RNA Interference Prevents Autosomal-Dominant Hearing Loss

Seiji B. Shibata,1,2,8 Paul T