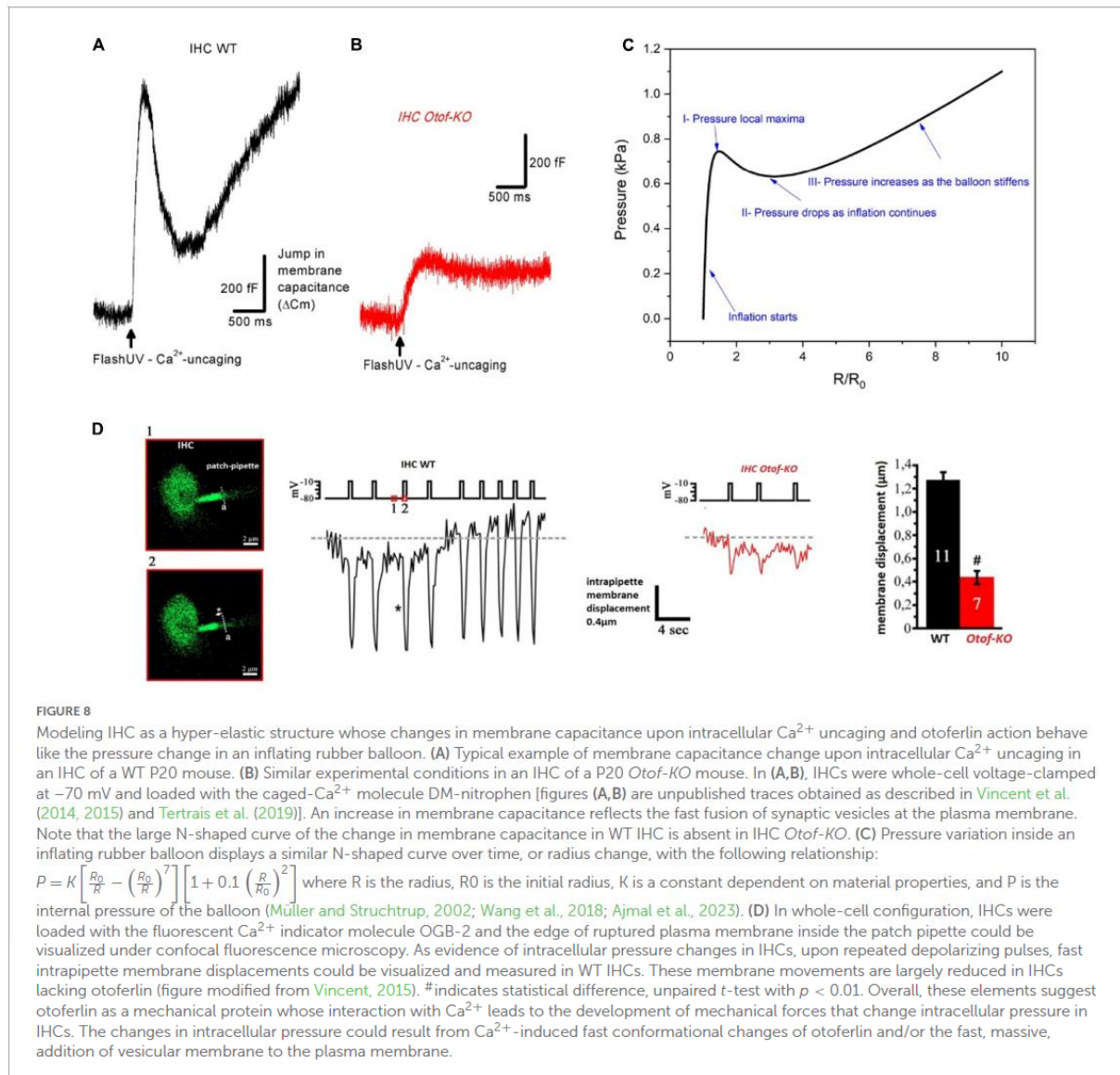
Mammalian auditory inner hair cell (IHC) ribbon synapses have to deal with the great challenge of encoding an extremely wide range of sound intensities, with a dynamic range of more than 100 dB. To perform this challenging task, IHCs have partitioned their synaptic output sensitivity and dynamic range. Indeed, each IHC forms synapses with a pool of 10 to 30 afferent nerve fibers, among which spontaneous activity, acoustic threshold, and dynamic range vary widely. One of the most important questions in auditory neuroscience is to elucidate the functional mechanisms that dictate the synaptic diversity of each type of sensory synapses within a single IHC. Variation in the voltage-gating and spatial



organization of  $\text{Ca}_v1.3$  calcium channels at each presynaptic active zone has been proposed to determine the firing specificity of the auditory nerve fibers (Özçete and Moser, 2021). Other factors, such as a mechanical tuning of each ribbon, could also participate in the firing frequency characteristic of each auditory fiber (Figures 7–9).

Otoferlin has been proposed to control IHC intracellular hydrostatic pressure and in turn exocytosis, presumably via interactions with a synaptic F-actin network (Vincent et al., 2015) and the  $\text{Ca}^{2+}$  channels  $\text{Ca}_v1.3$  at the ribbon (Vincent et al., 2017). Otoferlin is essential for synchronous multivesicular release at IHC active zones; this process underlying fast transient  $\text{H}^+$ -inhibition of  $\text{Ca}_v1.3$   $\text{Ca}^{2+}$  channels (Vincent et al., 2018).

Remarkably, there is a good linear correlation between the size of the hair cells and their number of synaptic ribbons per cell when comparing the ears of various vertebrates (Figure 7). In



IHCs from mice lacking otoferlin, the number of synaptic ribbons is proportionally reduced with the cell size (Tertrais et al., 2019). Also in old P360 C57BL/6J mice, the reduced size of the IHCs is proportionally associated with a lower number of synaptic ribbons (Peineau et al., 2021). This correlation suggests that the number of ribbons may influence membrane exocytosis, and in turn, cell size, possibly by modifying the intracellular hydrostatic pressure. Numerous studies in various cell type have shown a reciprocal link between cell biomechanics, the submembrane actomyosin network, and exocytosis (Wang and Galli, 2018). Auditory IHCs seem to be able to sense the biomechanical properties of the environment since their exocytotic properties are sensitive to changes in intracellular hydrostatic pressure (Vincent et al., 2014). Membrane addition during hair cell synaptic exocytosis can be visualized by confocal-fluorescent microscopy (Hudspeth and Issa, 1996), indicating membrane deformation and a possible local change in hydrostatic pressure.

We propose that otoferlin, by controlling membrane fusion, regulates plasma membrane rigidity and tension of its underlying actomyosin meshwork, whose mechanical properties are likely essential to maintaining active membranous synaptic ribbons. Note that upon UV-flash-photolysis allowing ultrafast intracellular  $Ca^{2+}$  uncaging from DM-nitrophen, the change in IHC membrane capacitance (produced by massive vesicle fusion to the plasma membrane) behaves like an inflating rubber balloon (Figures 8A–C), suggesting a non-monotonic relation (non-linear stress-strain response) between the elasticity of the IHC plasma membrane and the intracellular pressure. Changes in IHC intracellular pressure can directly be visualized during exocytosis when monitoring membrane displacement during repetitive depolarizing steps (Figure 8D; Vincent, 2015). It is tempting to propose the synaptic ribbons as pulsating microspheres that are activated by  $Ca^{2+}$ -otoferlin interactions with the membranous F-actin network; these mechanical interactions being the source generator (hydrostatic

force) triggering vesicle membrane fusion. In this model, the mechanical oscillatory frequency of the ribbons would essentially depend on their mass, similarly to a spring-block oscillator, a factor that would then determine the firing frequency of the postsynaptic afferent nerve fibers (Figure 9). In this context, it is interesting to note that amphibian hair cells have been shown to display frequency selectivity in synaptic exocytosis, presumably due to a tonotopic variation of  $\text{Ca}^{2+}$  buffers (parvalbumin 3 and calbindin-28K) that tunes a regime of spontaneously oscillatory vesicle release (Patel et al., 2012).

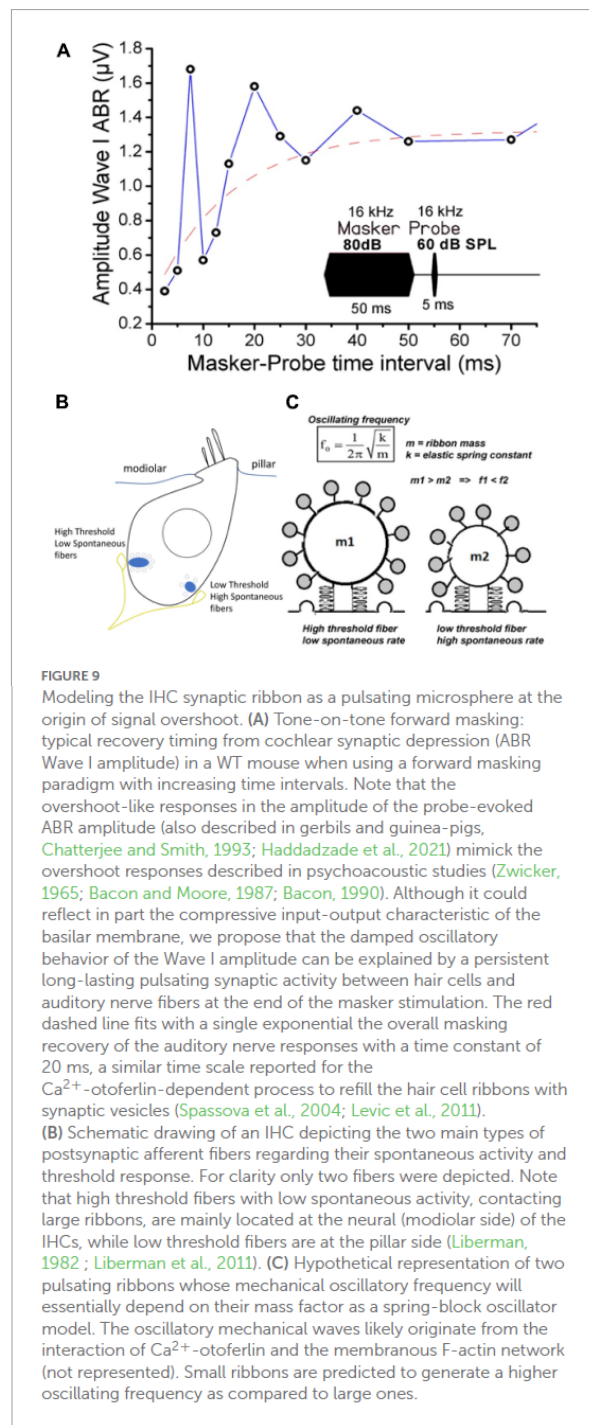
## 5.4. The ribbon synapse: an unconventional synapse

Intriguingly, some proteins usually present in glutamatergic synapses of the central nervous system, such as synaptophysin, synaptotagmin, and complexins, are absent from IHC synapses, suggesting that an unconventional mechanism of neurotransmitter release may be involved (Safieddine and Wenthold, 1999; Uthaiyah and Hudspeth, 2010; Nouvian et al., 2011). However, some classical neuronal SNARE proteins, such as SNAP-25, are essential for normal exocytotic function of the IHC ribbon synapses (Calvet et al., 2022). Also, molecular interactions of otoferlin with Soluble NSF Attachment Protein Receptor (SNARE) proteins, such as syntaxin-1 and SNAP-25, but not synaptobrevin-1 (VAMP-1) have been shown in protein-protein interaction studies *in vitro* (Ramakrishnan et al., 2014; Hams et al., 2017; Table 1). At the IHC synapse level, mutations in genes encoding proteins other than otoferlin are also implicated in auditory synaptopathies leading to congenital deafness vesicular glutamate transporter (VGLUT3) (Ruel et al., 2008), L-type voltage-dependent  $\text{Ca}^{2+}$  channel  $\text{Ca}_v1.3$  (CACNA1D) (Platzer et al., 2000; Baig et al., 2011), and Usher proteins such as harmonin (Gregory et al., 2011) and the tetraspan protein clarin-1 (Dulon et al., 2018).

## 6. Ferlin family

Otoferlin belongs to the ferlin protein family, which in humans has five members in addition to otoferlin (fer1L2): dysferlin (fer1L1), myoferlin (fer1L3), fer1L4, fer1L5, and fer1L6 (Lek et al., 2010; Redpath et al., 2016). The first ferlin identified was Fer-1, in *Caenorhabditis elegans*. Fer-1 is a fertilization factor required for the fusion of specialized vesicles with the plasma membrane during spermatogenesis (Achanzar and Ward, 1997). The main common features of ferlins are:

- The presence of multiple C2 domains that share a high sequence similarity of about 100 amino acids, and most often allow binding to  $\text{Ca}^{2+}$ , phosphatidylinositol 4,5-bisphosphate (PIP2) or phosphatidylserine (PS).
- The presence of "FerA" domains also allows binding to phospholipid membrane.
- Their anchoring to the membrane by a single C-terminal domain (tail-anchored proteins).



**FIGURE 9** Modeling the IHC synaptic ribbon as a pulsating microsphere at the origin of signal overshoot. (A) Tone-on-tone forward masking: typical recovery timing from cochlear synaptic depression (ABR Wave I amplitude) in a WT mouse when using a forward masking paradigm with increasing time intervals. Note that the overshoot-like responses in the amplitude of the probe-evoked ABR amplitude (also described in gerbils and guinea-pigs, Chatterjee and Smith, 1993; Haddadzade et al., 2021) mimic the overshoot responses described in psychoacoustic studies (Zwicker, 1965; Bacon and Moore, 1987; Bacon, 1990). Although it could reflect in part the compressive input-output characteristic of the basilar membrane, we propose that the damped oscillatory behavior of the Wave I amplitude can be explained by a persistent long-lasting pulsating synaptic activity between hair cells and auditory nerve fibers at the end of the masker stimulation. The red dashed line fits with a single exponential the overall masking recovery of the auditory nerve responses with a time constant of 20 ms, a similar time scale reported for the  $\text{Ca}^{2+}$ -otoferlin-dependent process to refill the hair cell ribbons with synaptic vesicles (Spassova et al., 2004; Levic et al., 2011). (B) Schematic drawing of an IHC depicting the two main types of postsynaptic afferent fibers regarding their spontaneous activity and threshold response. For clarity only two fibers were depicted. Note that high threshold fibers with low spontaneous activity, contacting large ribbons, are mainly located at the neural (modiolar side) of the IHCs, while low threshold fibers are at the pillar side (Lieberman, 1982; Lieberman et al., 2011). (C) Hypothetical representation of two pulsating ribbons whose mechanical oscillatory frequency will essentially depend on their mass factor as a spring-block oscillator model. The oscillatory mechanical waves likely originate from the interaction of  $\text{Ca}^{2+}$ -otoferlin and the membranous F-actin network (not represented). Small ribbons are predicted to generate a higher oscillating frequency as compared to large ones.

Their common role would therefore concern  $\text{Ca}^{2+}$ -triggered membrane fusion and trafficking, in endocytic, secretory, and lysosomal pathways (McNeil and Kirchhausen, 2005; Jiménez and Bashir, 2007; Lek et al., 2012). Dysferlin and myoferlin mutations cause muscle diseases: limb-girdle muscular dystrophy type 2B (LGMD2B), Miyoshi myopathy (Dysferlin), and muscular

dystrophy with cardiomyopathy (Myoferlin) (Bashir et al., 1998; Liu et al., 1998).

Dysferlin (Fer1L1) is ubiquitously expressed, with high expression in the brain, heart and skeletal muscle (Anderson et al., 1999). Dysferlin is composed of seven C2 domains, the DysF domain and the FerA domain preceding the FerB domain (Figure 10). Dysferlin acts as a primary emergency regulator of membrane repair in a calcium-dependent manner following membrane damage to myofibres (Bansal et al., 2003; Barthélémy et al., 2018). Dysferlin is also involved in  $\text{Ca}^{2+}$  metabolism regulation in injured muscle fibers (Hofhuis et al., 2017). Dysferlin can be cleaved by calpain (1 and 2; calcium-dependent protease) in response to calcium influx following a membrane micro-lesion (Lek et al., 2013). Cleavage releases a “mini-dysferlin-C72” (with C2E, C2F, and TMD). Mini-dysferlin-C72 is selectively recruited and is thought to be a minimal configuration required for physiological membrane repair function (Krahn et al., 2010; Lek et al., 2013; Redpath et al., 2014). Otoferlin and myoferlin can also release a module with the last two C-terminal domains and the TMD after enzymatic cleavage of calpain *in vitro* (Redpath et al., 2014; Piper et al., 2017). This structure carries a high structural similarity to the synaptotagmin family. Moreover, a phylogenetic study reveals that these two C-terminal C2 domains are the most evolutionarily conserved domains of the ferlin family: there is 90% sequence similarity of C2-EF domains between mammals and mollusks, suggesting a key function (Lek et al., 2010).

Like otoferlin, the C2 domains of dysferlin bind to phosphatidylserine (PS), and phosphatidylinositol 4,5-bisphosphate in a  $\text{Ca}^{2+}$ -dependent fashion (Therrien et al., 2009). Dysferlin interacts with caveolin 3 and MG53, which play an important role in maintaining dysferlin within the plasma membrane and enabling effective muscle membrane repair (Matsuda et al., 2001; Hernández-Deviez et al., 2008; Cai et al., 2009). Dysferlin also interacts with annexin A1 and A2, involved in membrane trafficking and actin organization (Lek et al., 2012),  $\text{Ca}_v1.1$  L-type calcium channels (Ampong et al., 2005), vinculin, which acts as a link between actin filament and plasma membrane (de Morrée et al., 2010), and syntaxin 4, a protein that facilitates the docking and fusion of glucose transporter type 4 (GLUT4) vesicles with the plasma membrane (Evenson et al., 2010; Codding et al., 2016; Drescher et al., 2023).

In the central nervous system, dysferlin has been observed to accumulate in endothelial cells near sclerosis lesions (Hochmeister et al., 2006), as well as within amyloid-beta plaques in people with Alzheimer's disease (Galvin et al., 2006). In addition, in humans, certain genetic variations of the Dysf gene have been associated with an increased risk of developing Alzheimer's disease, and Dysf mRNA expression has been shown to be increased in the brains of people with Alzheimer's disease (Chen et al., 2015). Interestingly, OTOF was identified as a downregulated gene in a human-mouse chimeric model of Alzheimer's disease using genome-wide expression analysis, suggesting an essential function of otoferlin in the central nervous system (Espuny-Camacho et al., 2017).

Myoferlin (Fer1L3) is also ubiquitously expressed in skeletal and cardiac muscle and in the placenta (Davis et al., 2000). Myoferlin is involved in the fusion of myoblasts, in repair and regeneration, and in muscle cell membrane growth (Doherty et al., 2005). Myoferlin may be involved in the maintenance of transverse tubule function (Demonbreun et al., 2014). Between the C2-C and

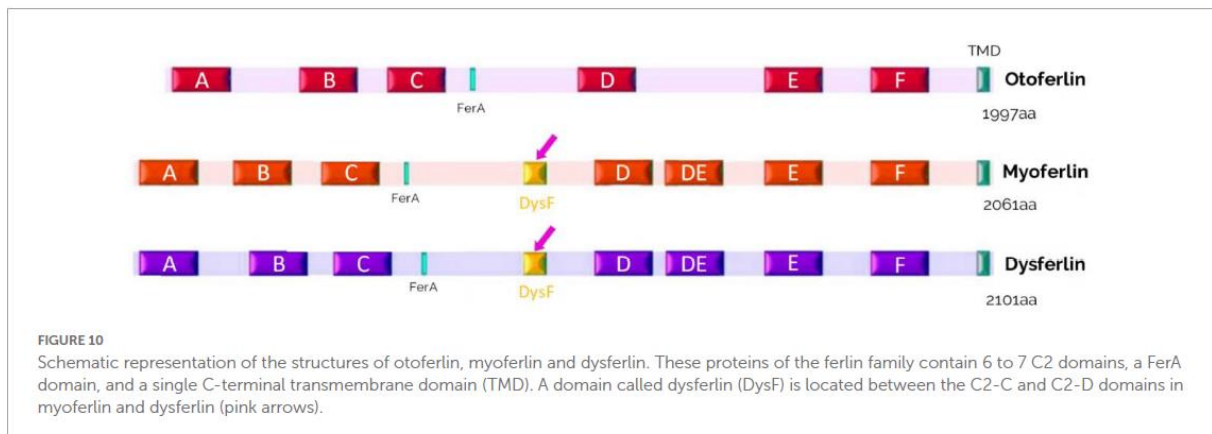
C2D, a domain consisting of two long  $\beta$ -sheets is called the dysferlin (DysF) domain, and a FerA domain, consisting of  $\alpha$ -helices, is located before the FerB domain (Figure 10). The exact role of the DysF domain is unknown but must be significant, as mutations in this domain cause myopathies (Sula et al., 2014). Full information about the ferlin family is available in a recent review by Peulen et al. (2019).

## 7. Otoferlin-disease management and prospects

Auditory Neuropathy Spectrum Disorder (ANSD) is most often suspected after hearing loss is detected at birth, especially if otoacoustic emissions, temporal bone MRI, and CT scans are normal. This deafness may be detected later if the otoacoustic emissions at birth are falsely reassuring. The diagnosis is confirmed by molecular genetic testing, which shows biallelic pathogenic variants in OTOF. Two phenotypes are possible: OTOF-related ANSD, with severe to profound bilateral hearing loss (> 70 dB), and temperature-sensitive ANSD (TS ANSD), with normal to moderate hearing loss (0–69 dB) at normal body temperature and worsening to profound hearing loss during hyperthermia. In these patients, prevention of hyperthermia by early use of antipyretics for infection and avoidance of exposure to high temperatures is important (Azaiez et al., 2021).

As we have seen above, in OTOF-related ANSD, the defects are presynaptic at the level of the hair cells. In these situations, the cochlear nerve is intact and functional. This is a prerequisite for cochlear implantation. It is currently the only option for restoring hearing in patients with severe to profound hearing loss. Early implantation is recommended after the diagnosis of OTOF-related auditory neuropathy with severe to profound hearing loss. It involves surgery. A foreign body is implanted under the skin, and an electrode array is placed in the cochlea for life (De Seta et al., 2022). It requires the wearing of an external magnetic processor. The risk of infection or failure requiring re-intervention and the limited autonomy of the device are significant limitations of this therapy. We can also point out the potential limitations of speech recognition in noise. In OTOF-related ANSD, audiometric results are known since 2005 (Loundon et al., 2005). They can be considered of high quality since the thresholds for sound perception in silence from 500 to 2000 Hz vary on average from 25 to 45 dB SPL, depending on the series (Loundon et al., 2005; Rouillon et al., 2006; Zhang et al., 2016; Chen et al., 2018; Zheng and Liu, 2020). Regarding speech recognition, which is the main goal of cochlear implantation, discrimination scores are mostly >90%. There is no evidence that cochlear implantation outcomes are correlated with distinct OTOF genotypes (Zheng and Liu, 2020).

Gene therapy for congenital deafness has been expanding in recent years, with the aim of achieving a therapy with better hearing outcomes without the limitations of cochlear implant. A truncated form of the OTOF gene was shown to rescue hearing and balance in zebrafish (Chatterjee et al., 2015). Since then, several studies have been interested in genetic therapy using recombinant dual AAV vectors (adeno-associated viruses) to encode full-length otoferlin (Akil et al., 2019; Al-Moyed et al., 2019; Rankovic et al., 2020). These studies gave very encouraging results by showing significant



restoration of hearing in mice. Recently, recombinant dual AAV vectors encoding for the whole human cochlear otoferlin were used to restore hearing in mice to near wild-type levels for 6 months (Tang et al., 2022). This raises the prospect of treatment by gene therapy in humans for DFNB9. The main limitations are the early age of AAV treatment used in most of these studies, which would correspond to an *in utero* AAV injection in humans, and the weak restoration of the ABR wave-1 amplitude, which could limit speech intelligibility and discrimination, particularly in noise, as the main current defect known for cochlear implants. The weak restoration of wave-1 amplitude during AAV gene therapy is likely due to an incomplete rescue of the loss of ribbon synapses in the absence of functional otoferlin (Stalmann et al., 2021). Note that a reduction in spiral ganglion neurons with apoptosis is also a secondary defect caused by a lack of otoferlin in IHCs (Tsuzuki et al., 2023). Currently, several biotech companies (SENSORION, NCT05402813 and AKOUOS–NCT05572073) are in the pre-clinical phase for the development of gene therapy for otoferlin mutations in humans.

## 8. Otoferlin and cancer

### 8.1. Ferlins

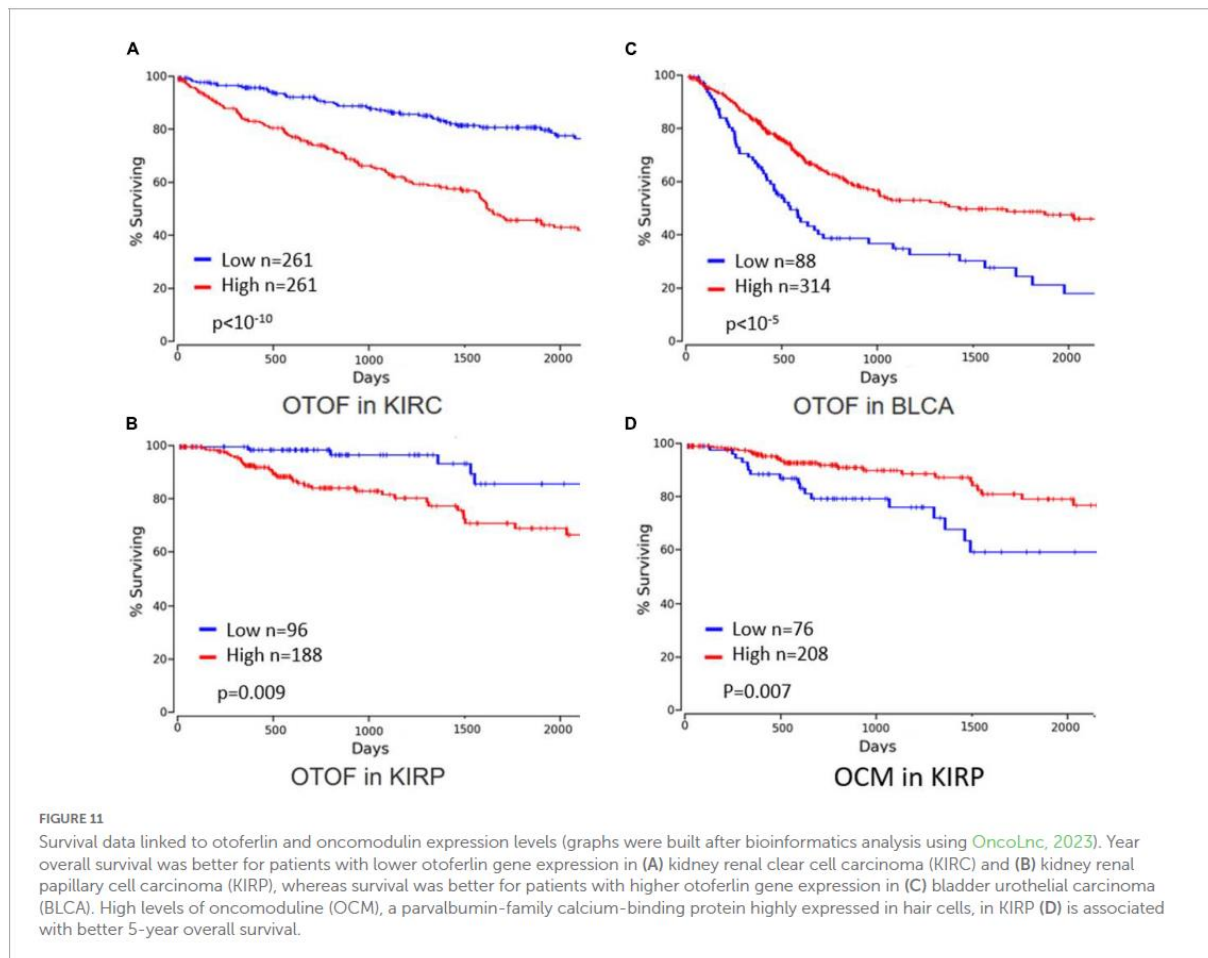
As we have seen, ferlins are a group of proteins that play an important role in several membrane-related processes that are crucial for cell survival and signaling. These processes include endocytosis, exocytosis, recycling, and membrane repair (Bansal et al., 2003; Roux et al., 2006; Bernatchez et al., 2009; Redpath et al., 2016). The link between these processes and cancer is through their effect on cell  $Ca^{2+}$  signaling and development, which allows tumors to form and cancer cells to adapt to a hostile environment. Despite their importance, ferlins have not been extensively studied in the context of cancer. However, recent research has shown that all ferlin genes are modulated in different types of cancer (Peulen et al., 2019). Myoferlin and fer114 genes are more commonly upregulated (Barnhouse et al., 2018; You et al., 2020). Myoferlin has been found to be highly expressed in several types of cancer, including kidney, liver, pancreatic, breast, and head and neck squamous cell carcinomas (Wang et al., 2013; Kumar et al., 2016; Blomme et al., 2017; Hermanns et al., 2017; Song et al., 2017).

### 8.2. Myoferlin

The literature on ferlins in cancer only started about 10 years ago, although the proteins have been described for over 20 years. Most of the work has focused on myoferlin, which appears to be the ferlin with the most important role in cancer. The fact that ferlin expression can be either a good prognostic factor (breast cancer) or a poor prognostic factor (kidney, head and neck cancer) is one of the most surprising elements.

In the study of Yadav et al. (2017) which shows that when cells are treated with IL-6, myoferlin dissociates from EHD2 and binds to activated STAT3, a protein involved in cell signaling. The study found that depletion of myoferlin did not affect STAT3 phosphorylation, but blocked its translocation to the nucleus. The study also found that myoferlin knockdown significantly reduced IL-6-mediated tumor cell migration, tumor sphere formation, and the population of cancer stem cells *in vitro*. In addition, myoferlin knockdown significantly reduced IL-6-mediated tumor growth and metastasis. In 211 patients with head and neck squamous cell carcinoma (HNSCC), Kumar et al. (2016) investigated the association of myoferlin with disease progression and patient outcome. The results showed that nuclear myoferlin expression is associated with poor overall survival and an increased risk of death, as well as tumor recurrence, perineural invasion, extracapsular spread, a higher T stage, and distant metastasis. The study also found a direct association between nuclear myoferlin expression and IL-6 and an inverse association with HPV status. Patients with both nuclear myoferlin expression and high levels of IL-6 and those with HPV-negative/myoferlin-positive tumors had the worst survival. These results suggest that nuclear myoferlin expression independently predicts poor clinical outcomes in these patients.

By modeling the effect of myoferlin on tumor cell invasion through altered regulation of metalloproteinase production, Eisenberg et al. (2011) identified a role for myoferlin in promoting invasive behavior in breast cancer cells. The identification of myoferlin as a key regulator of EGFR (Epidermal Growth Factor Receptor) activity through inhibition of non-clathrin endocytosis in breast cancer cells was important in understanding the molecular mechanisms involved in cancer growth (Turtoi et al., 2013). The production of myoferlin-silenced tumor cells has provided another element that may help explain the role of ferlin: these tumors lacked functional blood vessels, an effect that may be due to a reduction in



VEGFA exocytosis (Fahmy et al., 2016). This may explain its strong association with cancer prognosis, as an independent prognosis factor in kidney (Song et al., 2017).

### 8.3. Otofelin

Currently, there is limited information available on otofelin. Involvement of IL-6 in inner ear damage during noise trauma or cisplatin treatment has been reported (So et al., 2007; Wakabayashi et al., 2010), but a possible link with otofelin function during disease progression has not yet been explored. Using OncoLnc, a tool for exploring correlations between the expression of mRNAs, mRNAs, and lncRNAs, we analyzed 5-year overall survival in 21 cancer types (Anaya, 2016). In three cancers, we found a significant impact of otofelin expression on survival: in renal clear cell, papillary cell carcinoma, and in bladder urothelial carcinoma (Figure 11). Interestingly, otofelin expression was either a protective or a risk factor depending on the histology of the cancer. Given the differences in 5-year survival, it seems appropriate to consider using otofelin expression as a prognostic biomarker to guide therapeutic decisions.

In clear cell renal cell carcinoma, Cox et al. (2021) showed in a study of 79 patients that otofelin was an indicator of tumor

staging and a prognostic biomarker for cancer-specific survival. In 25 patients with oral squamous cell carcinoma, Kraus showed complete suppression of otofelin expression in malignant tissue (Kraus et al., 2022). Taken together, these results suggest a role for otofelin in the carcinogenesis of these tumors, which deserves investigation to confirm and understand its exact role, especially as it appears to vary according to tumor site. Studies are needed to ensure that otofelin is an independent prognostic factor for overall survival. This could lead to new cancer therapies targeting this protein, which are already underway for myoferlin (Zhang et al., 2018; Li et al., 2019; He et al., 2021).

### 8.4. Oncomodulin

Hair cells, notably OHCs, highly express another protein, which has also been used as a prognostic marker in cancer: oncomodulin (OCM). OCM is a small EF-hand  $\text{Ca}^{2+}$ -binding protein (CaBP) of approximately 12 kDa. It belongs to the parvalbumin family. It is the  $\beta$ -isoform of parvalbumin. It shares at least 53% sequence identity with alpha-parvalbumin (PVALB) (Climer et al., 2019). OCM has an unusually restrictive post-embryonic expression pattern in mammals, mostly restricted to subsets of sensory hair cells in the inner ear and more recently found in certain subtypes



of immune cells. Oncomodulin is essential for normal hearing function since its deletion in mice leads to progressive deafness due to alterations in the mechanical amplification function of OHCs (Tong et al., 2016). We recall that OHC lateral mechanical stiffness and motility have long been recognized as regulated by calcium acting on the actin-based cortical cytoskeleton (Dulon et al., 1990). Oncomodulin, acting as a CaBP, may be essential in sculpting calcium signals important for F-actin remodeling and membrane mechanics in OHCs, a role somewhat similar to that of otoferlin in IHCs, as proposed in our review.

The initial discovery of OCM as an oncoprotein in cancer tissue and its similarity to calmodulin as a CaBP led to the term "oncomodulin" (Durkin et al., 1983). OCM was considered oncogenic because no expression was detected in normal post-embryonic tissue. However, after its initial discovery, OCM was identified as a major protein in sensory cells of the cochlea. Studies have suggested that OCM may play a role in cell proliferation, and recently, Yin et al. (2006) identified oncomodulin as a potent macrophage-derived growth factor for retinal ganglion cells (RGCs) and other neurons. Oncomodulin also stimulates the outgrowth of peripheral sensory neurons.

## 9. Discussion-conclusion

Otoferlin, a protein mainly produced in the brain and inner ear, plays a key role in hearing. More than 220 mutations causing DFNB9-type deafness have been identified so far. Many of these mutations alter the folding, stability, or function of the protein, resulting in severe to profound hearing loss associated with a defect in synaptic transmission between the IHC and the nerve fibers of the auditory nerve.

An intriguing question is: why have auditory and vestibular hair cells selected otoferlin as a key  $\text{Ca}^{2+}$ -sensor to control exocytosis of synaptic vesicles instead of the classical synaptotagmins found in central synapses? The need to encode tiny graded microphonic potentials spanning over a large range of amplitude (several dozens of dB) by graded multivesicular release at each ribbon synapses and the requirement to sustain an extremely high rate of vesicle release by an efficient vesicular replenishment are probably the two main factors explaining the use of a large multi-C2  $\text{Ca}^{2+}$  sensing protein such as otoferlin. Another important property of otoferlin is its capability to function as a high affinity large-range  $\text{Ca}^{2+}$  sensor as compared to synaptotagmins. In our review, we propose otoferlin as a  $\text{Ca}^{2+}$ -dependent mechanical interactor between the membranous F-actin network and the synaptic vesicles at the hair cell ribbon. This protein would confer  $\text{Ca}^{2+}$ -dependent oscillatory movement of the ribbon, whose resonant frequency would depend on their size (mass), tuning the firing frequency of the postsynaptic fibers.

In this context, it is important to note that otoferlin belongs to the ferlin protein family in humans: dysferlin, myoferlin, fer1L4, fer1L5, and fer1L6, all of which being expressed in mechanically active or contracting cells. These proteins share common features such as the presence of multiple C2 domains, FerA domains, and membrane anchoring through their C-terminal domain, and are thought to play a role in calcium-triggered membrane fusion and trafficking. Although otoferlin is expressed in the central

nervous system, in particular in the cortex and amygdala, its role in brain tissues remains unknown. Interestingly, a genome-wide expression analysis of a human-mouse chimeric model of Alzheimer's disease identified OTOF as a downregulated gene, suggesting a critical function of otoferlin in the central nervous system. Dysferlin has been observed to accumulate in endothelial cells adjacent to sclerotic lesions and within amyloid-beta plaques in individuals with Alzheimer's disease. These findings highlight the need for further research into the role of ferlin proteins in the central nervous system, particularly their potential involvement in neurodegenerative diseases such as Alzheimer's disease.

Also, otoferlin, like many of the other ferlins, plays a crucial role in membrane-related processes that are important for cell survival and signaling. Studies have suggested a link between ferlins and cancer through their effect on cell signaling and development, allowing tumors to form and cancer cells to adapt to a hostile environment. Despite their importance, ferlins have not been extensively studied in the context of cancer. Data show that otoferlin expression is significantly associated with survival in specific cancer types, including clear cell and papillary cell renal carcinoma, and urothelial bladder cancer. These results suggest a role for otoferlin in the carcinogenesis of these tumors, which needs further investigation to confirm and understand its exact role, especially as it appears to vary according to tumor site. This could lead to new cancer therapies targeting this protein.

## Author contributions

J-CL and DD wrote the manuscript. Both authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Comme nous avons pu le voir, l'otoferline, a plusieurs fonctions indispensables pour l'audition, notamment en tant que senseur calcique ayant une implication dans la fusion membranaire.

Cependant, le rôle exact de son domaine transmembranaire est mal connu. Son rôle est probablement majeur, puisqu'une mutation entre le domaine C2F et le domaine trans membranaire en C-ter (p.R1939Q) cause une surdité sévère à profonde. Il s'agit de la mutation la plus fréquemment retrouvée au Japon (Matsunaga et al., 2012). Pour étudier cette mutation, nous avons utilisé la technologie CRISPR-Cas9 *in vivo*, et créé un modèle murin qui permet l'expression de cette endonucléase spécifiquement dans les CCI.

Article n°2 La diminution spécifique de l'expression de l'otoferline dans les cellules ciliées grâce à l'utilisation de CRISPR-Cas9 *in vivo* chez la souris provoque une surdité associée à une dégénérescence des synapses à ruban.

## Résumé

Des mutations du gène OTOF, codant pour l'otoferline, sont à l'origine de la surdité profonde non syndromique de transmission autosomique récessive DFNB9. Cette protéine qui contient plusieurs domaines C2 est un capteur de calcium essentiel qui déclenche la libération des vésicules synaptiques (exocytose) au niveau des synapses à ruban des cellules ciliées internes. Fait intéressant, cette protéine s'est également révélée essentielle au maintien et à la survie des synapses à ruban de cellules ciliées par des mécanismes encore inconnus. Pour déterminer le rôle de l'otoferline dans ce dernier processus, nous avons créé un modèle de souris dans lequel l'expression de l'otoferline dans les cellules ciliées a été diminuée à plusieurs niveaux, à un stade postnatal tardif. Les conséquences sur les seuils auditifs et le maintien des synapses à ruban des cellules ciliées ont été étudiées.

Méthodes : Des souris knock-in CRISPR/Cas9 H11LSL-Cas9, qui ont une expression de l'endonucléase CRISPR associated protein 9 (Cas9) dirigée par un promoteur CAG qui est dépendante de la Cre recombinase (The Jackson Laboratory), ont été croisées avec des souris Myo15-Cre<sup>+/+</sup> (Myo15<sup>tm1.1(cre)Ugds</sup>) pour obtenir des souris avec une expression spécifique de la Cas9 dans les cellules ciliées. Chez des souris P2-P4 anesthésiées, des virus adéno-associés recombinants (AAV2/8) portant une séquence codant pour des ARN guides spécifiques de l'otoferline suivie d'une séquence GFP ont été micro-injectés dans la cochlée à travers la membrane de la fenêtre ronde. Les contrôles étaient constitués d'une part de souris exprimant la Cas9 dans les cellules ciliées ayant eu des injections d'AAV2/8 contenant des ARN guides aléatoires non spécifiques et d'autre part de souris n'exprimant

pas la Cas9 ayant eu des injections de l'AAV2/8 contenant l'ARN guide spécifique de l'otoferline. Les AAV2/8 contenant les ARN guides spécifiques de l'otoferline (ARNg-Otof) et les ARN guides aléatoires non spécifiques de contrôle ont été conditionnés et titrés par Penn Vector Core. Les potentiels évoqués auditifs (ABR) et les produits de distorsion des oto-émissions acoustiques (DPOAE) ont été enregistrés chez des souris anesthésiées à P18-P30 à l'aide du système BioSigRZ-RZ6 de Tucker-Davies Technologies. L'otoferline et les rubans synaptiques ont été visualisés en immunofluorescence par microscopie confocale à haute résolution.

Résultats : Les souris CRISPR/Cas9 H11LSL-Cas9/Myo15-Cre<sup>+/-</sup> qui ont eu une injection d'AAV contenant des ARNg-Otof avaient une augmentation moyenne de 35 dB des seuils d'ABR en tonal et au clic par rapport aux souris contrôles ( $p < 0,001$ ), tandis que les DPOAE étaient normales. Chez ces souris, le taux moyen de transduction du gène rapporteur GFP (et des ARNg associés) par AAV2/8 dans les cellules ciliées a été évalué en microscopie à fluorescence à 82 % dans les cellules ciliées internes (CCI) et à 36 % dans les cellules ciliées externes (CCE). Chez ces souris injectées avec des ARNg-Otof, nous avons constaté une diminution mosaïque de l'expression de l'otoferline dans les CCI le long de la cochlée. Nous avons pu établir une bonne corrélation entre le niveau d'expression de la protéine otoferline et le nombre de rubans synaptiques par CCI ( $r = 0,82$   $p < 0,001$ ). La taille des CCI était également positivement corrélée au niveau d'expression de l'otoferline ( $r = 0,69$   $p = 0,01$ ).

Conclusion : Par cette preuve de concept, nous montrons que l'outil de modification du génome CRISPR-Cas9 peut fonctionner *in vivo* dans la cochlée. Grâce à cette technologie, nous avons pu moduler à la baisse le niveau d'expression de l'otoferline spécifiquement dans les cellules ciliées, en postnatal, et montrer que cette protéine est essentielle pour le maintien des synapses à ruban.

*Manuscrit en cours de finalisation*

# Mice with hair cell-specific down expression of otoferlin by CRISPR-Cas9 technology *in vivo* shows hearing loss associated with ribbon synapse degeneration

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## Abstract

Mutations in the *OTOF* gene, encoding otoferlin, cause profound nonsyndromic recessive deafness, DFNB9. This large multi-C2 protein is an essential calcium-sensor triggering synaptic vesicle release (exocytosis) at the inner hair cell ribbon synapses. Remarkably, this protein has also been found to be essential for the maintenance and survival of the hair cell ribbon synapses by mechanisms that remain unknown. To decipher the role of otoferlin in this later process, we created a mouse model in which the hair cell expression of otoferlin was down modulated at various levels along the cochlear partition at late postnatal stages. The consequences on hearing thresholds and the maintenance of the hair cell ribbon synapses will be studied.

Methods: H11LSL-Cas9 CRISPR/Cas9 knock-in mice, which have Cre recombinase-dependent expression of CRISPR associated protein 9 (Cas9) endonuclease directed by a CAG promoter (Jackson Laboratory), were crossed with Myo15-Cre<sup>+/+</sup> mice (Myo15<sup>tm1.1(cre)Ugds</sup>) to obtain offspring mice with a specific cas9 expression in hair cells. On these anesthetized P2–P4 offspring mice, a recombinant adeno-associated virus (AAV2/8) carrying a sequence encoding for otoferlin-specific gRNAs followed by a GFP sequence was microinjected in the cochlea through the round window membrane. Control mice were injected with AAV2/8 containing nonspecific random gRNAs or injected in mice that did not express cas9. The virus containing the otoferlin-specific gRNAs and control random gRNAs were packaged and titrated by Penn Vector Core. Auditory brainstem responses (ABRs) and distortion product otoacoustic emissions (DPOAEs) were recorded in anesthetized mice at P30 by using the BioSigRZ and RZ6



system of Tucker-Davies Technologies. Otoferlin and synaptic ribbons were specifically visualized under high resolution confocal immunofluorescence microscopy.

Results: H11LSL-Cas9 CRISPR/Cas9-Myo15-Cre+/- mice injected with AAV containing Otoferlin-specific gRNAs displayed a mean increase of 35 dB in tone and click ABR thresholds as compared to control mice ( $p < 0.001$ ) while DPOAEs remained normal. In these mice, the mean rate of hair cell AAV transduction of the reporter gene GFP (and the associated gRNAs) was evaluated under fluorescence microscopy at 82% in IHCs and 36% in OHCs. Remarkably, in these Otoferlin-gRNAs injected mice, we found a mosaic decrease in the otoferlin expression in IHCs along the cochlear partition. We could establish a good correlation between the expression level of protein otoferlin and the number of synaptic ribbons per IHCs ( $r = 0.82$ ,  $p < 0.001$ ). The size of the IHCs were also positively correlated to the level of otoferlin expression ( $r = 0.69$ ,  $p = 0.01$ ).

Conclusion: We demonstrated as a proof of concept that CRISPR-Cas9 technology can work *in vivo*. By this technology we could down modulate the expression level of otoferlin in postnatal hair cells and show that this protein is indeed essential for the maintenance of the synaptic ribbon

## Introduction

Otoferlin is a large multi-C2 protein, an essential calcium-sensor triggering synaptic vesicle release (exocytosis) at the inner hair cell (IHC) ribbon synapses (Roux et al., 2006; Beurg et al., 2010; Michalski et al., 2017; Leclère and Dulon, 2023). This protein of 1997 amino-acid contains a single C-terminal transmembrane domain (TMD) which anchors the protein to the vesicular membrane and six C2 domains (A-F) which are directed toward the IHC cytosol. The main function of C2 domains is to target membrane surfaces after  $Ca^{2+}$ -binding (Corbalán-García and Gómez-Fernández, 2014). Otoferlin has a FerA domain, with its own  $Ca^{2+}$ -dependent activity (Harsini et al., 2018). The long isoform of otoferlin is encoded by 48 exons, of which exons 6 and 31 can be alternatively spliced.

Otoferlin is known for its interactions at the presynaptic active zone of inner hair cells (IHC), where it interacts with essential SNARE (soluble N-ethylmaleimide-sensitive-factor attachment protein receptor) proteins, including SNAP25 (Roux et al., 2006; Calvet et al., 2022), as well as Cav1.3  $Ca^{2+}$  channels (Vincent et al., 2017, 2018). Otoferlin enables  $Ca^{2+}$ -triggered exocytosis of synaptic vesicles containing glutamate - the primary excitatory

neurotransmitter in the cochlea - at inner hair cell (IHC) synapses located along the ribbon structure. The presynaptic ribbons - an electron-dense structure approximately 300-350 nm wide, with 15 to 20 per IHC - face individual auditory nerve boutons, which contain ionotropic AMPA (glutamate) receptors composed of GluA2, GluA3 and GluA4 subunits (Rutherford et al., 2023). This protein has also been found to be essential for the maintenance and survival of the hair cell ribbon synapses by mechanisms that remain unknown. It is important to note that otoferlin is not involved in the normal electromechanical amplification of sound by the outer hair cells (OHCs), as evidenced by the measurement of distortion products in otoacoustic emissions (DPOAEs).

Mutations in the OTOF gene, which encodes otoferlin, have been identified as the cause of a recessive and profound form of deafness in humans, termed DFNB9 (Yasunaga et al., 1999, 2000). DFNB9 is classified as an auditory cochlear synaptopathy (Moser and Starr, 2016). This specific type of deafness, which accounts for approximately 2% of cases of non-syndromic prelingual deafness, has significant clinical implications as it is the leading cause of auditory neuropathy spectrum disorder (ANSO) (Iwasa et al., 2019). The genetic landscape of this condition has been extensively studied, leading to the identification of approximately 220 mutations associated with DFNB9-associated deafness (Azaiez et al., 2021).

Remarkably, one of the most common OTOF mutations in Japan (p.R1939Q) is located at the junction between the calcium-binding C2F domains and the C-terminal transmembrane domain (TMD) (Matsunaga et al., 2012).

To study this mutation at the IHC level, we used CRISPR-Cas9 technology in an *in vivo* setting. This approach allowed us to bypass the need to generate a mutant mouse line, which would have been more time consuming and costly. The aim was to achieve Cas9 expression exclusively within the hair cells, both to reduce potential side effects and to establish a method that could be used to study other mutations or proteins within the inner ear. To this end, we used a mouse strain in which Cas9 expression depends on cross-breeding with a second strain that expresses Cre-recombinase under the control of the myosin 15 gene promoter, which is specific for hair cells (Caberlotto et al., 2011). The gRNA (targeting exon 48), delivered by an AAV that facilitates the action of Cas9, was injected intracochlearly in mice at P2-P4.

Using this technique to create a mouse model in which the TMD is precisely targeted to exon 48 using CRISPR-Cas9 technology in postnatal mice served a dual purpose. It provided proof of concept for studying mutations in inner hair cells and allowed us to assess the impact of the otoferlin exon 48 mutation on both auditory function and the maintenance and survival of hair cell ribbon synapses.

## Methods

### Animals

We used two mice line: the  $Myo15^{tm1.1(cre)Ugds}$  mice (Caberlotto et al., 2011), carrying the cre-recombinase gene driven by the myosin-15 promoter, which is specific of hair cells; and B6.129-Igs2<sup>tm1(CAG-cas9\*)Mmw</sup> /J knock-in mice (Jackson Laboratory; mouse strain 027632) having a loxP-flanked STOP (LSL) cassette preventing expression of the downstream Cas9 sequence. This endonuclease was directed by a CAG promoter. By crossing these two mice line we generated mice with a specific hair cell expression of Cas9 (**Fig. 1**). The excision of the LSL in the genome of the hair cells was verified by PCR and the expression of Cas9 was verified by RT-PCR from IHCs (data not shown).

### gRNA design and viral vector constructs

The endonuclease activity of the Cas9 is guided by a guide RNA (gRNA) with sequence homology to a given locus, where it induces a double stranded break (DSB). We designed a target sequence in exon 48 of the OTOF gene, followed by a GFP sequence. The DSB induced by Cas9 is repaired through non-homologous end joining (NHEJ), an error-prone process that produces insertions/deletions, and will conduct to the disruption on the target exon 48. The Cas9 edited OTOF gene then would lead to truncated forms of Otoferlin which do not carry the TMD part. In order to design the gRNA targeting exon 48 and to minimize off-targeting effect, we used a computational CRISPR design tool (<http://crispor.tefor.net>; Haeussler et al., 2016). We chose two gRNA with a high specificity score of 92 (gRNA1), and 97 (gRNA2) (ranges from 0-100 and measures the uniqueness of a guide in the genome, the higher the specificity score, the lower are off-target effects in the genome) in the reverse strand: 'ACTTGTACCGGGTGCAGATG 5' and 'AACCAGACGAATGCCGTGTC 5' with PAM sequence CCT, corresponding to nucleotide 5754 or 5806 of the OTOF gene. The analysis showed a low probability of interference with off-targets. There were only four (gRNA1) or two (gRNA2)

possible off-targets that had 3 mismatches (Table 1). The two gRNA were delivered in a 1:1 ratio by intracochlear injection of Adeno-associated-virus (AAV) through the round window of P2-P4 mice (see paragraph below). The two murine gRNA sequence were each cloned into an AAV-U6/TO-gRNA(exon47)-CMVTetR- P2A-eGFP-KASH-pA plasmid (pJEP10; Addgene). Plasmids containing gRNA were subcloned into AAV2 genome and packaged in AAV8 capsid (Penn Vector Core; Pennsylvania) as described in (Emptoz et al., 2017). The gRNA expression was driven by the ubiquitous U6 promoter and the eGFP reporter gene, allowing a monitoring of transfected IHCs, was driven by the CMV promoter.

gRNA	Gene Symbol	Sequence - PAM	Predicted function	Location	CFD score
2	Slamf1	GACGAGACAATGCCGTGTC AGG	lymphocyte activation	chr1	0.257
1	Arid1B	ACTAGTACCGAGTGCAGAGG AGG	unknown	chr17	0.119
1	Tshz3	AATTGTATCGGATGCAGATG AGG	regulation of transcription	chr7	0.154
1	Fank1	ACTTGTAACCGGTGCAGAGG AGG	fibronectine	chr7	0.065
1	Agbl1	ACTTGTAGCCTGTGCAGATG AGG	proteolysis	chr7	0.025
2	Etv1	CACGAGACGAATGCCTTGTC AGG	regulation of transcription	chr12	0.000

**Table 1** : Off-targets identified. The table recapitulates gene where off-target have been found, ranked by CFD score (Doench et al., 2016) from most to least likely. The presence of these off-target in the inner ear was found in SHIELD database (<https://shield.hms.harvard.edu/>)

#### Round window membrane injection

Experiments were performed in accordance with the guidelines of the Animal Care Committee of the European Communities Council Directive (86/609/EEC) and were approved by the ethics committee of the University of Bordeaux (animal facility agreement number C33-063-075). The AAV2/8 vector containing the gRNA sequence targeting OTOF was injected in the cochlea of P2-P4 mice at a titer of  $5.75 \times 10^{13}$  genome copies (gc)/ml. Two types of control mice were used in the study: mice expressing Cas9 in IHC were injected with AAV2/8 containing non-specific random gRNA, and mice without Cas9 expression were injected with OTOF gRNA. Intracochlear viral transduction was carried out as previously described (Emptoz et al., 2017; Dulon et al., 2018). Mice were anesthetized using hypothermia exposure. ENT surgeon performed surgery (JCL). A left post-auricular incision was made to expose the otic bulla and

to visualize the cochlea. A glass micropipette containing 1 $\mu$ l of AAV2/8 vector preparation was inserted through the round window membrane (RWM) and injected into the cochlea. The pipette was removed, the hole in the membrane was covered with connective tissue, and the incision was sealed with biological glue (3M Vetbond). Standard post-operative care was applied.

#### Auditory Brainstem Responses

To record ABRs (Auditory Brainstem Responses, which represent the sound-evoked synchronous firing of the auditory cochlear nerve fibers) as well as distortion product otoacoustic emissions (DPOAEs, which reflect the amplification provided by OHCs), mice were anesthetized with intraperitoneal injection of xylazine (6 mg/ml) and ketamine (80 mg/ml) mixture (Rompun, #KP091XW and Vibrac, #4C14) diluted in physiological saline. The mouse body temperature was kept constant at 37°C. Tests were conducted at P18 and P30. For stimulus generation and data acquisition, we used a TDT RZ6/BioSigRZ system (Tucker-Davis Technologies). ABR signals were averaged after the presentation of a series of 512 stimulations. ABR thresholds were defined as the lowest stimulus for recognizable wave-I and II. The amplitude of ABR wave-I was estimated by measuring the voltage difference between the positive (P1) and negative (N1) peak of wave-I. Pure tone stimuli were used at frequencies of 4, 8, 16, 24 and 32 kHz. Sound intensities of 10 to 90 dB SPL, in 10 dB step, were tested.

Distortion product otoacoustic emissions (DPOAEs), which originate from the electromechanical activity of the outer hair cells (OHCs), were tested by using two simultaneous continuous pure tones with frequency ratio of 1.2 ( $f_1 = 12.73$  kHz and  $f_2 = 15.26$  kHz). DPOAEs were collected with the TDT RZ6/BioSigRZ system designed to measure the level of the “cubic difference tone”  $2f_1 - f_2$ .

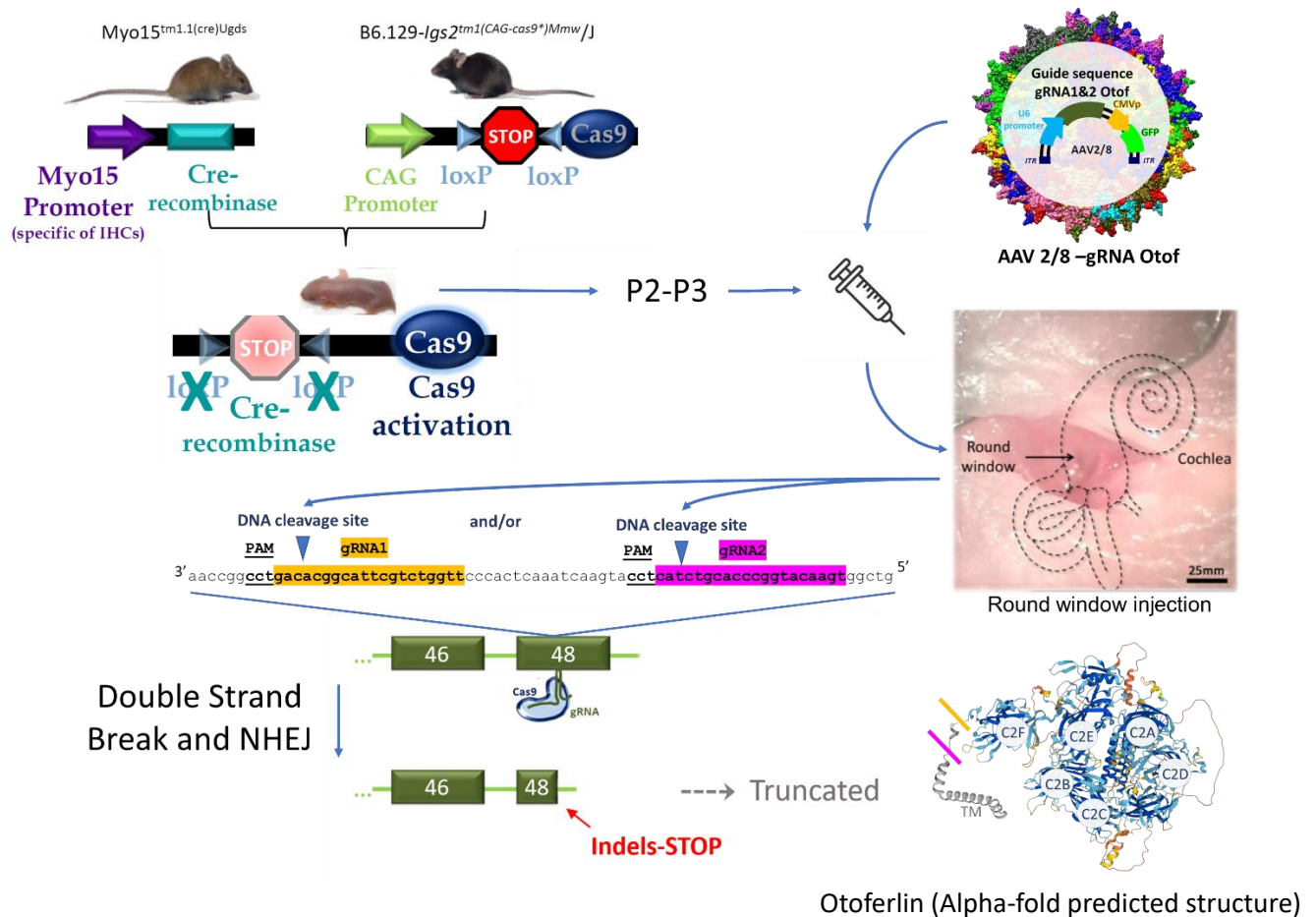
#### Tissue preparation and immunocytochemistry

Mice were deeply anesthetized with isoflurane (Vetflurane, Virbac). Cochleas were dissected and prepared as previously described (Vincent et al., 2014). Inner ears (cochleae) were fixed by incubation in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4, at 4°C during 12h and washed with cold phosphate buffered saline (PBS). They were then incubated 48h in PBS solution containing 10% EDTA pH 7.4 at 4°C. The middle part of the organ of Corti (area encoding between 8 and 16 kHz) was then dissected and the tectorial membrane

removed. The tissue was first incubated with PBS containing 30% normal horse serum and triton X100 0.5 % for 1 h at room temperature. Synaptic ribbons were labeled with anti-CtBP2 (1/200 Guinea pig monoclonal antibody, Synaptic system, cat # 192104). Otoferlin was labeled with a N-ter mouse monoclonal antibody (1/200 AbCam, cat # ab53233). Explants of Organ of Corti were incubated with primary antibodies during 12h at 4°C. Fluorescent secondary antibodies 1/500 were then used: anti-Goat Donkey polyclonal Fluoprobes 546H (Molecular Probes, cat# A21123), anti-Guinea pig Donkey polyclonal Fluoprobes 647H (Jackson, cat# 706-605-148). Actin-F was also used to visualize hair cells (1/100, Phalloidin Fluoprobe 405, Interchim, Montlucon, France; cat # FP-CA9870). For Image Acquisition, organ of Corti samples were analyzed using a confocal laser scanning microscope Leica SP8 with a 63X oil immersion objective (NA = 1.4) and white light laser (470–670 nm) (Bordeaux Imaging Center). Phalloidin was imaged by using a diode laser at 405 nm. For 3D-stack reconstruction of IHCs, 25–30 images (0.3  $\mu\text{m}$  thickness) were acquired (Vincent et al., 2014). Ribbon volumes were calculated using the 3D-Objects Counter Plugin of ImageJ, and count was confirmed with DIVA viewer (El Beheiry et al., 2020).

#### Data Analysis

Data were analyzed using Origin 9.1 (OriginLab). Means were expressed  $\pm$  SEM. Data were compared either using two-tails Student's t-test for normally distributed data or Mann-Whitney test for non Gaussian distributed data. Otherwise, for multi-parametric analysis, we used a two-way ANOVA. In the graphs \* indicates statistical significance with  $p < 0.05$ , \*\* indicates  $p < 0.005$ , and \*\*\* indicates  $p < 0.001$ .



**Figure 1.**  $Myo15^{tm1.1(cre)Ugds}$  mice carrying the cre-recombinase gene driven by the myosin-15 gene promoter, were crossed with  $B6.129-Igs2^{tm1(CAG-cas9^*)Mmw/J}$  knock-in mice having a loxP-flanked STOP cassette preventing expression of the downstream Cas9 sequence. The offspring resulting from such a cross have a specific expression of Cas9 in hair cells. In these later mice, specific gRNA (gRNA1 and gRNA2 targeting exon 48 of the OTOF gene) were delivered to hair cells by AAV intracochlear injection through the round window at P3. The Cas9 endonuclease activity guided by the gRNA induces a double stranded break (DSB), repaired through non-homologous end joining (NHEJ), an error-prone process that produces insertions/deletions on the target exon 48, leading to mutated or truncated forms of the otoferlin transmembrane domain.

## Results

### CRISPR/Cas9 targeting of exon 48 of OTOF gene in mouse cochlear hair cells

We crossed  $Myo15^{tm1.1(cre)Ugds}$  and  $B6.129-Igs2^{tm1(CAG-cas9^*)Mmw/J}$  knock-in mouse lines to obtain mice expressing Cas9 specifically in cochlear hair cells (**Figure 1**). Cas9 expression was verified by RT-PCR from dissected organs of Corti (Supplementary data). A single intracochlear injection of an AAV vector containing a specific gRNA targeting exon 48 of the OTOF gene was performed in mice on postnatal days P2-P3. These injected mice were named CRISPR-OTOF-gRNA mice. The Cas9 endonuclease activity directed by this specific gRNA would generate hair cells without functional otoferlin. The efficacy of AAV-gRNA-eGFP hair cell transduction, assessed at P15-P30 by counting eGFP-expressing hair cells under confocal fluorescence microscopy, showed a high percentage of transduced IHCs (82.4%) and a lower percentage of OHCs (36.2%) (Supplementary Data).

## Decrease of auditory function in CRISPR-Otof-gRNA injected mice

Auditory brainstem response (ABR) measurements were performed to assess the auditory function of CRISPR-Otof-gRNA at P18 and P30. No significant difference was found between P18 and P30. These mice had a significant increase in ABR thresholds compared to control mice injected with AAV2/8 with non specific random gRNA (**Figure 2A**). At all frequencies from 4 to 32 kHz, there was a 10-25 dB increase in threshold compared to controls ( $p < 0.001$ ). The effect on hearing was less pronounced at lower frequencies, particularly at the apex of the cochlea, where there was an increase of approximately 10 dB at 4 kHz. In addition, five wild-type control mice were injected with OTOF gRNA and did not show a significant increase in ABR compared to mice injected without gRNA. While the latency of the ABR wave I did not show significant changes, at least at low sound intensities, its peak amplitude was significantly reduced in CRISPR-Otof mice (**Figure 2B**).

Distortion product of otoacoustic emissions (DPOAEs), which assess the electromechanical activity of outer hair cells (OHCs), remained unaffected in CRISPR-Otof-gRNA mice (**Figure 2C**). This auditory phenotype, characterised by abnormal ABRs and normal DPOAEs, suggested the presence of auditory hair cell synaptopathy, indicating a defect in synaptic transmission between inner hair cells (IHCs) and auditory nerve fibers. Notably, a similar auditory phenotype was observed in older CRISPR-Otof-gRNA mice, around 2-3 months of age (data not shown), suggesting that the hearing loss was not due to developmental delay, but rather permanent. Interestingly, evaluation of DPOAEs at P40 revealed a decline in OHC function (data not shown), suggesting a deleterious effect of loss of IHC function, probably due to reduced stimulation of OHCs by efferent nerve fibers.

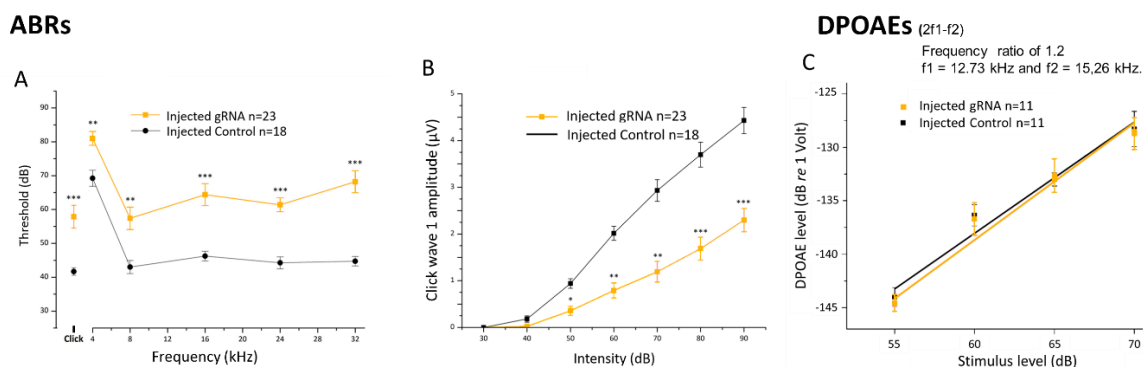


Figure 2: Auditory brainstem responses (ABRs), representing the sound evoked neural output of the cochlea, were measured in P18-P30 mice. In CRISPR-Cas9/AAV-Otof gRNA injected mice (orange), we observed an increase of ABR thresholds (A), as compared to control mice (injected with AAV-with random gRNA, black), averaging 25 dB at all hearing frequencies, and a large decrease in wave 1 amplitude (B). By contrast, distortion-product otoacoustic emissions (DPOAEs), which probe outer hair cells (OHCs) function, remained unaffected in CRISPR-Cas9/AAV-Otof gRNA injected mice at P16 (C), indicative of an IHC synaptopathy.



## Mosaic reduction of otoferlin expression, and loss of synaptic ribbon in CRISPR-Otof-gRNA injected mice

The variability in otoferlin expression levels between AAV-transfected inner hair cells (IHC) (Figure 3A) and the contralateral control ear (Figure 3B) can be attributed to the stochastic nature of the repair processes following a double-strand break. On average, the mean fluorescence intensity of otoferlin in the AAV-transfected IHCs, measured at  $1456 \pm 371$ , was significantly lower than that observed in the control group, which averaged  $2253 \pm 117$  ( $p < 0.001$ ).

It is worth noting the wide range of otoferlin fluorescence intensities observed in the AAV-transfected cells. The intensity of otoferlin fluorescence could vary from completely normal to completely absent (Figure 3A). In contrast, the IHCs from the control mice showed a consistent and overall homogeneous level of otoferlin fluorescence intensity (**Figure 3B**).

In CRISPR-OTOF-gRNA mice, a significant reduction in the number of synaptic ribbons per cell was observed, with an average decrease from 14 to 6 ribbons per cell ( $p = 0.004$ ) (**Fig 3C,D**). This decrease in ribbon density was more pronounced in the basal and middle regions of the cochlea compared to the apex (9 vs 3 respectively,  $p = 0.002$ ) (Supplementary Data). These findings closely match the audiometric results shown in **Figure 2A**, where a lower increase in hearing threshold was observed at lower frequencies, coded at the apex of the cochlea.

Moreover, the maximum distance between synaptic ribbons was significantly shorter in the CRISPR-OTOF-gRNA mice than in control ( $5.68$  vs  $8.49 \mu\text{m}$  respectively,  $p < 0.001$ ) (Supplementary Data). This effect was also more observed in IHCs located in the middle part of the cochlea compared to those in the apex ( $4.84$  vs  $5.92$   $p = 0.03$ ). This spatial redistribution of synaptic ribbons further increases the concentration of these structures towards the basal end of inner hair cells.

## Number of ribbons per IHC and the size of IHC are correlated to otoferlin expression in CRISPR-Otof-gRNA injected mice.

When comparing otoferlin expression with the number of synaptic ribbons per inner hair cell (IHC), a strong correlation was observed ( $r = 0.82$ , 95% CI 0.73-0.87,  $p < 0.001$ ) (**Fig4A**). In addition, a decrease in the length of the synaptic area of IHCs, measured from the centre of the nucleus to the basal side, was found in CRISPR-Otof-gRNA mice compared to the control group ( $10.81$  vs.  $8.24 \mu\text{m}$  respectively,  $p = 0.004$ ). Similarly, the size of the IHCs showed a positive correlation with the level of Otoferlin expression ( $r = 0.69$ , 95% CI 0.60-0.77,  $p < 0.01$ ) (**Fig4B**).

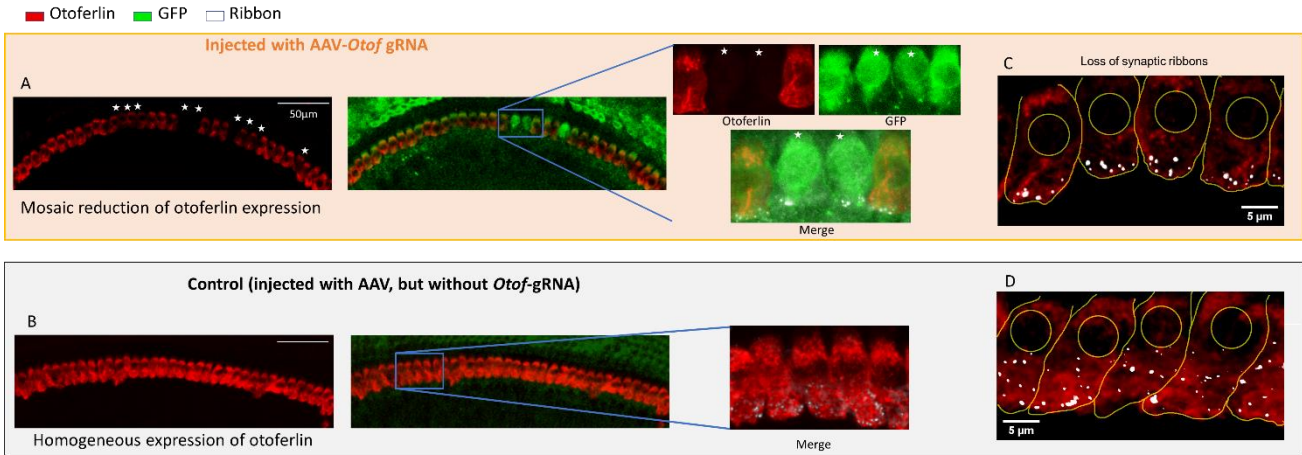


Figure 3 : The random nature of the repairs after the double strand break caused heterogeneity in the expression level of otoferlin in AAV transfected IHC (A) as compared to control contralateral ear (B). All types of otoferlin intensity were noted for the AAV transfected cells, from normal otoferlin fluorescence intensity to no fluorescence at all (A, C) whereas in control IHCs, otoferlin fluorescence intensity was homogeneous (B, D). In CRISPR-Cas9/AAV-Otof gRNA injected mice, the synaptic ribbons were less numerous and more concentrated at the cell basal pole (C,D). (Red: otoferlin N-ter antibody; Green: viral-GFP; White: ribbon, anti-CtBP2 antibody)

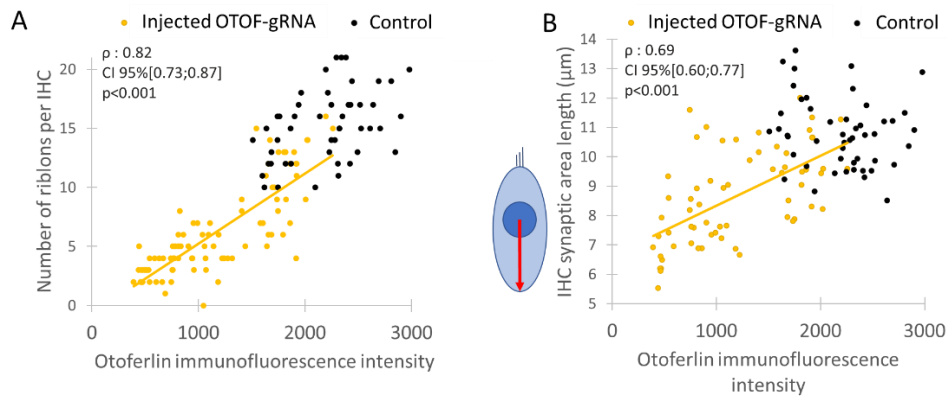


Figure 4 The data from CRISPR-OTOF-gRNA injected mice are represented in yellow, and the data from control injected mice are shown in black. Assessment of synaptic ribbon number based on otoferlin immunofluorescence intensity in inner hair cells (IHC) is shown in (A). Each data point represents an IHC, and its position on the graph reflects both the intensity of otoferlin immunofluorescence and the number of synaptic ribbons. The graph shows a strong positive correlation between otoferlin expression and the number of synaptic ribbons, as indicated by the upward sloping trend line ( $r=0.82$ , CI95 0.73;0.87  $p < 0.001$ ). This suggests that as otoferlin expression increases, the number of synaptic ribbons in IHCs increases and vice versa. Data on the size of inner hair cells (IHCs) based on their otoferlin expression levels are shown in (B). In the same way, each data point corresponds to an individual IHC and the position of the point on the graph represents the intensity of otoferlin immunofluorescence and the size of the IHC synaptic area, measured from the centre of the nucleus to the basal side. It shows a positive correlation between IHC size and otoferlin expression, with cells expressing lower levels of otoferlin tending to be smaller ( $r=0.69$ , CI95 0.60;0.77,  $p < 0.001$ ).

## Discussion

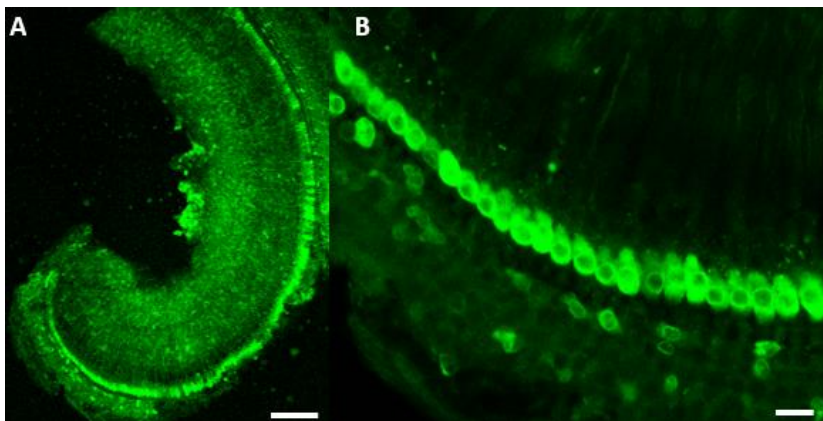
We demonstrated as a proof of concept that CRISPR-Cas9 technology can work *in vivo* in quiescent sensory auditory hair cells. By using a cross of mice expressing Cre under the control of myosin XV and Cas9 floxed, meaning that it requires myosin XV expression to be functional, we were able to achieve specific Cas9 expression within sensory hair cells. Other studies have also used *in vivo* gene editing. Using an adeno-associated virus (AAV) vector encapsulating Cas9 and sgRNA, Xue et al. successfully restored hearing in mice with autosomal dominant hearing loss associated with myosin VI abnormalities (Xue et al., 2022). The effect was observed for more than 5 months. More recently, Tao et al. demonstrated the efficacy of CRISPR-Cas9 via a cationic lipid to correct hearing loss by targeting outer hair cells (Tao et al., 2023). The same team had previously shown similar results in inner hair cells (Gao et al., 2018a). The drawback of these techniques is the lack of cellular specificity of the vector used, whether it's an AAV or a cationic lipid, unlike the method we used. Although not applicable in a clinical rescue context, our approach seems particularly promising for the specific study of hair cell proteins without the risk of collateral effects on other cell types, which could complicate or bias the interpretation of the results.

We have shown that CRISPR-Cas9 targeting of the transmembrane domain of otoferlin *in vivo* in mice results in a phenotype similar to auditory hair cell synaptopathy (reduced ABR wave amplitudes with normal DPOAEs). Successful gene editing of otoferlin was previously tested in sheep embryos, targeting exons 5 and 6 to create a sheep knockout model, with injections performed at the embryonic stage, directly into the cytoplasm of each zygote (Menchaca et al., 2020). Furthermore, downregulation of otoferlin expression in postnatal mouse auditory hair cells demonstrates that this protein is indeed essential for the maintenance and organisation of synaptic ribbons in IHC. These findings confirm those obtained by Manchanda et al. in a zebrafish model, where truncation of the TM domain of otoferlin resulted in a reduction in the number of ribbons (Manchanda et al., 2021). Similar results were observed in the study by Roux et al. in *Otof*<sup>-/-</sup> mouse models, showing a 62% reduction in the number of ribbons compared to *Otof*<sup>+/+</sup> mice (Roux et al., 2006). Interestingly, in our study, the expression level of Otoferlin correlated with the number of ribbons per IHC and the size of the IHC synaptic area. This correlation suggests that otoferlin not only affects

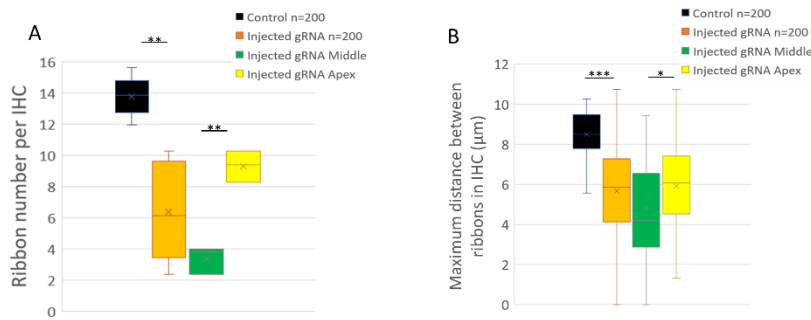
the number of synaptic ribbons, but also plays a role in shaping the overall structure of the synaptic area. This structural adaptation may facilitate more efficient signal transmission, thereby contributing to the quality of auditory information processing. Taken together, all results highlight the significant impact of CRISPR-Cas9-mediated OTOF gene editing targeting the TMD on synaptic ribbon distribution and provide valuable insights into the underlying mechanisms contributing to the observed hearing loss.

We demonstrate as a proof of concept that CRISPR-Cas9 technology can work *in vivo*. By this technology we could down modulate the expression level of otoferlin in postnatal hair cells and show that this protein is indeed essential for the maintenance of the synaptic ribbon.

Supplemental data :



**Fig6** Evaluation of GFP reporter gene transduction efficiency in a mouse injected with the OTOF-gRNA virus, at a scale of 50µm (A) and 10µm (B).



**Fig 7** Comparison of the number of ribbons per IHC (A) and the maximum distance between ribbons in each IHC (B) in CRISPR-OTOF-gRNA mice (with details at the middle and apex) and control mice. CRISPR-OTOF-gRNA mice had a reduction in the number of synaptic ribbons per IHC, with a significant decrease from an average of 14 to only 6 ribbons per cell ( $p=0.004$ ) (A). This reduction was particularly accentuated in the basal and middle regions of the cochlea, as opposed to the apex ( $p=0.002$ ). There was also a reduction in the maximum distance between synaptic ribbons, which was significantly shorter compared to the control group ( $p<0.001$ ). Furthermore, this reduction was more pronounced in IHCs located in the middle part of the cochlea compared to those located in the apex ( $p=0.03$ ).

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Partie II

Traiter les surdités génétiques : stratégies utilisant  
les vecteurs synthétiques



Dans la prise en charge étiologique des mutations de l'otoferline, les avancées les plus importantes concernent l'utilisation de vecteur viraux adéno-associés (AAV). Le problème majeur initial était la taille limitée du transgène qui pouvait être transporté. L'utilisation de double vecteur AAV a permis de surmonter la limite de la taille du transgène encapsidé dans un unique AAV. Ce double vecteur AAV a permis une récupération de l'audition *in vivo* sur modèle murin ayant une surdité causée par une mutation de l'otoferline (Akil et al., 2019a; Al-Moyed et al., 2019). Deux vecteurs recombinants différents étaient utilisés, l'un contenant la portion 5' et l'autre la portion 3' de l'ADNc de l'otoferline (~ 6kb). Plusieurs sociétés pharmaceutiques ont développé des produits de thérapie génique ayant conduit à des études pré-cliniques sur modèle murin et primates non humains (Lahlou et al., 2023). Compte tenu des résultats prometteurs, deux essais cliniques ont été approuvés par la Food and Drug Administration (FDA) et vont être menés aux Etats-Unis ainsi que dans certains pays de l'Union Européenne. En France, le traitement élaboré par l'Institut Pasteur et le groupe pharmaceutique Sensorion®, repose également sur un double vecteur AAV. Une demande d'essai clinique a été déposée au premier semestre 2023. Dans le but de rendre les AAV plus spécifiques et de limiter l'immunogénicité systémique, l'utilisation de capsides synthétiques s'est également développée ces dernières années (Grimm et al., 2008; El Andari and Grimm, 2021; Wagner et al., 2021).

Malgré toutes les optimisations mises en place, des limites à l'utilisation des AAV subsistent. Actuellement, peu d'informations sont disponibles sur la manière dont l'oreille interne humaine réagit à l'administration de gènes par AAV. Il a été mis en évidence la présence d'une immunité innée et acquise au sein de cochlées de modèles murins après injection d'AAV (Ishibashi et al., 2023). L'utilisation de l'AAV comme vecteur pour traiter l'amyotrophie spinale, maladie neuromusculaire mortelle d'origine génétique, a constitué une avancée majeure. Cependant, il est important d'être conscient de ses effets secondaires potentiels, en particulier l'insuffisance hépatique aiguë, qui a été observée chez certains jeunes patients, entraînant deux décès (Arjomandnejad et al., 2023).

Un deuxième facteur limitant doit être soulevé. Dans les différentes études pré-cliniques publiées sur modèle murin, le recul sur l'efficacité des thérapeutiques n'est que de 9 mois maximum avec, dans certains cas, un déclin des résultats auditifs dans le temps (Omichi et al., 2019; Bankoti et al., 2021). Une ré-administration compte tenu de l'immunogénicité des AAV semble difficilement envisageable.

Ainsi, bien que les méthodes d'administration, la posologie et le sous-type d'AAV puissent varier, l'utilisation de ce type de vecteur aux effets secondaires potentiellement graves et à l'efficacité éventuellement limitée dans le temps chez des nouveau-nés, soulève des problèmes éthiques. Ces considérations éthiques sont d'autant plus présentes qu'il existe actuellement des traitements fonctionnels (appareillage auditif et implant cochléaire) qui sont efficaces, bien qu'imparfaits. Paradoxalement, on peut considérer que le développement de l'implant cochléaire a probablement

freiné d'une certaine manière le développement de la thérapie génique, puisqu'il en constitue une alternative efficace et peu iatrogène.

Partant de ce constat, il nous a paru plus opportun de nous concentrer sur le développement de vecteurs ayant le meilleur profil de tolérance, notamment immunologique. Une revue évaluant les différents types de vecteurs non viraux utilisés dans l'oreille a donc été réalisée en ce sens, en prenant en compte également l'efficacité de ceux-ci et leur applicabilité clinique ultérieure.

### Article n°3 Thérapie génique pour les surdités : Etat des lieux et perspectives utilisant les vecteurs non viraux

#### Résumé

Les possibilités thérapeutiques actuelles pour les patients atteints de surdité génétique sont uniquement fonctionnelles et reposent principalement sur les aides auditives et les implants cochléaires. Toutefois, ces dispositifs ont des limites en termes de gain, en particulier dans les environnements acoustiques bruyants. Ceci explique l'intérêt croissant pour le développement de traitements étiologiques afin d'améliorer non seulement la compréhension, mais aussi la qualité de vie des patients. La thérapie génique peut être un traitement étiologique prometteur pour restaurer complètement la fonction auditive, en complément ou en remplacement des thérapeutiques actuelles. Le succès de la thérapie génique repose sur l'apport efficace de gènes thérapeutiques ou de modifications génétiques aux cellules de l'oreille interne afin de réparer ou de remplacer les gènes défectueux, afin de restaurer une fonction auditive normale. Les vecteurs viraux et les vecteurs non viraux constituent les deux principales approches de la thérapie génique.

Les vecteurs viraux recombinants, en particulier les virus adéno-associés (AAV), sont actuellement les plus utilisés pour la thérapie génique concernant les surdités, en raison de leurs excellentes capacités de diffusion notamment concernant la transfection des cellules ciliées internes. Cependant, leur utilisation est limitée par la taille des transgènes qu'ils peuvent transporter et par des problèmes d'immunogénicité, en particulier chez les nouveau-nés.

Les vecteurs non viraux, tels que les lipides et polymères cationiques, offrent une alternative intéressante. Ils présentent des avantages tels qu'une faible immunogénicité, une cytotoxicité moindre et la possibilité de transporter des transgènes de taille en théorie illimitée. Deux articles récents démontrent une récupération auditive significative *in vivo* en les utilisant chez des modèles de souris sourdes. Cependant, leur efficacité dans le transfert de matériel génétique est actuellement inférieure à celle des vecteurs viraux. Par ailleurs, les méthodes physiques, principalement l'électroporation, offrent un contrôle précis et une bonne efficacité. Cette technique cible principalement les cellules de

surface, comporte un risque de dommages cellulaires et nécessite l'insertion d'électrodes, ce qui suggère une utilisation future possible en combinaison avec l'implantation cochléaire.

L'objectif de cette revue est de fournir des informations précises sur l'état actuel de la recherche sur les vecteurs non viraux pour la thérapie génique de l'oreille interne et ainsi de contribuer au développement de nouvelles approches de thérapie génique efficaces pour les surdités d'origine génétique.

*Manuscrit en cours de finalisation*

# Gene Therapy for Hearing Loss: Current Status and Future Prospects of Non-Viral Vector Delivery Systems

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## Abstract

Current treatment options for patients with genetic deafness are exclusively functional, based on hearing aids and cochlear implants. However, these devices have limitations, particularly in noisy acoustic environments. Therefore, there is a growing interest in exploring etiological treatments to improve not only understanding but also the quality of life of those affected. Gene therapy may be a promising aetiological treatment to fully restore auditory function, potentially surpassing or complementing the capabilities of existing functional systems. The success of gene therapy relies on the efficient delivery of therapeutic genes or genetic modifications to the cells of the inner ear to repair or replace defective genes and restore normal hearing function. Two primary approaches to gene therapy are recombinant viral vectors and non-viral delivery vehicles.

Recombinant viral vectors, particularly adeno-associated viruses (AAV), have dominated gene therapy in the cochlea due to their excellent diffusion properties and compatibility with inner hair cells. However, they are limited by the size of the transgenes they can carry and raise concerns about immunogenicity, particularly in neonates.

Non-viral vectors, such as cationic lipids and polymeric nanoparticles, offer an attractive alternative. They have advantages such as low immunogenicity, minimal cytotoxicity and the ability to carry transgenes of unlimited size. Two recent articles demonstrate significant auditory recovery *in vivo* using murine models. However, their efficiency in transferring genetic material currently lags behind that of viral vectors. Physical methods, primarily electroporation, offer precise control and good efficiency. This technique primarily targets surface cells, carries a risk of cell damage and requires the insertion of electrodes, suggesting a possible future use in combination with cochlear implantation.

This review will provide valuable insights into the current state of research on non-viral vectors for inner ear gene therapy and inform the development of new and effective gene therapy approaches for hearing loss.

## Introduction

In developed countries, approximately 1 in 500 newborns has a severe-to-profound hearing loss caused by genetic factors (Smith et al., 2005). It is estimated that approximately 50-60% of all cases of hearing loss in children are due to genetic factors. The prevalence of genetic hearing loss is higher in certain populations, such as those with a history of consanguinity or in certain ethnic groups. There are over 400 genetic syndromes associated with hearing loss, and over 100 genes have been identified as causing non-syndromic hearing loss.

Current treatments consist of two types of functional compensatory systems: hearing aids, which amplify sound, and cochlear implants, which encode sound as electrical impulses that are delivered to the auditory nerve through an array of electrodes (Purcell et al., 2021). Although these devices usually allow for the development of speech and proper communication, they have certain limitations in terms of speech intelligibility in noise (Müller and Barr-Gillespie, 2015). They do not restore hearing to a completely normal state, hence the interest in developing etiologic treatments to improve understanding but also quality of life (Géléoc and Holt, 2014a).

Gene therapies are being developed with the aim of fully restoring hearing and achieving better results than the current functional systems, or perhaps to be used as a supplement. Although the isolated nature of the cochlea may appear to be an advantage in avoiding the proliferation of therapies, surgical access to the cochlea remains limited, with the risk of worsening hearing loss. Various options to limit this risk of hearing loss are being investigated (Lee et al., 2020). A therapeutic gene is delivered to the cells in the inner ear to replace or repair a defective or missing gene, thereby restoring the normal inner ear function (Delmaghani and El-Amraoui, 2020). Negative charges on nucleic acids and cell membranes limit the ability of nucleic acids to cross the plasma membrane. The successful introduction of genetic material into cells requires efficient and safe vectors. The vector must be capable of compacting nucleic acid and protecting it during its journey from the point of administration to the nucleus. An ideal vector should be specific for the target tissue, non-immunogenic, have few side effects, and induce significant and functional expression of the therapeutic transgene. There are two main approaches to gene therapy: recombinant viral vectors, which exploit the natural ability of viruses to penetrate host cells, and non-viral carriers, which can be further divided into chemical and physical approaches.

Currently, viral vectors, in particular Adeno-Associated Virus (AAV), are the mainstay of gene therapy because of their excellent diffusion within the cochlea and inner hair cells. Their first weakness

is the size of the transgene they can carry (4.7kb). The use of dual AAVs has made it possible to overcome this size limitation and is being used for the otoferlin gene (Ghosh et al., 2011; Akil et al., 2019b; Al-Moyed et al., 2019). However, the problem remains for larger genes. The second weakness is immunogenicity, especially since the target population is likely to be neonates (Kho, 2000). Currently, there is limited information on how the human inner ear responds to gene delivery by AAV (Ishibashi et al., 2023). The use of AAV as a vector to treat spinal muscular atrophy, a life-threatening disease, has been a major breakthrough. However, it is important to be aware of its potential side effects, in particular acute liver dysfunction, which has been observed in some young patients, resulting in two deaths (Philippidis, 2022). Although the methods of administration, dosage, and AAV subtype may vary, the use of this vector with potential side effects in treating deafness raises ethical considerations, especially given the availability of current functional treatments that, while potentially improved, have proven effective.

The alternative is non-viral vectors. Synthetic vectors, such as cationic lipids or polymeric nanoparticles, offer many advantages over viral vectors, in particular low immunogenicity and no significant cytotoxicity or cellular damage (Scheper et al., 2009). In addition, it should be noted that siRNA molecules alone can diffuse from the middle ear to the inner ear across the round window membrane (RWM), where they were able to induce a silencing response without histologic damage to the inner ear (Mukherjea et al., 2010).

Synthetic vectors have many advantages over viral vectors, such as well-controlled production, high stability, low immunogenicity, low production costs, and the size of the transgene they can carry is theoretically unlimited. However, their main limitation is that their ability to transfer genetic material is still lower than that of viral vectors. The efficiency of synthetic vectors varies depending on intrinsic factors such as the chemical structure of the vector, the charge ratio for lipid or polymer, and the characteristics of the associated auxiliary lipid if exist (Belmadi et al., 2015b). It also varies depending on extrinsic factors encountered during the gene delivery process, including cellular barriers and the extracellular environment, depending on the delivery route.

Another option for gene transfer without the use of viral vectors is physical methods, including electroporation. This technique uses high-intensity electrical pulses to disrupt and permeabilise the cell membrane (Mehier-Humbert and Guy, 2005). This allows nucleic acids to enter the cells. This technique has several advantages, including precise control over the site of gene transfer and high efficiency. However, its efficiency is primarily applicable to surface cells. It can potentially cause cell damage due to the electrical pulses and requires the insertion of electrodes. As a result, in the medium term, it could be used in conjunction with cochlear implantation to treat severe to profound deafness.

The aim of this literature review is to provide an overview of the current state of research on non-viral vectors for inner ear gene therapy. Specifically, this review will explore the various types of non-viral vectors, including lipid-based nanoparticles, polymeric nanoparticles, and physical methods. The advantages and limitations of each technique type will be discussed, as well as the potential future directions.

## 1. Lipid-Based NanoParticles

Lipid-based NanoParticles (LBNPs) consist of a hydrophobic core and a hydrophilic outer layer, often forming a lipid bilayer structure or vesicle. By encapsulating genetic material, they facilitate specific targeting by diffusion. Currently, LBNPs are under investigation as gene therapy vectors in various clinical settings. In 2018, patisiran [Onpattro] became the first drug to be approved by the FDA using LBNP (Adams et al., 2018). It encapsulates siRNAs within an LBNP for targeted delivery to the liver, where it modulates or inhibits the production of disease-causing proteins in hereditary transthyretin amyloidosis, an autosomal dominant inherited disorder. More recently, LBNPs have been used in messenger RNA vaccines against Covid-19 (Wang et al., 2021). Their successful use on a very large scale (hundreds of millions of people worldwide) in these vaccines, with generally limited side effects, has naturally increased interest in their development (Klein et al., 2021).

### 1.1 Cationic Lipid

Cationic lipids are composed of a positively charged (cationic) hydrophilic head and a hydrophobic tail. The positively charged portion enables both the formation of lipid-DNA/RNA complexes due to the negative charge of nucleic acids, and interaction with negatively charged cell membranes. Their head groups can contain tertiary amines, allowing them to transform from neutral to positively charged particles under acidic pH conditions. The hydrophobic part can promote fusion with the cell membrane and the release of DNA/RNA into target cells. They have the ability to form liposomes, which appear as spherical vesicles composed of a double layer of phospholipids enclosing an aqueous environment. Synthetic cationic lipids used for transfection can be coupled with auxiliary lipids to enhance the destabilisation of the endosome after endocytosis, resulting in the release of DNA into the cytoplasm (Wrobel and Collins, 1995).

Ref.	Vector	Trans-gene	Animal	Transfection age	Delivery	Analysis delay (days)	Targeted cells	Effic.	ABR	Tox.
(Wareing et al., 1999)	Liposome (DDAB)	$\beta$ -gal	guinea pig	adult	RWM, cochleostomy +/- minipump	1,3,7,14	spiral limbus, spiral ligament, Reissner's membrane, spiral ganglion neurons	-	-	-
(Zheng and Gao, 2000)	Lipofectamine	Math1-eGFP	Rat	P3	Utricles	6-8	Hair cells, Supporting cells	-	-	-
(Jero et al., 2001a)	Liposome (POPC/CHIM/DDAB)	eGFP	Mouse	adult	gelatin sponge - RWM	3	Spiral limbus, spiral ligament, organ of corti, Reissner's membrane, spiral ganglion cells	39%	-	-
(Jero et al., 2001b)	Liposome (POPC/CHIM/DDAB)	eGFP	Mouse	adult	gelatin sponge - RWM / cochleostomy	3	Spiral limbus, spiral ganglion cells, Reissner's membrane	-	-	-
(Staecker et al., 2001)	Liposome (DOTMA)	eGFP	Mouse	P90	cochleostomy	3	Spiral ganglion neurons, stria vascularis, supporting cells, mesenchymal cells	47% (SGN)	-	Yes
(Maeda et al., 2007)	Liposome (DOTAP)	GJB2-eGFP	Mouse	adult	Cochleostomy - RWM	3,5,12	Claudius cells, pillar cells, OHC, spiral limbus, spiral ligament	-	Normal	-
(Zou et al., 2008)	Liposome (Labrafac/Lipoid/Solutol)	NA	Rat	adult	gelatin sponge - RWM	0-7	spiral ganglion cells, IHC, OHC pillar cells, spiral ligament, stria vascularis,	-	-	-
(Zhang et al., 2011)	Lipofectamine 2000	Math1-eGFP	Rat	P1-P5, adult	organ of corti explants		Fibrocytes, spiral ganglion neuron, hair cell like cells	3%	-	-
(Zuris et al., 2015)	Lipofectamine 2000	Cre	loxP-Tomato mice	P0	cochleostomy	5	OHC	91%	-	Yes
	Lipofectamine 2000	Cas9:sgRNA	Atoh1-GFP mice	P2	cochleostomy	5-10	OHC	13-20%	-	No
	Lipofectamine RNAiMAX	Cre	loxP-tdTomato mice	P0	cochleostomy	5	OHC	33%	-	No
(Gao et al., 2018)	Lipofectamine 2001	Cas9:sgRNA	Mouse	P0-2, P40	cochleostomy (base, middle and apex-middle turn)	14	IHC	21%	Better	-
(Tao et al., 2023)	Lipofectamine 2000	Cas9:sgRNA	Mouse	P0-2	cochleostomy (base, middle and apex-middle turn)	28-56	IHC	21%	Better	-
							OHC	28%	Better	-

**Table 1** : Summary of cationic lipid-mediated transfection methods and results in inner ear cells (Ref. : reference, Effic: Efficiency, ABR: Auditory Brainstem Response, Tox.: toxicity, RWM: round window membrane, OHC: outer hair cell, IHC: Inner hair cell, SGN: spiral ganglion neuron).



Cationic lipids have been used in this type of application for about two decades (Wareing et al., 1999; Zheng and Gao, 2000) (Table 1). An interesting aspect is their potential use in adult animals. Several routes of administration were tested, including cochleostomy and application to a gelatin sponge in contact with the round window membrane (Jero et al., 2001; Zou et al., 2008). Peak efficacy was around 3 days, but few long-term analyses were performed. All cell types could be transfected, with varying efficiencies, but up to 39% transfection could be achieved (Jero et al., 2001). Auditory tests were not affected by the surgery or these compounds, except when used at high concentrations (Staecker et al., 2001; Maeda et al., 2007). The use of commercially available agents, such as Lipofectamine (Invitrogen, Ghent, Belgium), which are easy to use, has greatly popularised access to this type of vector. Although efficacy results vary widely (3 to 91% transfection), this type of cationic lipid has since become the most widely used (Zhang et al., 2011; Zuris et al., 2015).

One of the advantages of using cationic lipid lies in their potential for genome editing technologies. The goal is to directly modify the genetic mutation responsible for deafness in order to restore hearing. One promising approach involves the delivery of Cre recombinase and Cas9:sgRNA complexes to the organ of Corti using commercial vectors like RNAiMAX and Lipofectamine 2000, as demonstrated by Zuris et al. (Zuris et al., 2015). In their study, these vectors were applied to mice between P0 and P2, with a specific focus on targeting outer hair cells. Recombination efficiency was determined to be 33% with RNAiMAX, while Lipofectamine 2000 resulted in a higher efficiency of 91%. However, it's important to note that Lipofectamine 2000 also induced cellular toxicity in outer hair cells, although the extent of this toxicity was not quantified. The study did not evaluate the impact of these interventions on hearing. Furthermore, there were no available data to determine the effects on distant cells. Genome modification to restore hearing or reduce hypoacusis in progressive deafness is theoretically easier to achieve in autosomal dominant disorders. Building upon this premise, Gao et al. targeted Beethoven, a mouse model of dominant progressive hearing loss (Vreugde et al., 2002; Gao et al., 2018a). They administered cationic lipid-encapsulated Cas9-gRNA complexes targeting the mutant allele TMC1 (transmembrane channel-like 1) via cochleostomy to neonatal mice (P0-P2). This intervention resulted in a significant reduction in progressive hearing loss, an increase in hair cell survival, and a decrease in auditory brainstem response (ABR) thresholds in the injected ears compared to those that remained untreated or received injections of complexes targeting another gene. Additionally, acoustic reflex responses were enhanced in the injected Beethoven mice compared to their uninjected counterparts.

Recently, the same team published an article on a mouse model carrying a dominant hearing loss mutation affecting the OHC, known as Oblivion (Tao et al., 2023). They showed that *in vivo* delivery of CRISPR-Cas9 ribonucleoprotein complexes using Lipofectamine 2000 at an early age could specifically

edit the *Obl* allele in this mouse model. Genome editing *in vivo* resulted in survival and functional restoration of outer hair cells, leading to partial recovery of hearing (average ABR thresholds 26 dB). Interestingly, the most significant improvement was seen at frequencies below 32 kHz. However, these results were not sustained over time and declined significantly after 16 weeks.

Furthermore, using a double mutant mouse model with both the Beethoven and Oblivion mutations causing digenic genetic hearing loss, delivery of Cas9-sgRNA targeting both mutations slightly improved hearing (average ABR thresholds 10dB). While these gains are promising, especially considering the technical complexity of the correction, they need to be further improved to be of clinical significance. These results suggest that liposome-mediated delivery of these ribonucleoprotein-CRISPR complexes may be a strategy for restoring hearing in cases of dominant mutations in outer hair cell genes and digenic mutations in auditory hair cells.

Finally, a hybrid technique using Lipofectamine 2000 as a vector for Adeno-Associated Virus (AAV) was tested (Casey et al., 2020). This approach did not improve transfection efficiency compared to the virus alone. The relevance of the immune response remains to be evaluated.

## 1.2 Other Lipid

Another type of lipid was used in the inner ear: neutral lipids (Miwa et al., 2021). These neutral lipids were loaded with BDNF mRNA to reduce the degeneration of spiral ganglion neurons in adult guinea pigs. They were applied to the round window using a gelatin sponge. The number of spiral ganglion neurons at J14 was higher in the lipid nanoparticle-treated group compared to the saline control group, despite an overall decrease in the total number of spiral ganglion neurons. In addition, although no toxicity was observed in inner hair cells (IHC), significant toxicity was observed in outer hair cells (OHC), resulting in severe hearing loss.

## 2. Polymeric Nanoparticles

The use of polymer nanoparticles is a more recent development in inner ear gene therapy (Tan et al., 2008; Zhang et al., 2011a). They may have the ability to cross membranes such as the round window membrane. Polymers have the advantage of allowing a wide range of modifications, including the design of block and comb polymers. However, their elimination or biodegradation can potentially lead to toxic side effects (Li et al., 2015). This limitation may limit their use in clinical practice. In this context, we will review the key findings of studies on this topic, focusing on PLGA (Polylactic-co-glycolic acid), PHEA (polyhydroxyethyl aspartamide), and PEI (Polyethyleneimine) and amino-acid (**Table 2**).

Ref.	Vector	Trans-gene	Animal	Transfection age	Delivery	Analysis delay (days)	Targeted cells	Effic.	ABR	Tox.
(Tan et al., 2008)	Polyethylenimine (PEI)	eGFP	Guinea pig	adult	cochleostomy middle turn minipump	7	vestibular and tympanic fibrocytes, Reissner's membrane mesenchymal and epithelial cells, and spiral ligament fibrocytes	-	-	-
(Zhang et al., 2011a)	Hyperbranched polylysine nanoparicles	eGFP	Rat	P1-P5; adult	<i>in vitro</i> and gelatin sponge - RWM	1	OHC, IHC, supporting cells, spiral ganglion, stria vascularis, spiral ligament	9%	-	No
(Du et al., 2013)	poly(lactic-co-glycolic acid) PLGA	<i>Hes1</i> <i>Hes5</i>	Mouse	P3	<i>in vitro</i>	1,2,3	OC cells	45%	-	-
(Wu et al., 2013)	Polyethylenimine (PEI)	Atoh1-eGFP	Rat	adult	gelatin sponge - RWM	7	OHC and IHC	0%	Lower	-
	CM-β-CD modified activated PAMAM	Atoh1-eGFP	Rat	adult	gelatin sponge - RWM	7	OHC	48%	As control	-
(Kim et al., 2015)	poly(2-hydroxyethyl aspartamide) (PHEA)	Nile red	C57/BL6 mice	1 month	gelatin sponge - RWM	1	IHC IHC, supporting cells	82%	As control Lower DPOAE	- Yes
(Youm et al., 2017)	poly D,L-lactic-co-glycolic acid (PLGA)	MAPK1 siRNA	Mouse	P3	<i>in vitro</i>	2	OHC	-	-	No
(Du et al., 2018)	poly(lactic-co-glycolic acid) PLGA	siRNA	Guinea pig	young adult	cochleostomy basal turn osmotic pump	21-64	OHC, IHC	-	Better	No
(Yang et al., 2018)	cationique polyethyleneglycol	-	C57/Bl6	1 month	Middle ear	1-7	OHC, IHC	-	Normal	No
(Miwa et al., 2021)	vitamin E-scaffold SS-cleavable proton-activated lipid-like material (ssPalm)	eGFP BDNF	Guinea pig	adult	gelatin sponge - RWM	28	Spiral ganglion neuron	Yes	Normal	Yes

**Table 2** : Summary of polymeric nanoparticles transfection methods and results in inner ear cells (Ref.: reference, Effic: Efficiency, ABR: Auditory Brainstem Response, Tox.: toxicity, RWM: round window membrane, OHC: outer hair cell, IHC: Inner hair cell)

## 2.1 PLGA (Polylactic-co-glycolic acid)

PLGA nanoparticles have the ability to transport compounds across the round window membrane (RWM) into the perilymph (PL), thereby increasing the local bioavailability of the encapsulated compound within the inner ear. After crossing the RWM and reaching the cochlea, these PLGA are readily internalised by the cellular components of the organ of Corti (Youm et al., 2017). This ability enables them to achieve gene silencing in a dose-dependent manner, even within the sensory epithelia of the cochlea (Du et al., 2013; Cai et al., 2014). For example, Du et al. used poly(lactide-co-glycolide) PLGA nanoparticles to deliver siRNA targeting the Hairy and Enhancer of Split 1 (Hes1) gene, a key player in the Notch signalling pathway. These nanoparticles had previously been used in the organs of Corti of P3 mice, with a transfection efficiency of approximately 45% (Du et al., 2013). In young adult guinea pigs exposed to acoustic trauma, a micropump was implanted at the basal turn of the cochlea 72 hours after the trauma and left in place for 24 hours (Du et al., 2018). The results showed that hearing was restored for up to 9 weeks in the treated group. In particular, siRNA targeting the Hes1 gene resulted in partial restoration of outer hair cell (OHC) and inner hair cell (IHC) counts. In addition, the Hes1 siRNA group showed the presence of immature hair cells. These results suggest that the use of PLGA nanoparticles for siRNA delivery targeting the Hes1 gene may have potential as a therapeutic strategy for hearing restoration.

## 2.2 PHEA (Polyhydroxyethyl aspartamide)

In their study, Yoon et al. developed a graft copolymer by conjugating an oligoarginine peptide (Arg8) to poly(amino acid) (poly(2-hydroxyethyl-L-aspartamide; PHEA)) (Yoon et al., 2015a). *In vivo* testing was performed on mice at P30, where 20  $\mu$ L of the PHEA-g-C18-Arg8 complex was placed on the round window using a gelatin sponge. After 24 hours of evaluation, nanoparticles were observed in both inner hair cells (IHCs) and supporting cells, mainly concentrated in the modiolus and lateral wall tissues. Expression of a GFP reporter gene was detected only in the modiolus and lateral wall, but not in the hair cells.

## 2.3 PEI (Polyethyleneimine) and Dendrimer

A dendrimer-based nanocarrier was developed and tested *in vivo* (Wu et al., 2013). This nanocarrier, called CMAP dendriplex (Na-carboxymethyl-beta-cyclodextrin-modified activated polyamidoamine dendrimers) was associated with a plasmid containing Atoh1 and eGFP. The experimental procedure involved the application of this nanocarrier to the round window of adult rats, coupled with an absorbable gelatin sponge. Analysis was performed seven days after application. The efficiency of transfection, as indicated by the expression of GFP, reached 48  $\pm$  7% for the IHC group and 82  $\pm$  10% for the OHC group. However, expression was not assessed beyond the first week. In particular, the researchers highlighted the deterioration of the auditory brainstem response (ABR)

when polyethyleneimine (PEI) was used. In contrast, no significant difference in ABR deterioration was observed between the CMAP dendriplex and the control group.

Linear polyethyleneimine (PEI) was also tested in guinea pigs via cochleostomy with placement of an osmotic pump (Tan et al., 2008). Cells transfected *in vivo* included vestibular and tympanic fibrocytes, Reissner's membrane mesenchymal and epithelial cells, and spiral ligament fibrocytes. Notably, no transfection was observed in the organ of Corti or the vascular stripe. No inflammatory response was observed. However, the effect of the treatment on hearing was not reported.

## 2.4 Amino-acid

Hyperbranched polylysine nanoparticles (HPNPs) have been evaluated for their potential in gene delivery in the inner ear (Zhang et al., 2011a). These nanoparticles comprise dendritic polymers characterised by their partially branched or irregular structures. Both gelatin sponge and HPNPs were applied to the round window membrane of adult rats. The results were assessed 24 hours after application. The presence of HPNPs was detected in the organ of Corti, supporting cells, stria vascularis and spiral ganglion cells. Auditory brainstem responses (ABRs) were not evaluated in this study. There was no evidence of cytotoxicity from the use of HPNPs. There was no evidence of gene transfer within the cochlea.

## 3. Physical methods

Physical methods have proven to be promising approaches for targeted gene transfer into the inner ear (**Table 3**). Among these methods, two techniques stand out: electroporation and the "gene gun". Electroporation involves the application of pulsed electrical fields through the tissues of the inner ear to facilitate the entry of nucleic acids, while the 'gene gun' uses propulsion to deliver gene-carrying particles directly into target cells.

### 3.1 Electroporation

Electroporation is a technique that uses short, high-intensity electrical pulses to introduce DNA or RNA molecules into cells. These pulses temporarily create pores in the cell membrane, allowing the genetic molecules to enter the cell (Pinyon et al., 2014). This method is used to modify the genetic material of cells to achieve a variety of therapeutic goals, including correcting disease-causing genetic mutations, producing specific proteins to treat specific diseases, and regulating gene expression.

The understanding of the ability to transfer DNA using pulsed electric fields has evolved recently. Studies have shown that the term "electroporation" is not the most accurate because plasmid DNA is too bulky to enter cells through the temporary pores created in the plasma membrane. It is now understood that plasmid DNA, unlike small oligonucleotide DNA and RNA molecules, is internalised by

cells primarily through active processes. The importance of the train of electrical pulses that achieves this "electroporation" is to generate an electric field of sufficient intensity to physically bind negatively charged plasmid DNA to the cell surface facing the cathode, from which the pulses are emitted. This allows the DNA molecules to attach to the plasma membrane and subsequently be internalised by endocytosis and pinocytosis mechanisms (Escoffre et al., 2011; Rosazza et al., 2016). The majority of exogenous DNA is degraded within lysosomes and by endonuclease activity. However, some plasmid DNA is transported to nuclear pores, exiting transport vesicles and becoming extrachromosomal (episomal) DNA (Pinyon et al., 2019). This episomal DNA is then transcribed to produce mRNA encoding recombinant proteins of interest.

This method of gene transfer should be called "DNA electrotransfer" rather than "electroporation". It is made possible by the resistance of DNA to degradation and its ability to maintain expression for many months after detectable translation within a few hours. Key parameters for efficient expression of recombinant proteins using DNA electrotransfer are primarily related to the intrinsic biophysics of the gene delivery process. These parameters include the initial concentration of plasmid DNA and the intensity and duration of the electric field. Plasmid DNA is typically delivered to cells at uniform concentrations of  $\mu\text{g}/\mu\text{l}$ . To generate voltage gradients, the parameters of the electrical pulses used for gene delivery are typically on the order of several hundred volts for tens of milliseconds (Rosazza et al., 2016). However, the need for high voltages in conventional electroporation can potentially cause tissue damage due to irreversible dielectric breakdown and localised temperature increases (Lacković et al., 2009).

As shown in **Table 3**, the majority of studies have been conducted in mice. Cell transfection rates range from 26% to 98% (Woods et al., 2004; Pinyon et al., 2014; Takeda et al., 2019). All cell types can be transfected, including hair cells, supporting cells, vascular stripe cells and spiral ganglion cells (Gubbels et al., 2008; Brigande et al., 2009; Miwa et al., 2013). Gubbels shows that transfection is more effective at the base of the cochlea than in the middle. In the three studies that examined auditory thresholds, the technique did not induce hearing loss. No toxicity was reported. Immune response was not evaluated. One of the advantages of this technology is that it can be applied to mouse embryos, allowing the effects of correcting an abnormality to be assessed at an early age. This is particularly important for studying the role of growth factors. Animal models for in utero gene therapy are currently being developed. Several studies have shown that when plasmids are delivered to the otocyst of an embryonic mouse at 11 days (E11), proteins are expressed in a significant proportion of cells and persist for up to 30 days after delivery (Gubbels et al., 2008). In a proof-of-concept study, in utero electroporation was used to rescue auditory deficits by reintroducing *Gjb6* and to downregulate *Gjb6* expression using short strand RNAs (Miwa et al., 2013). The prospect of correcting genetic mutations

Ref.	Method	Trans-gene	Animal	Transfection age	Delivery	Analysis delay (days)	Targeted cells	Effic.	ABR	Tox.
(Zheng and Gao, 2000)	Electrop.	pRK5-Math1-eGFP	Mouse	P0-P1	organ of corti	4-8	OHC IHC	-	-	-
(Belyantseva et al., 2003)	Gene gun (gold particle)	eGFP-MyoXVa	Mouse/rat	P3-P5	organ of corti	1-4	OHC, IHC, vestibular hair cells	-	-	-
(Woods et al., 2004)	Electrop.	Math1-eGFP	Mouse	E12.5-E17.5	organ of corti	2	OHC, IHC, Kölliker's organ	98%	-	-
(Belyantseva et al., 2005)	Gene gun (gold particle)	eGFP-MyoXVa	Mouse/rat	P1-P4	organ of corti	0-4	OHC, IHC, vestibular hair cells	-	-	-
(Gubbels et al., 2008)	Electrop.	Atoh1(Math1)-GFP	Mouse	E11.5	E11.5 mouse otocyst	7-14	OHC, IHC, Pillar cell, Inner Phalangial Cell, Border Cell, Deiters cells, Claudius' cells, Hensens' cells	50%	-	-
(Brigande et al., 2009)	Electrop.	EF1-alpha-GFP	Mouse	E11.5	E11.5 mouse otocyst	6	Supporting cells, pillar cells, OHC	-	-	-
(Puligilla et al., 2010)	Electrop.	pCMV-Sox2.EGFP	Mouse	E13 - P3	organ of corti	6	Non sensory epithelial cells	39%	-	-
(Xiong et al., 2012)	Electrop.	G-CaMP3-GFP, HA-TMHS-GFP	Mouse	P0-P8	organ of corti	7	OHC, IHC	-	-	-
(Zhao et al., 2012)		mCherry-actin	Mouse Frog	P4 Adult	organ of corti organ of corti	1,2 0,1	OHC, IHC OHC, IHC Non sensory epithelial cells	15% 3% 76%		
(Miwa et al., 2013)	Electrop.	pCMV-Cx30-eGFP, pCMV-eGFP plasmid	Cx30 -/- mouse	E11.5	Embryonic day 11.5 mouse otocyst	7,38	Spiral limbus, organe of corti, OHC, IHC, supporting cells, stria vascularis, spiral ligament, spiral ganglion neurons	-	Better	-
(Pinyon et al., 2014)	Electrop. (cochlear implant)	pCMV-BDNF-IRES-GFP	Guinea pig	adult	RWM (cochlear implant) in vitro and in vivo	4,7,14	Spiral ganglion neurons; mesenchymal cells lining scala tympani and scala vestibuli	43% (Mesenchymal cells)	Better	No
	Electrop.	pCMV-Neurog1.EGFP	"	"	"	"	"	26%	-	-
	Electrop.	pCMV-NeuroD1.EGFP	"	"	"	"	"	73%	-	-
(Xiong et al., 2014)	Electrop.	SANS-eGFP, eSPIN-eGFP	Mouse	P0-P8	organ of corti	5	OHC, IHC	-	-	-
(Takeda et al., 2019)	Electrop.	pCMV-Slc26a4-eGFP	Mouse	E11.5	Embryonic day 11.5 mouse otocyst	2,7	Organ of corti, Spiral Ganglion, Lateral Wall, Utricular Macula, Saccular Macula, Endolymphatic sac, Brain	56%	Normal	No

**Table 3** : Summary of gene transfer physical methods and results in inner ear cells. (Ref. : reference, Effic: Efficiency, ABR: Auditory Brainstem Response, Tox.: toxicity, electrop.: electroporation, RWM: round window membrane, OHC: outer hair cell, IHC: inner hair cell)

in human embryos in utero could potentially be considered in the future, provided that a number of technical barriers can be overcome, but such use to solve functional problems remains highly controversial, as the risk to the foetus cannot be completely eliminated (Müller and Barr-Gillespie, 2015).

### Cochlear implant-mediated gene delivery

The use of near-field electroporation through the placement of a cochlear implant represents a hybrid technique with a rather tempting principle (Pinyon et al., 2019). It could potentially combine current functional treatment with neurotrophic or even aetiological treatment (Pinyon et al., 2014). In this study, electrodes inserted into the cochlea of guinea pigs delivered electrical pulses to the surrounding tissue. This caused a temporary opening of the cell membrane, allowing genetic material to enter the mesenchymal cells that line the scala tympani. Once in the cell nuclei, the transferred BDNF gene produced brain-derived neurotrophic factor (BDNF). This facilitated neurite regeneration within the cochlea, thereby correcting hypoacusis in a deaf guinea pig model (animals with damaged hair cells). As cochlear implants are already widely used in patients with severe to profound deafness, the adjunctive use of electroporation could enable the use of gene therapy as a complementary means of treating hypoacusis. This could help to improve patient understanding, particularly in noisy environments, which is a significant limitation of current therapeutic approaches.

### 3.2 Gene gun

The "gene gun" is a gene transfer method in which small particles coated with genes are accelerated at high speeds towards target cells (Uchida et al., 2009). Gold and tungsten particles are commonly used as gene carriers because of their high density and small diameter, which allow them to effectively penetrate cells. Particle bombardment relies on the use of compressed gas, such as helium, to propel these particles towards the target cells (Belyantseva, 2009). An advantage of this technique is that it is not dependent on the specific characteristics of the target cells, including surface receptors or their electrical potential. It also allows precise control of the gene transfer area. However, this technique can potentially alter cells due to the impact of the accelerated particles. Finally, the use of compressed gas to accelerate particles requires specific equipment.

Belyantseva et al. used a gold-particle gene gun to transfect hair cells from shaker 2 (Myo15a(sh2)) deaf mice with eGFP-Myo15a *in vitro*, and were able to restore the normal pattern of hair bundles by recruiting endogenous Whirlin to the tips of stereocilia (Belyantseva et al., 2005).

The method has since been refined to allow transfection of hair cells from the basal side, thereby reducing the morphological changes induced by the technology (Zhao et al., 2012). However, to the best of our knowledge, no publication has yet tested this procedure *in vivo*.



#### 4. Prospects and challenges

Currently, the non-viral vectors that appear closest to clinical application are cationic lipids. Combined with a genome editing system, they have restored hearing in mice with autosomal dominant deafness for at least 8 weeks (Gao et al., 2018; Tao et al., 2023). The loss of hearing after 4 months is the subject of debate as to its origin. Two main hypotheses can be proposed: firstly, that the injection, although neonatal, is too late and, secondly, that due to the low number of edited cells (approximately 20%), the overall degeneration prevents them from maintaining sufficient auditory thresholds. Nevertheless, in order to obtain these results, the injections were performed in mice at an early age, which in terms of development would correspond to an in-utero injection in humans. In addition, the injections are made through 3 separate cochleostomies, the morbidity of which would probably not be negligible in humans. With regard to the specificity of cationic lipids, we have seen that it is low, which is both an advantage and a disadvantage in certain cases to limit the risk of off-target effects associated with genome-modifying agents. Several targeting methods have been tested to improve the selection of transfected cells and to enhance nuclear import (Tu and Kim, 2008; Midoux et al., 2009; Le Guen et al., 2021). In addition, as mentioned in the introduction, biocompatibility and the potential presence of an immune response need to be assessed before any clinical application.

Nanoparticles, a more recent development, have been tested in different age groups with mixed results. Tolerance profiles are generally good, with no toxicity reported in several studies (Zhang et al., 2011a; Youm et al., 2017; Du et al., 2018; Yang et al., 2018). To our knowledge, only one study reports auditory benefits. The gain is approximately 10 dB following the use of a nanoparticle (PLGA) coupled to a transgene (siRNA targeting *Hes1*) in a guinea pig model exposed to acoustic trauma (Du et al., 2018). The results show a sustained gain of hearing up to 9 weeks after surgery. Although moderate, these results are encouraging, particularly for the treatment of hearing loss due to acoustic trauma. However, the need for a cochleostomy may remain an obstacle to clinical use in this indication.

Physical methods show good results in terms of effectiveness on the organ of Corti, especially in the short term. Data beyond 7 days are limited (Gubbels et al., 2008; Miwa et al., 2013; Pinyon et al., 2014). A major advantage of this technique is that it can be used at an embryonic stage, allowing intervention before cochlear maturation. In a model of connexin 30 deafness, Miwa et al. were able to restore hearing thresholds (about 50 dB) by transferring the wild-type gene for this connexin (Miwa et al., 2013). Although this success is theoretically not transferable to humans, it confirms the justified interest in gene therapy, especially for connexin-related deafness. Furthermore, the study by Pinyon et al. is promising because it allows an improvement of the implant performance after the transfer of a

neurotrophic factor by electroporation (Pinyon et al., 2014). It should be noted that no transgene expression was detected after 10 weeks. Although performance is improved, the auditory gain associated with electroporation of the neurotrophic factor has not been measured with ABR. Furthermore, the risks associated with the use of electrical pulses should be further investigated.

In conclusion, non-viral vectors hold great promise for treating the genetic causes of hearing loss. While progress has been made in animal models, overcoming challenges related to long-term efficacy, potential toxicity, and clinical translation is essential to make these therapies available to deaf patients.

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Au vu des conclusions de cette revue, nous nous sommes concentrés sur les nanoparticules lipidiques. Ces vecteurs ont plusieurs avantages par rapport aux vecteurs viraux, notamment une production bien contrôlée et de faible coût, une bonne stabilité et une faible immunogénicité. La taille du transgène qu'ils peuvent transporter est quant à elle théoriquement illimitée (Belmadi et al., 2015a). Cependant, leur principale limite est une efficacité à transférer du matériel génétique inférieure à celle des vecteurs viraux. L'efficacité des vecteurs synthétiques varie en fonction de la structure chimique du vecteur, le rapport de charge (RC) du lipide (ratio entre les charges positives du vecteur synthétique et les charges négatives de l'ADN) ou du rapport de masse (RM) du polymère (ratio entre la masse du polymère par rapport à la masse de l'ADN), et les caractéristiques du potentiel co-lipide associé. Elle varie également en fonction de facteurs extrinsèques rencontrés au cours de la délivrance du gène, notamment les barrières cellulaires et l'environnement extracellulaire.

Un lipide cationique, la Lipofectamine 2000 (Thermo Fisher Scientific) a été utilisée comme vecteur du complexe CRISPR-Cas9 *in vivo* sur lignée de souris mutées et a permis une restauration fonctionnelle de l'audition après action de la nucléase (Gao et al., 2018b; Tao et al., 2023). Il est actuellement utilisée comme témoin positif *in vitro* pour la recherche de nouveaux vecteurs synthétiques efficaces (Yoon et al., 2015). Concernant les cellules transfectées par la Lipofectamine 2000 au sein de l'organe de Corti, différents auteurs décrivent une transfection effective mais partielle des cellules ciliées externes (CCE) et cellules ciliées internes (CCI) de l'ordre de 20 à 30% (Zuris et al., 2015; Gao et al., 2018; Tao et al., 2023). Il est important de noter que d'autres types cellulaires que les cellules ciliées peuvent être impliqués dans les surdités (Petit et al., 2023) (**Figure 1C**). La capacité de transfection du vecteur dans d'autres cellules d'intérêt, notamment les fibrocytes et neurones du ganglion spiral serait quant à elle nettement plus faible, de l'ordre de 3 à 6% (Zhang et al., 2011b).

Pour améliorer les résultats de transfection en terme d'efficacité et de nombre de type cellulaire ciblés, nous avons opté pour la sélection de vecteurs non viraux ayant démontré leur efficacité *in vivo* dans d'autres contextes, notamment dans l'épithélium respiratoire (Picquet et al., 2005; Belmadi et al., 2015b; Ghanem et al., 2023). Bien que cela ne soit pas la cible, il convient de noter que cet épithélium respiratoire est également présent dans l'oreille moyenne.

L'objectif de notre étude était de sélectionner un vecteur synthétique permettant d'améliorer la capacité de transfection des cellules ciliées internes de la Lipofectamine 2000, et de cibler d'autres types cellulaires impliqués dans les surdités d'origine génétique.

## Article n°4 BSV163: un nouveau vecteur synthétique pour la thérapie génique de l'oreille interne ?

### Résumé

Introduction : Les surdités d'origine génétique représentent 80 % des surdités congénitales et plus de 500 syndromes de surdité ont été décrits. Les traitements actuels sont fonctionnels (implants cochléaires, aides auditives) et ne permettent pas de traitement étiologique. Les lipides cationiques ont permis de restaurer l'audition dans des maladies autosomiques dominantes en ciblant les cellules ciliées internes ou externes. Cependant, leur efficacité de transfection est faible. L'objectif de cette étude était de tester et de sélectionner un ou des vecteurs synthétiques capables d'augmenter l'efficacité de transfection des cellules de l'organe de Corti, en améliorant le taux de transfection des cellules ciliées, et/ou en permettant la transfection d'autres cellules d'intérêt impliquées dans des surdités génétiques.

Méthode : Les expériences ont été réalisées sur des explants d'organe de Corti prélevés sur des rats âgés de 3 jours. Les explants ont été mis en culture avec des vecteurs associés à des plasmides codant pour la luciférase et l'eGFP. Une deuxième série d'expérience a été réalisée sur des cochlées entières, qui ont ensuite été disséquées pour l'analyse des explants. L'analyse quantitative de la transfection a été réalisée par des mesures de luminescence. La viabilité cellulaire a été analysée en mesurant la teneur en ATP du pool de cellules.

Résultats : Le vecteur liposome cationique BSV163 associé au co-lipide DOPE (1/1) avait la meilleure efficacité de transfection des vecteurs synthétiques testés. Son efficacité était comparable à la Lipofectamine 2000. Le BSV163/DOPE avait une localisation préférentielle de transfection au niveau des neurites et de la bandelette sillonnée, contrairement à la Lipofectamine 2000, qui permettait une transfection des cellules ganglionnaires spirales. Le vecteur KLN47 était efficace dans les explants de l'organe de Corti mais pas sur cochlées entières.

Conclusion : Le vecteur BSV163/DOPE était le plus performant des vecteurs synthétiques testés, montrant une efficacité similaire à celle de la Lipofectamine 2000, mais avec une préférence distincte pour la localisation de la transfection, en ciblant principalement les cellules de la bandelette sillonnée et les neurites.

*Manuscrit en cours de préparation*



## **BSV163: A new synthetic vector for inner ear gene therapy ?**

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### ABSTRACT

Genetic hearing loss accounts for 80% of congenital deafness and over 500 deafness syndromes have been described. Current treatments are functional (cochlear implants, hearing aids) and do not allow etiological treatment. Cationic lipids have allowed the restoration of hearing in autosomal dominant diseases by targeting inner or outer hair cells (IHC or OHC). However, their transfection efficiency is low. The aim of this study was to test and select a synthetic vector capable of increasing the transfection efficiency of organ of Corti cells. This could be achieved by improving the transfection rate of IHC and OHC or by allowing the transfection of other cells of interest involved in genetic hearing loss.

Experiments were carried out on organ of Corti explants taken from rat pups at 3 days of age. The explants were exposed to vectors associated with plasmids coding for luciferase and eGFP. The second part was performed on whole cochleae, which were then dissected to obtain explants for analysis. Quantitative analysis of transfection was performed using luminescence measurements. Cell viability was analysed by measuring the ATP content of the cells pool.

The cationic liposome vector BSV163 associated with the co-lipid DOPE (1/1) showed the highest transfection efficiency of the synthetic vectors tested, comparable to Lipofectamine 2000. In particular, it revealed a differential transfection localisation, with a preference for targeting neurites and the spiral limb, in contrast to Lipofectamine 2000, which showed a preference for transfection in spiral ganglion cells. The KLN47 vector was effective in organ of Corti explants but not in whole cochlea.

The BSV163/DOPE vector emerged as the most promising of the synthetic vectors tested, showing similar efficacy to Lipofectamine 2000 but with a distinct transfection localisation preference, primarily within the spiral limbus and neurites.

## 1. Introduction

Genetic deafness accounts for 80% of congenital deafness, with over 500 deafness syndromes described (Tanaka-Ouyang et al, 2017). Current treatments, such as cochlear implants and hearing aids, are functional but do not provide an aetiological cure. While the majority of patients (90%) achieve understanding in quiet environments, understanding speech in noisy environments and prosody can be particularly challenging for some patients (Géléoc and Holt, 2014). Although effective and continually improving, these methods do not fully restore normal hearing, highlighting the need for aetiological treatments to improve patients' understanding and quality of life.

Early studies in inner ear gene therapy initially explored the use of synthetic vectors (nanoparticles, polymers and cationic lipids), which are safer and easier to produce (Felgner, 1996; Lasic and Ruff, 1998; Belmadi et al., 2015a). At the same time, interest in viral vectors grew due to their increased efficiency in targeting specific tissues, in particular adenoviruses targeting inner cochlear hair cells (Van De Water et al., 1999; Hinrich Staecker et al., 2001). Currently, viral vectors are the most widely used because of their efficacy. However, their potential side effects, including immunological reactions, and potential limitations in long-term efficacy raise ethical considerations (Lahlou et al., 2023). Non-viral vectors, in particular cationic lipids, have emerged as potential alternatives. Recent studies have demonstrated their efficacy in restoring hearing in mouse models of autosomal dominant genetic deafness affecting both inner and outer hair cells (Gao et al., 2018; Tao et al., 2023). However, the efficacy of the vector used, Lipofectamine 2000 (ThermoFisher Scientific), remained low for inner hair cells (IHC) and outer hair cells (OHC), ranging from 20% to 30% (Zuris et al., 2015), and even lower for other cells of interest, such as fibrocytes and spiral ganglion neurons, around 3% to 6% (Zhang et al., 2011).

To identify more efficient vectors within the organ of Corti, we selected non-viral vectors that have demonstrated *in vivo* efficacy in other applications related to the respiratory epithelium (Picquet et al., 2005; Belmadi et al., 2015a; Ghanem et al., 2023). This epithelium is also present in the middle ear, allowing for potential future treatments with delivery to the middle ear without cochleostomy, a procedure associated with the risk of hearing loss.

The primary objective was to obtain a non-viral vector that is more effective than Lipofectamine 2000 *in vitro* within the organ of Corti, allowing improved transfection of hair cells and/or targeting other cells of interest involved in genetic deafness.

## 2. Methods

### 2.1 Animals

Newborn rats (Wistar Han, JanvierLabs®), aged 3 to 5 days, were euthanised for other research projects. Sacrifice was performed by decapitation away from littermates in a biosafety level 2 containment area (Central Animal Facility, University of Western Brittany (UBO)), in accordance with the current regulations on animal experimentation in France. This project was approved by the UBO Animal Care Committee under registration number 2019-28-10-018.

### 2.2 Cochlear Dissection and Culture

All instruments were sterilised by either autoclaving or soaking in 70% ethanol prior to use. A sterile Swann-Morton #19 blade (ST19/0224) was used to make a longitudinal incision between the two cerebral hemispheres, followed by a transverse incision behind the eyes. The cerebral hemispheres were removed together with the skin and subcutaneous tissue. Whole cochlear dissections were then performed using microsurgical instruments under microscopic guidance in a sterile Petri dish containing sterile Dulbecco's modified Eagle's medium (DMEM) at a refrigerated temperature (4°C) to maintain tissue integrity throughout the procedure. The vestibular portion was sectioned and the stapes removed to allow opening of the oval window. The round window was opened by gently removing the membrane covering it and an incision was made at the apex of the cochlea. The culture medium was prepared using 43 ml sterile cold Dulbecco's modified Eagle's medium (DMEM) (4°C), 1.6 ml 1.0 M 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) and 5 ml fetal bovine serum (FBS). The dissected cochleae were placed in sterile 96-well plates under a laminar flow hood with 150 µL of culture medium. The cochleae were maintained at 37°C in a humidified incubator in an atmosphere containing 5% carbon dioxide (CO<sub>2</sub>) prior to transfection.

### 2.3 Dissection and Coating of Explants for Transfection

The coating solution, for a final volume of 1.5 mL, was composed of 17.5 µL of 2 M NaOH, 1465 µL of 0.1 M NaHCO<sub>3</sub> at pH 8.1, filtered to 0.2 µm, and 17.5 µL of pure Corning® CellTak™. This

was applied to a plate (150  $\mu$ L/well) and maintained at +37°C for 12 hours. The coating solution was then removed, followed by rinsing with sterile water and drying under laminar flow for 3 hours.

For fresh cochlear explants, the entire cochlea was finely dissected in a sodium phosphate buffer (PBS) containing 0.9% saline, pH 7.4. The spiral ligament, Reissner's membrane, and spiral ganglion were removed to retain only the organ of Corti. The explants were placed in a well after coating with PBS.

## 2.4 Transfection

### 2.4.1 Plasmids Used

Two types of plasmids were used in all experiments: a CpG-optimised plasmid encoding the luciferase gene; pGL3control (5.2 kb), and another plasmid encoding both luciferase and green fluorescent protein (GFP); pEGFP-Luc (6.4 kb). The plasmids were amplified in *E. coli* GT115 or DH5 $\alpha$  before extraction by alkaline lysis and purification using the NucleoBond<sup>®</sup> purification kit.

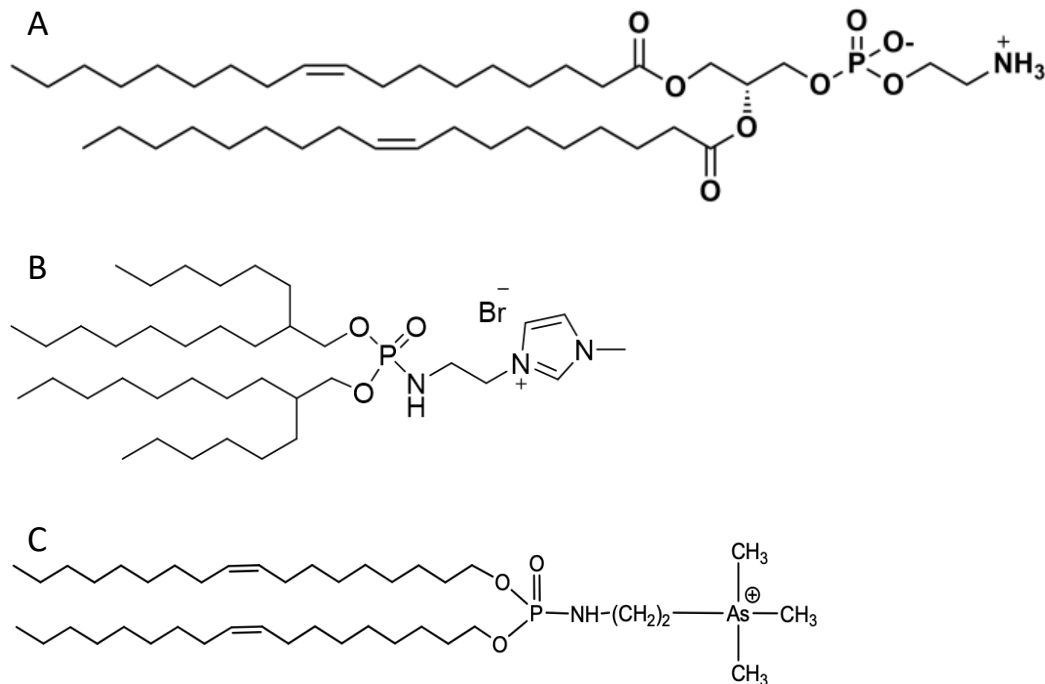
### 2.4.2 Tested Vectors

The mass ratio (MR) for polymers was the mass of polymer relative to the mass of DNA. The charge ratio (CR) for cationic liposomes represented the ratio between the positive charges of the synthetic vector and the negative charges of the DNA.

All compounds were synthesised by the UMR CNRS 6521 - Molecular Electrochemistry and Analytical Chemistry, "Organic Chemistry Synthesis Materials" team. Cationic polymer vectors were tested on organ of Corti explants: linear polyethyleneimine (IPEI) at MR3, branched polyethyleneimine (bPEI) at MR3 and MR5 (Supplementary Figure 2).

Cationic liposomes were tested on organ of Corti explants and whole cochleae. The O,O'-dioleoyl-phosphoramidate arsonium vector KLN47) was used at CR4 and CR6 (Picquet et al., 2005) (**Fig 1A**). The Brest Synthetic Vector 163 (BSV163) was mixed with dioleoylphosphatidylethanolamine (DOPE) in a 1:1 molar ratio (Ghanem et al. 2023) (**Fig 1BC**). BSV163 combined with DOPE was also combined with polyethylene glycol (PEG) for transfection on explants. The loading ratio and the amount of DNA associated with BSV163 were adjusted during the experiments to determine the optimal combination. Lipofectamine

(Invitrogen) 2000 and 3000, commercial compounds containing DOSPA (2,3-dioleoyloxy-N-2-(spermincarboxamido)ethyl-N,N-dimethyl-1-propanamium) formulated with DOPE (3/1), were used at a dosage of 0.5  $\mu\text{L}$ /well associated with 0.25  $\mu\text{g}$  of plasmid pGL3 or pEGFP-Luc, unless otherwise indicated.



**Figure 1** : Chemical composition of KLN47 (A), BSV163 (B) and DOPE (C).

#### 2.4.3 Transfection, Luminescence Reading, and Cell Viability

Transfections were performed by direct deposition of plasmid/vector complexes onto whole cochleae in their culture medium. Plasmids pGL3 or pEGFP-Luc were contacted with the different vectors for 30 min before transfection. Lipoplexes were prepared in OptiMEM™ (Gibco) and polymers in HEPES. Charge ratios (CR) and the amount of DNA associated with the vector were modulated to determine the optimal dosages for the most efficient vectors. Whole cochleae and explants for luminescence measurements were lysed 24 hours after transfection with 100  $\mu\text{L}$  of 0.5% phospholipase B (PLB). Luminescence measurement, corresponding to quantitative transfection analysis, was performed using a multimode microplate reader (Mithras2 LB) and protein levels were assessed using the BCA protein assay kit (Interchim). Luciferase expression was expressed as relative luminescence units per mg of total protein (RLU/mg of protein). Cell viability was assessed using the ViaLight™ kit to determine adenosine-triphosphate (ATP) content, which reflects the number of living cells regardless of

their transfection status. Results were expressed as a percentage of viability relative to untransfected cochlear cells.

## 2.5 Fixation of Transfected Whole Cochleae and Subsequent Explant Dissection

After 24 hours of incubation at 37°C, transfected whole cochleae were placed in 4% paraformaldehyde (PFA) at 4°C for 6 hours. After rinsing, the cochleae were dissected in PBS to obtain explants. The explants were placed on a plate with a coating solution according to the protocol described previously (Section 2.3).

## 2.6 Optical and Fluorescence Microscopy

Explants on slides and cochlear sections on slides were observed in brightfield and fluorescence using direct fluorescence optical microscopy Axiostar, coupled with an AxioCam camera and AxioVision analysis software (Carl Zeiss Meditec). Explants in solution were observed by optical microscopy with fluorescence Olympus IX71 x20 coupled with a Retiga R3 camera and Ocular™ software (QImaging).

## 2.7 DNA Complexation and Relaxation

DNA complexation was achieved by adding pGL3 plasmid (25 ng/μL) to Lipofectamine 2000, BSV163/DOPE, and KLN47 based formulations at different charge ratios (CR) for cationic liposomes and varying amounts of solution for Lipofectamine. The amount of plasmid was titrated to investigate potential saturation of the vector with DNA. The respective mixtures were incubated for 30 minutes at room temperature. Excess dextran sulphate (20 mg/ml) was added to half of the mixtures. All mixtures (with and without dextran sulphate) were incubated at 37°C for 30 minutes to determine the relaxation capacity of the vector. Electrophoresis was then performed in a 0.8% agarose gel at 100 V, 90 mA and stained with ethidium bromide (EtBr). A UV transilluminator (Fisher Bioblock) was used to visualise the plasmid after 15 minutes of migration

## 2.8 Clarification

In order to accurately visualise the transfected cell types and perform 3D analysis, a clarification process was performed to render the cochlea transparent. The cochleae were first perfused with 4% paraformaldehyde in 0.1 M sodium potassium phosphate buffer, pH 7.4. They were then postfixed by immersion in 4% paraformaldehyde and placed at 4°C for 6 hours.

A careful incision was made in the cochlear shell above the basal turn of the scala vestibuli to facilitate solution exchange during immunolabelling. Decalcification was achieved by immersion in a 10% sodium salt of ethylenediaminetetraacetic acid (EDTA) solution in PBS, pH 7.4, for 4 days at 4°C with agitation. After decalcification, cochleae were incubated in Image-iT fx (Molecular Probes, Eugene, OR) for 30 minutes and then transferred to blocking solution for 4 hours. Actin-F was used to visualise hair cells (1/100, Phalloidin Fluoprobe 405, Interchim, Montluçon, France; cat # FP-CA9870). DAPI (ThermoFisher Scientific #62248) was used to label DNA at a concentration of 0.5 µg/ml. These reagents were applied in a blocking solution and incubated for 3 days. After incubation, the samples were washed with PBS. The cochleae were then transferred to 70% ethanol for 12 hours. Dehydration continued in 95% ethanol for 30 minutes, followed by two changes of absolute ethanol for 2 hours each. The inner ears were cleaned in a 1:1 mixture of methyl salicylate with benzyl benzoate (MSBB) and absolute ethanol for 4 hours, followed by three changes of 100% MSBB for 2 hours, 4 hours and overnight. The cochleae were rinsed through multiple changes of absolute ethanol for 24 hours.

This multi-step clarification process was undertaken to prepare the samples for detailed 3D analysis.

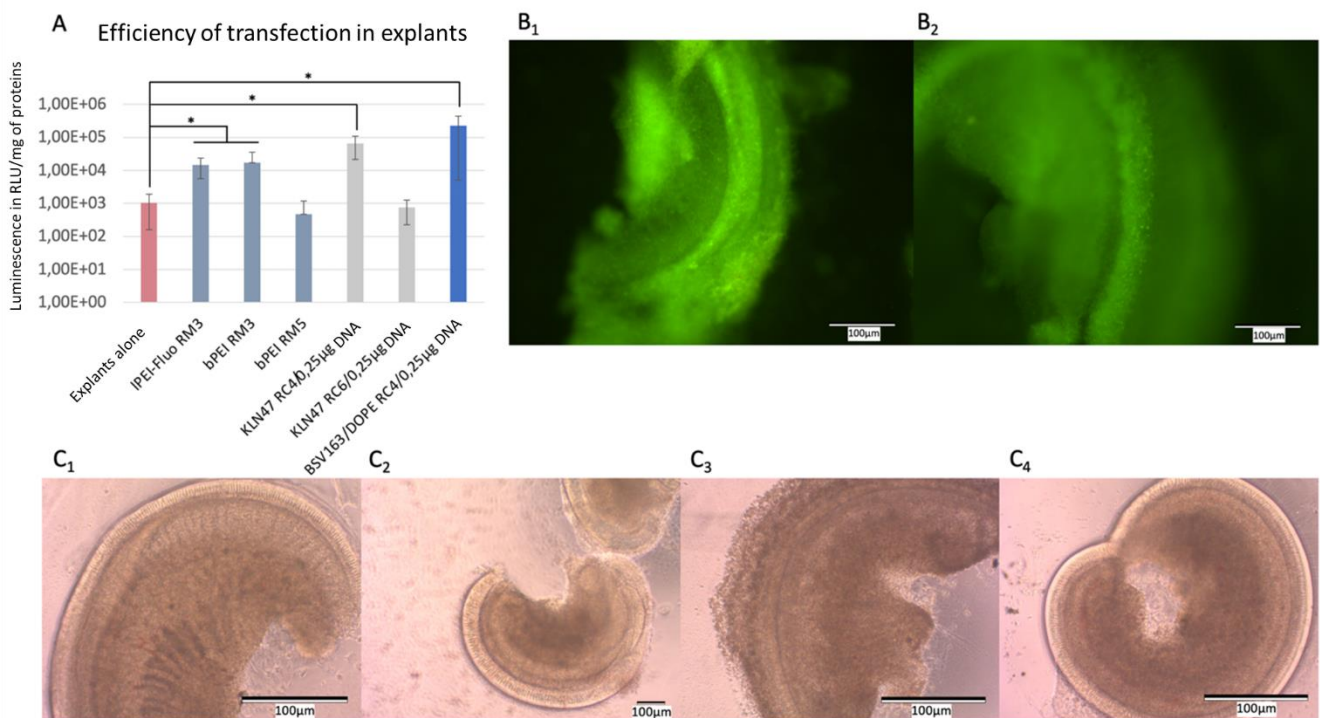
## 2.9 Statistical Analysis

Data were analyzed using SPSS 25 software. For quantitative data, a Student's t-test was performed. Results were presented as the mean of at least triplicates and were considered statistically significant for a p-value < 0.05 (\* corresponding to a p-value < 0.05, \*\* corresponding to a p-value < 0.01, \*\*\* corresponding to a p-value < 0.001).

## 3. Results

The vectors were first tested in organ of Corti explants, a method that facilitates transfection and allows toxicity assessment. For each compound tested, an average of three wells was calculated. For each vector, the amount of DNA was determined (0.25 µg of pEGFP-Luc plasmid per well). Luminescence from explants alone was compared to those in contact with the tested

vectors (**Fig 2A**). Both KLN47 and BSV163/DOPE, at CR4, showed the ability to transfect cells within the organ of Corti without specifying their nature. KLN47 at CR6 showed no transfection efficiency. The polymers (IPEI and bPEI) at RM3 effectively transduced the explants, but bPEI at RM5 did not. Overexpression of eGFP was more pronounced in the spiral ganglion, inner hair cells and supporting cells for BSV163 (**Fig 2B<sub>1</sub>**). It was more intense in the supporting cells and outer hair cells for KLN47 (**Fig 2B<sub>2</sub>**). To assess toxicity, evaluation under white light 24 hours after transfection showed comparable aspects between the negative control and explants transfected with BSV163/DOPE CR4 and KLN47 CR4 (**Fig 2C**). However, there was clear toxicity in explants transfected with IPEI, particularly in the supporting cells and outer hair cells, with cellular separation (**Fig 2C<sub>3</sub>**).



**Figure 2** A) Luciferase expression after the application of various vectors associated with the pEGFP-Luc plasmid. Results were expressed in relative light units per milligram of total protein (Relative Light Units - RLU/mg of protein) as the mean  $\pm$  standard deviation of three wells. ("Explants alone" represented non-transfected explants). Student's t-test was performed to compare the different vectors to the negative control (\* indicates  $p < 0.05$ ). B) GFP expression after vector application; BSV163/DOPE (B<sub>1</sub>) and KLN47 CR4 (B<sub>2</sub>). C) Appearance of explants after transfection in white light; BSV163/DOPE CR4 (C<sub>1</sub>), KLN47 CR4 (C<sub>2</sub>), IPEI RM3 (C<sub>3</sub>), negative control (C<sub>4</sub>). Scale bar = 100  $\mu$ m.

For transfections on whole cochleae, an average of three to nine cochleae was calculated for each vector tested. Similarly, each vector was associated with a fixed amount of DNA (0.25  $\mu$ g of pGL3 or pEGFP-Luc plasmid per well). BSV163/DOPE at RC4 was identified



as the most efficient synthetic vector in terms of quantitative luminescence on whole cochlea compared to cells alone ( $1.36 \times 10^5$  vs.  $9.98 \times 10^3$  RLU/mg protein;  $p=0.01$ ) (**Fig. 3A**). Transfection efficiency was better for BSV163/DOPE alone than when combined with PEG (135820 vs. 9781 RLU/mg protein;  $p=0.008$ ). On the whole cochlea, KLN47 showed no quantitative transfection efficiency in luminescence at RC4 or RC6. The binding capacity of the different compounds to DNA was evaluated in water by mixing different amounts of DNA (between 0.25  $\mu\text{g}$  and 1  $\mu\text{g}$ ) with different concentrations of cationic lipids and different amounts of Lipofectamine (**Fig 3B**).

Complete DNA complexation was demonstrated by the absence of DNA migration in the agarose gel. KLN47 showed excellent DNA complexation at RC4 and RC6 with 0.25  $\mu\text{g}$  of associated DNA and effective relaxation in the presence of dextran sulphate. For BSV163/DOPE, complexation was less pronounced than for KLN47, but DNA relaxation was still visible. With RC2, complexation was greater than with RC1, and there was rapid vector saturation as the amount of plasmid was increased with RC1 and RC2. For Lipofectamine 2000, excellent complexation and relaxation was observed regardless of the amount of plasmid and vector used. Surprisingly, cell viability was relatively variable between cochleae (**Fig 3C**). There was no major toxicity of the vectors, except for one cochlea out of a triplicate associated with the KLN47 vector at RC4, which had a viability of 13%. In terms of qualitative analysis and localisation of vector transfection, neurites and the spiral limbus between the spiral ganglion on the inside and the inner hair cells on the outside were preferentially transfected by the BSV163/DOPE vector (**Figure 3D<sub>1</sub>**). Using Lipofectamine 2000, the spiral ganglion was transfected inside the organ of Corti explant (**Figure 3D<sub>2</sub>**).

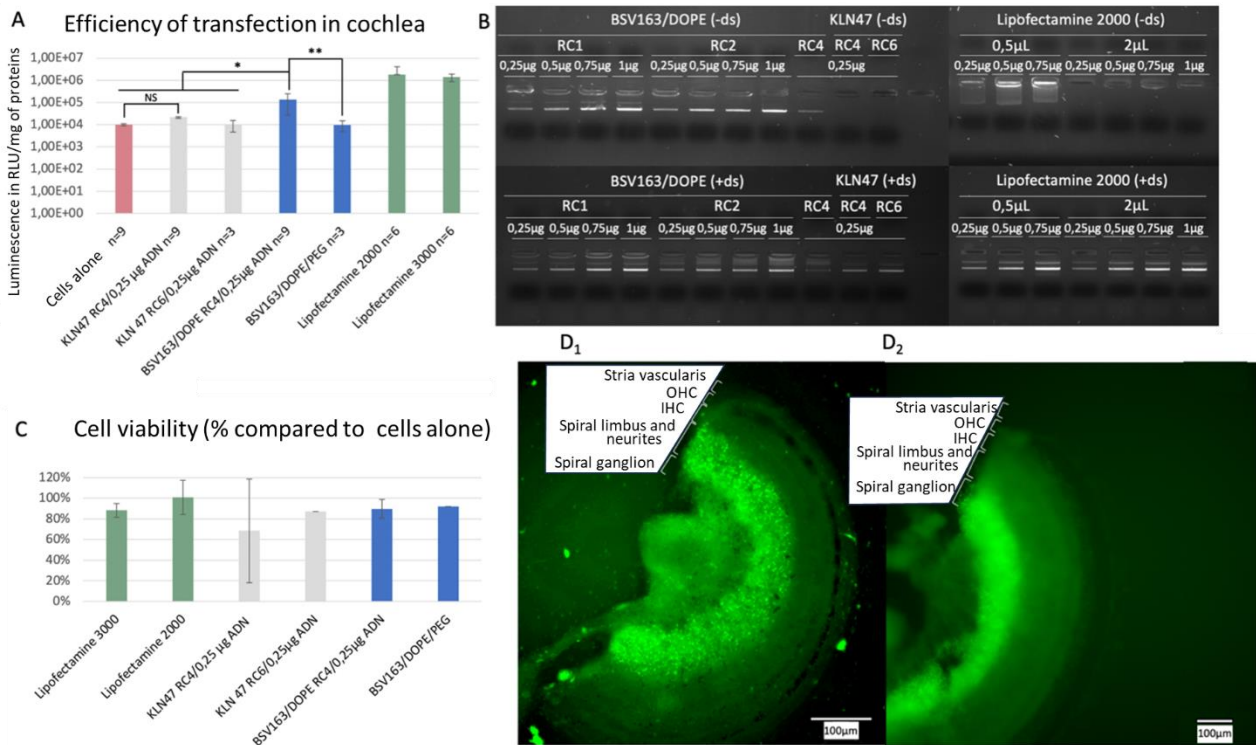


Figure 3. A) Luciferase expression after deposition of different vectors associated with the pGL3 plasmid on whole cochlea. Results are expressed in relative light units per milligram of total protein (RLU/mg protein) as the mean  $\pm$  standard deviation of three to nine wells. Student's t-test was performed to compare BSV163/DOPE RC4 with the negative control and with KLN47 RC4, RC6 and BSV163/DOPE/PEG. KLN47 RC4 was compared to the negative control ('Cells alone' corresponds to non-transfected cochleae, NS = not significant). B) Electrophoresis performed on different lipoplexes, at different charge ratios (RC) for cationic lipids and at different amounts for Lipofectamine ( $\mu$ L). Titration of the amount of pGL3 plasmid to demonstrate DNA complexation and relaxation. Complete DNA complexation was demonstrated by the absence of DNA migration in the agarose gel. Experiments were performed in the presence (+ds) or absence (-ds) of dextran sulphate. C) Cell viability 24 hours after transfection. Non-transfected cochleae were used as negative control and results are expressed as percentage of viability relative to these controls. The average viability was calculated from the results of at least three wells. D) GFP expression in fixed explants after dissection of transfected cochleae under an inverted fluorescence microscope; BSV163/DOPE RC4 (D1), Lipofectamine 2000 (D2). Scale bar = 100  $\mu$ m.

Considering the results obtained with BSV163/DOPE on whole cochleae, transfections were performed with different amounts of vector and DNA to determine the optimal conditions for efficiency. The results are shown in **Figure 4**. BSV163 with RC2 combined with 0.75 $\mu$ g pGL3 plasmid was the most effective combination compared to cells alone ( $4.22 \times 10^5$  vs.  $2.42 \times 10^3$  RLU/mg protein;  $p=0.02$ ) (**Fig 4A**). There was no significant difference compared to lipofectamine. Given these results with RC2 combined with 0.75 $\mu$ g of plasmid and the

convincing results from previous transfections with RC4 combined with 0.25µg of plasmid (**Fig. 3A**), transfections were repeated at these doses (RC2/0.75µg of DNA, RC4/0.25µg of DNA) (**Fig. 4B**). RC4/0.75µg DNA, tested on a sample of fifteen cochleae, resulted in a significantly higher transfection compared to cells alone ( $9.2 \times 10^4$  vs.  $5.69 \times 10^3$  RLU/mg protein;  $p=0.004$ ). For RC2/0.75µg, assessed in a sample of eleven cochleae, the difference was not statistically significant compared to cells alone, with a high degree of variability noted ( $p=0.1$ ).

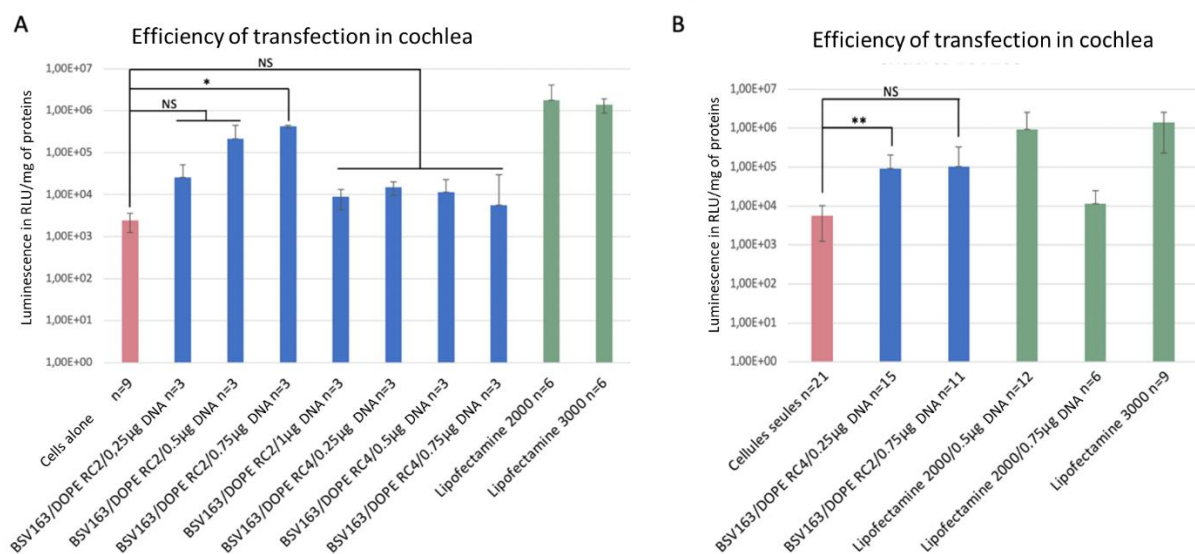


Figure 4. A) Luciferase expression after transfection with BSV163/DOPE and Lipofectamine in combination with the pGL3 plasmid. Results are expressed as relative light units per milligram of total protein (RLU/mg protein) as the mean  $\pm$  standard deviation of three to nine wells. A Student's t-test was performed to compare the negative control with different combinations (RC and DNA amount) of BSV163/DOPE. B) Luciferase expression over several litters after transfection with BSV163/DOPE and Lipofectamine in combination with the pGL3 plasmid. Results are expressed as relative light units per milligram of total protein (RLU/mg protein) as the mean  $\pm$  standard deviation of three to twenty-one wells. Student's t-test was performed to compare BSV163/DOPE RC4/0.75µg DNA and BSV163/DOPE RC2/0.25µg DNA with the negative control ('Cells alone' corresponds to non-transfected cochleae, \*  $p<0.05$ , \*\*  $p<0.01$ , NS: not significant).

#### 4. Discussion

The BSV163/DOPE (1/1) vector was the most effective vector tested on both organ of Corti explants and whole cochleae. There was no difference between BSV163/DOPE and KLN47 on explants (Figure 3A), but a difference was evident on whole cochleae (Figure 4A). There was no difference in transfection efficiency compared to Lipofectamine 2000.

Regarding transfection localisation analysis, BSV163/DOPE showed a preferential targeting of neurites and the spiral limbus. Lipofectamine 2000, on the other hand, showed a preference for transfection in the spiral ganglion cells, similar to the findings of Zhang et al. (Zhang et al., 2011). Therefore, compared to Lipofectamine 2000, BSV163/DOPE may be particularly relevant for deafness affecting the cells of neurites and spiral limbus. Three types of mutations leading to deafness are described in spiral limbus: those involving the WFS1 gene, which encodes the wolframine protein involved in Wolfram syndrome, which causes autosomal recessive deafness, type 1 diabetes, bilateral optic atrophy and neurological signs (Cryns et al., 2003); those involving the OTOA gene, which encodes the otoancorin protein required for limbal attachment of the tectorial membrane, essential for proper stimulation of inner hair cells (Kim et al., 2019); and finally those involving the COL11A2 gene, responsible for Stickler syndrome type 3, which combines deafness, dysmorphism, and joint and skeletal abnormalities without ocular abnormalities (Melkonniemi et al., 2000). These three genes could be potential targets for gene therapy with BSV163/DOPE.

The KLN47 vector showed transfection efficiency on organ of Corti explants with RC4. In the context of cystic fibrosis, the KLN47 vector was used *in vivo* in mouse models of lung pathology and was 25% more efficient than Lipofectamine 2000 (Picquet et al., 2005; Le Gall et al., 2010). Paradoxically, on whole cochleae, the results were reversed and it was 25% less effective.

On organ of Corti explants, polyethyleneimine, a cationic polymer, showed transfection efficiency whether in linear (lPEI) or branched form (bPEI) at RM3. However, the macroscopic appearance of the transfected explants at 24 hours indicated significant toxicity. The efficacy of PEI as a gene carrier has been demonstrated in several studies, especially in its hyperbranched form (Kurosaki et al., 2009; Tian et al., 2013; Belmadi et al., 2015a). However, several authors also reported cytotoxicity (Kurosaki et al., 2009; Tian et al., 2013), lack of biodegradability (Tian et al., 2013) and associated erythrocyte agglutination (Kurosaki et al., 2009).

Luminescence as a quantitative transfection validation tool has been widely used for synthetic vectors due to its objective nature. Several compounds that have shown *in vitro* efficacy in cell lines or primary cell cultures have demonstrated *in vivo* efficacy and are currently in clinical trials (Le Gall et al., 2010; Zhang et al., 2012; Alton et al., 2015; Belmadi et al., 2015b).

Furthermore, the variability in the use of primary cultures was significant, regardless of the method used. Several studies have been conducted on this topic in an attempt to standardise

practices and make them more reliable (Zhang et al., 2019). Another option has been the development of human induced pluripotent stem cell (hiPSC) organoids to overcome this variability (Van Der Valk et al., 2021; Nist-Lund et al., 2022).

The BSV163/DOPE vector was the most promising of the synthetic vectors tested. Although its efficiency was not superior to Lipofectamine 2000, the preferential localisation of its transfections in the spiral limbus and neurites could be useful for the treatment of deafness associated with Wolfram syndrome, Stickler syndrome type 3 and OTOA gene mutations.

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## Discussion générale

À travers ces articles, nous avons pu d'une part nous intéresser à l'otoferline, une protéine exprimée dans les CCI à l'origine de surdités d'origine génétique, et d'autre part aux vecteurs non viraux qui pourraient permettre de contribuer à la prise en charge des patients atteints de surdités d'origine génétique.

L'otoferline est une protéine multifonction globulaire constituée d'un domaine transmembranaire et de 6 domaines C2, ayant la capacité de fixer le calcium, située au niveau cochléaire et cérébral (Yasunaga et al., 2000). Dans la cochlée et plus précisément dans les cellules ciliées internes, elle intervient dans le contrôle de la fusion membranaire et notamment dans le cycle des vésicules synaptiques (amarrage, amorçage, fusion, endocytose, transport et maturation des vésicules) (Tertrais et al., 2019). En l'absence d'otoferline, l'exocytose des vésicules est absente (Vogl et al., 2015). En régulant la rigidité de la membrane plasmique et la tension du réseau d'actomyosine, elle permet probablement par ce biais le maintien de rubans membranaires synaptiques actifs. Bien que la littérature soit assez conséquente sur l'otoferline, plusieurs questions restent sans réponse.

Pourquoi n'y a-t-il pas dans les cellules ciliées de synaptotagmine, protéine senseur calcique présente dans les synapses centrales (Beurg et al., 2010)? Quelle est la plus-value ou la spécificité particulière de l'otoferline qui semble remplacer les synaptotagmines dans les cellules ciliées, est-ce lié à la fréquence de la stimulation, au type de libération multivésiculaire ? Nous proposons que l'otoferline agisse comme un senseur calcique intermédiaire entre le réseau d'actine-F et les vésicules synaptiques ; ce qui permettrait un mouvement oscillatoire dépendant du calcium, dont la fréquence de résonance dépendrait de sa masse, régulant ainsi la fréquence de décharge des fibres post-synaptiques.

Quel est le rôle de l'otoferline au niveau du système nerveux central, en particulier dans le cortex et l'amygdale ? Il n'y a pas de trouble neurologique décrit dans les surdités DFNB9 causées par des mutations du gène de l'otoferline. Est-elle remplacée par une autre protéine ? Pour autant, pourquoi son expression serait diminuée dans un modèle chimérique de maladie d'Alzheimer (Espuny-Camacho et al., 2017)?

Enfin, alors qu'elle intervient dans les processus membranaires de la survie cellulaire et de la signalisation, et que son expression dans plusieurs types de cancers est associée à la survie des patients, quel est son rôle exact dans la carcinogénèse ? Un élément intéressant est sa présence en tant que facteur de mauvais pronostic pour les cancers du rein (papillaire ou à cellules claires) ; et a contrario de bon pronostic pour les cancers de la vessie. Est-ce que son rôle dans ces deux types de



cancer est diamétralement opposé ? Ou est-ce en lien avec sa régulation ? En fonction de son rôle exact, pourrait-elle constituer une cible thérapeutique, soit en inhibant sa production ou au contraire en la stimulant ?

Le modèle murin que nous avons créé pour l'étude des protéines des cellules ciliées permet l'expression spécifique de l'enzyme Cas9 dans ces cellules d'intérêt. La genèse du projet provient d'une publication évaluant l'utilisation d'une Cre-recombinase (injectée avec un gRNA à l'aide d'un AAV), en utilisant un promoteur d'un type neuronal, afin d'exprimer spécifiquement l'enzyme Cas9 dans ce type neuronal (Platt et al., 2014). La tolérance de l'expression de la Cas9 *in vivo* avait auparavant été testée, et les auteurs ne notaient pas d'atteinte de l'ADN, du développement cellulaire ou de dysmorphie. Par analogie, nous avons utilisé une lignée de souris dont l'expression de la Cas9 dépend du croisement avec une seconde lignée exprimant la Cre-recombinase sous l'action du promoteur du gène de la myosine 15, spécifique des cellules ciliées (Caberlotto et al., 2011). Le croisement de ces lignées nous a permis d'obtenir des souris dont la Cas9 est exprimée spécifiquement dans les cellules ciliées. Ceci ouvre la possibilité de l'étude facilitée et spécifique de la fonction des protéines exprimées dans ces cellules, qui sont à l'origine de nombreuses surdités d'origine génétique (Petit et al., 2023). Cependant, il est tout de même nécessaire de noter qu'une limite de notre étude est l'absence de preuves génétiques de l'action de Cas9. Malgré plusieurs tentatives, cette action n'a pas pu être démontrée de manière concluante. Plusieurs explications sont possibles, notamment la fréquence relativement faible des indels, autour de 1% dans d'autres articles (Tao et al., 2023), et la possibilité de délétions plus importantes (Kosicki et al., 2018; Cullot et al., 2019). Néanmoins, l'utilisation de souris contrôle ayant soit le même phénotype et une injection d'un virus contenant un guide ARN, ou avec un phénotype sauvage et injectées avec le virus contenant l'ARNg ciblant l'otoferline, nous permet tout de même de conclure sur l'efficacité de notre méthode.

En ciblant l'otoferline, et en particulier son domaine trans membranaire, dont le rôle exact est méconnu, grâce à un guide ARN spécifique, nous avons pu montrer que cette partie est indispensable au développement et au fonctionnement normal des cellules ciliées internes, et donc à l'audition. Lorsque la Cas9 provoque une cassure double brin provoquant la production d'otoferline sans domaine transmembranaire, il existe une diminution de son expression de façon mosaïque le long de la cochlée.

Dans notre étude il existe une corrélation entre l'expression de l'otoferline et le nombre de rubans synaptiques d'une part, ainsi qu'entre l'expression de l'otoferline et la taille cellulaire d'autre part. Il existe vraisemblablement des interactions entre l'otoferline et les protéines ribeye, composant les rubans synaptiques, et les composés du cytosquelette (Vincent et al., 2015), d'autant que nous montrons également que la répartition des rubans est variable en fonction de l'expression de l'otoferline. Ils se répartissent de manière plus concentrée au pôle basal en cas de diminution de

l'expression de l'otoferline. Cela suggère un rôle majeur de l'otoferline dans la formation, l'organisation et/ou le maintien de la zone active, élément clé de la qualité de transmission du signal.

Par ailleurs, des résultats préliminaires ont montré chez les souris injectées avec le gRNA spécifique, une détérioration progressive des DPOAE débutant plus d'un mois après l'injection, excluant une cause traumatique chirurgicale des CCE. Plusieurs hypothèses peuvent être envisagées, notamment la présence d'un « rétro contrôle organisationnel », qui, un peu à la manière de la concurrence sensorielle au cours du développement, permettrait une priorisation des zones fonctionnelles au détriment des zones atteintes de surdité du fait de la réduction de l'activité des CCI.

De plus, chez les souris injectées avec le guide ARN ciblant l'otoferline, il existe une diminution de l'audition de l'ordre de 20dB. Afin d'améliorer ces résultats, il faudrait réduire l'effet mosaïque. Pour cela, une option serait d'utiliser des souris hétérozygotes pour l'allèle du gène OTOF (souris OTOF<sup>+/-</sup>). Avec les souris utilisées actuellement, il est nécessaire que les deux allèles du gène OTOF soient modifiés par la Cas9. Si l'on utilise des souris OTOF<sup>+/-</sup>, la Cas9 ne devra effectuer qu'une cassure double-brin sur l'allèle sauvage pour que l'effet soit évaluable, ce qui devrait doubler la probabilité de survenue, et ainsi majorer la perte auditive. Cela pourrait également faciliter l'obtention d'une preuve génétique, élément manquant de notre étude.

Dans notre étude, l'altération volontaire de l'audition, pour comprendre les mécanismes à l'origine de ce type de surdité génétique, est le principal témoin de l'efficacité de la méthode d'édition du génome. Bien que dans ce modèle la Cas9 soit exprimée de façon constitutive dans les cellules ciliées, cela suggère que le même procédé pourrait être utilisé cette fois-ci pour corriger une surdité congénitale, en injectant le guide ARN associé cette fois-ci à la Cas9. En extrapolant, nous pourrions espérer une récupération partielle de l'audition grâce à la modification du génome y compris pour des surdités de transmission de type autosomique récessive. Actuellement, l'amélioration des surdités d'origine génétique avec une méthode d'édition du génome n'a été montrée que pour des modèles de surdités de transmission autosomique dominante (Gao et al., 2018; Tao et al., 2023). Pour les surdités de transmission autosomique récessive, en supposant un gain auditif du même ordre de grandeur que la perte mesurée dans notre étude, l'utilisation de cette méthode de modification du génome ne pourrait actuellement être envisagée qu'en complément des dispositifs actuellement disponibles. Cependant, dans certains cas de surdités sévères, un gain de 20dB en complément de l'appareillage auditif pourrait avoir un intérêt en évitant le recours à une implantation cochléaire. La poursuite d'études ciblant la correction étiologique ce type de surdité est donc tout à fait souhaitable.

Dans la deuxième partie concernant le traitement étiologique des surdités génétiques, nous avons fait le choix de nous concentrer sur les vecteurs non viraux, bien que la grande majorité des études actuelles concernent l'utilisation de vecteurs viraux (Delmaghani and El-Amraoui, 2020). L'utilisation des vecteurs viraux, plus efficaces, nous paraît tout à fait légitime pour des études visant à comprendre les mécanismes à l'origine des surdités, comme cela a été le cas dans la première partie. Pour autant, leur profil de tolérance ne nous paraît à ce jour pas suffisamment documenté (Ishibashi et al., 2023a), d'autant plus que des effets secondaires létaux ont eu lieu dans le cadre de leur utilisation, justifiée, pour une maladie neurologique mettant en jeu le pronostic vital (Philippidis, 2022). Dans l'étude très récente de Zhang et al., 2023, après injection intra cochléaire de virus AAV, un nombre relativement élevé de particules virales ont été détectées dans le système nerveux central et le foie (Zhang et al., 2023). Les auteurs suggèrent la nécessité d'une évaluation minutieuse de la sécurité pour anticiper toute toxicité potentielle dans les futures études cliniques. Deux essais cliniques de phase I de thérapie génique pour les surdités liées à l'otoferline ont déjà débuté et montrent a priori une efficacité pour quatre enfants traités sur cinq (données non publiées). Pour autant, l'utilisation de vecteurs viraux chez des enfants atteints de surdité pour lesquels il existe des traitements fonctionnels (appareillage auditif et implant cochléaire) nous semble au vu des données actuelles éthiquement discutable, d'autant qu'il n'existe à notre connaissance qu'une seule étude publiée évaluant la tolérance et l'efficacité de ce type de vecteur chez le primate non humain. Pour ces raisons, nous nous sommes initialement concentrés sur les vecteurs non viraux, et en particulier sur les nanoparticules lipidiques. Leur profil de tolérance est nettement mieux connu, d'autant plus depuis leur utilisation dans les vaccins à ARN messenger contre le Covid-19 (Klein et al., 2021).

Ces nanoparticules lipidiques ont été utilisées avec succès pour restaurer l'audition de modèles de souris avec des surdités de transmission autosomique dominante, en utilisant une méthode d'édition du génome (Gao et al., 2018; Tao et al., 2023). Bien que ces résultats soient relativement encourageant, il existe encore plusieurs limites qui ne permettent pas de transposition chez l'homme à l'heure actuelle. Premièrement, l'injection est réalisée à un âge post natal précoce chez la souris, ce qui correspondrait en termes de développement à une injection in utero chez l'homme. Les injections réalisées plus tardivement chez la souris n'ont pas montré d'efficacité, suggérant des résultats similaires chez l'homme (Tao et al., 2023). Une compréhension des mécanismes à l'origine de cette perte d'efficacité sur des cochlées matures sera nécessaire pour la mise en place de mesures correctives. Deuxièmement, la récupération de l'audition ne persiste pas au-delà de 4 mois après injection, alors que la modification génomique devrait en théorie être pérenne. Cela pourrait être lié au faible nombre de cellules ayant eu une édition du génome, qui est estimée à 20%, qui serait initialement suffisante pour améliorer les seuils audiométriques, mais qui deviendrait insuffisante au fur et à mesure en raison de la dégénérescence cochléaire globale. Troisièmement, dans ces deux publications, trois

cochléostomies distinctes sont réalisées, ce qui pourrait avoir comme conséquence d'entraîner une diminution de l'audition résiduelle. L'amélioration de la diffusion des vecteurs pourrait permettre d'éviter ce type de geste, et en ce sens plusieurs outils non invasifs sont actuellement en développement, notamment par dépôt sur la fenêtre ronde et utilisation de laser (Jaffredo et al., 2023).

Malgré ces limites d'utilisation, nous avons tout de même concentré nos recherches sur les lipides cationiques, pour les raisons éthiques d'application clinique ultérieures énoncées précédemment. L'objectif était d'obtenir un vecteur au moins aussi efficace sur les cellules ciliées que le vecteur de référence actuel, la lipofectamine 2000 (Zuris et al., 2015), et/ou ciblant d'autres types cellulaires impliqués dans des surdités génétiques. Plusieurs formulations ont été testées, d'abord sur explants d'organes de Corti puis sur cochlées entières, pour des raisons de disponibilité. La plus efficace sur cochlées était le BSV163/DOPE, dont l'efficacité de transfection globale était similaire à la lipofectamine 2000. De façon assez surprenante, quel que soit le vecteur utilisé, nous n'avons obtenu qu'une transfection très modérée des cellules ciliées, inférieure à 5%, résultat comparable aux résultats de Zhang et al. (Zhang et al., 2011), mais inférieure aux 20-30% retrouvé dans d'autres études (Zuris et al., 2015; Gao et al., 2018; Tao et al., 2023). La cause de cet écart est potentiellement liée au modèle utilisé, puisque comme Zhang et al., nous avons utilisé la lipofectamine 2000 sur ratons et non sur modèles murins. Ce taux d'efficacité ne nous permet pas d'envisager un traitement étiologique pour des modèles OTOF<sup>-/-</sup>.

L'intérêt principal du BSV163/DOPE est son tropisme cellulaire différent de celui de la lipofectamine 2000, pour un niveau d'efficacité global identique. Les dissections d'explants ont montré une localisation préférentielle des transfusions au niveau de la bandelette sillonnée et des neurites, au contraire de la lipofectamine 2000. Cependant, la quantification exacte de la transfection de ces types cellulaires est complexe, et nécessite des coupes frontales. De multiples techniques de coupe ont été testées pour tenter de visualiser l'organe de Corti. Malheureusement la technique n'a pas permis d'obtenir des résultats exploitables malgré le suivi strict des protocoles disponibles (Hinrich Staecker et al., 2001; Gardella et al., 2003; Ishibashi et al., 2023b). L'ensemble de la structure du labyrinthe membranaire avait tendance à s'agglutiner à la coupe et la souplesse du labyrinthe osseux (cartilagineux à 3 jours de vie sur modèle murin) rendait les sections fines (entre 10 et 50µm) très friables. Pourtant, une section fine était indispensable pour ne pas risquer la superposition de structures cellulaires lors de la coupe. Nous nous sommes donc orientés vers une autre technique, la clarification. Bien que plus longue à mettre en place, elle permettra de rendre transparent le labyrinthe cochléaire cette technique devrait permettre de s'affranchir des difficultés liées coupes fines. Cela permettra d'affiner les résultats de localisation de transfection, grace notamment à l'association à l'immunomarquage par Phalloïdine et DAPI (MacDonald and Rubel, 2010; Risoud et al., 2017). Cette

technique sera suivie d'une imagerie confocale à balayage laser en fluorescence pour permettre une visualisation en trois dimensions et permettre une évaluation de l'efficacité le long de la cochlée.

Enfin, des tests *in vivo* seront nécessaires, avant d'envisager de l'utiliser sur des souris ayant une surdité d'origine génétique, afin de cibler notamment le syndrome de Stickler. La demande de d'autorisation de projet a été acceptée, et l'autorisation pour l'utilisation d'OGM vient d'être validée en novembre 2023 ouvrant la voie à des expérimentation *in vivo*.

## Conclusion

Les objectifs de ce travail étaient d'une part d'améliorer la connaissance de la physiopathologie d'un type de surdité d'origine génétique, lié à la protéine otoferline et en particulier concernant le rôle de son domaine trans membranaire, et d'autre part de tester de nouveaux vecteurs non viraux en vue de l'élaboration d'un traitement par thérapie génique. La première étude visait à recenser toutes les données disponibles concernant l'otoferline, une protéine multifonction fixant le calcium indispensable à l'audition, et qui a vraisemblablement un rôle dans la carcinogénèse, par son rôle dans la fusion membranaire. La deuxième étude visait à explorer le rôle de l'otoferline au niveau des synapses à ruban des cellules ciliées internes, en utilisant la technologie CRISPR-Cas9 *in vivo* spécifiquement dans ces cellules. Nous montrons l'efficacité du modèle qui permettra d'étudier le fonctionnement de protéines spécifiquement dans les cellules ciliées, en permettant une expression mosaïque. Nous obtenons une réduction moyenne de 20dB de l'audition chez les souris injectées avec le gRNA ciblant l'otoferline. Dans les cellules ciblées, il existe une corrélation entre l'expression de l'otoferline, et le nombre de rubans ainsi que la taille de la zone active. Par ailleurs, les rubans sont regroupés à la partie basale des cellules. Ceci montre le rôle majeur du domaine trans membranaire de l'otoferline dans le développement, l'organisation et le fonctionnement des CCI. Dans la troisième étude, afin de rechercher une alternative aux vecteurs viraux potentiellement pourvoyeurs d'effets secondaires graves, nous recensons les études utilisant des méthodes non virales de thérapie génique sur l'oreille interne, mettant en avant les nanoparticules lipidiques. La quatrième étude vise à évaluer l'efficacité de nouveaux vecteurs de type nanoparticule lipidique pour la thérapie génique de l'oreille interne. Nous montrons l'efficacité du BSV163/DOPE *in vitro*, qui cible préférentiellement les cellules de la bandelette sillonnée et les neurites. Si les résultats sont confirmés *in vivo*, cela pourrait devenir un candidat un traitement étiologique du syndrome de Stickler.

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**Titre :** Comprendre et traiter les surdités génétiques : étude de l'otoferline, et stratégies thérapeutiques utilisant des vecteurs synthétiques

**Mots clés :** Otoferline, Surdité, CRISPR-Cas9, Lipide cationique, Thérapie Génique

**Résumé :** Les objectifs de ce travail étaient d'une part d'améliorer la connaissance de la physiopathologie d'un type de surdité d'origine génétique lié à la protéine otoferline, et d'autre part d'explorer les vecteurs non viraux en vue de l'élaboration d'un traitement par thérapie génique. La première étude visait à recenser toutes les données disponibles concernant l'otoferline, une protéine multifonction fixant le calcium indispensable à l'audition, et qui a vraisemblablement un rôle dans la carcinogénèse, par son rôle dans la fusion membranaire. La deuxième étude visait à explorer le rôle de l'otoferline au niveau des synapses à ruban des cellules ciliées internes, en utilisant la technologie CRISPR-Cas9 *in vivo* spécifiquement dans ces cellules. Nous montrons l'efficacité du modèle qui permettra d'étudier le fonctionnement de protéines

spécifiquement dans les cellules ciliées. Nous obtenons une réduction moyenne de 20dB de l'audition chez les souris injectées avec le gRNA ciblant l'otoferline. Dans les cellules ciliées, il existe une corrélation entre l'expression de l'otoferline, et le nombre de rubans ainsi que la taille de la zone active. Dans la troisième étude, nous recensons les études utilisant des méthodes non virales de thérapie génique sur l'oreille interne, mettant en avant les nanoparticules lipidiques. La quatrième étude vise à évaluer l'efficacité de nouveaux vecteurs de type nanoparticule lipidique pour la thérapie génique de l'oreille interne. Nous montrons l'efficacité du BSV163/DOPE *in vitro*, qui cible préférentiellement les cellules de la bandelette sillonnée et les neurites.

**Title :** Otoferlin and Therapeutic Strategies with Synthetic Vectors for the Understanding and Treatment of Genetic Hearing Loss

**Keywords :** Otoferlin, Deafness, CRISPR, Cationic Lipid, Gene Therapy

**Abstract :** The aims of this work were to improve our understanding of the pathophysiology of a form of genetic deafness caused by the protein otoferlin, and to explore non-viral vectors for the development of a gene therapy-based treatment. The first study aimed to compile all available data on otoferlin, a multifunctional protein essential for calcium binding in hearing, which is thought to be involved in carcinogenesis through its involvement in membrane fusion. The second study aimed to investigate the role of otoferlin at the ribbon synapses of inner hair cells using CRISPR-Cas9 technology *in vivo*, specifically in these cells. We demonstrated the efficacy of the

model, which will facilitate the study of protein function specifically within hair cells. We observed an average hearing loss of 20 dB in mice injected with gRNA targeting otoferlin. In the targeted cells, there is a correlation between otoferlin expression and the number of ribbons and the size of the active zone. In the third study, we summarised studies using non-viral methods of gene therapy in the inner ear, focusing on lipid nanoparticles. The fourth study aimed to evaluate the efficacy of new lipid nanoparticle vectors for inner ear gene therapy. We demonstrated the *in vitro* efficacy of BSV163/DOPE, which preferentially targets spiral ganglion cells and neurites.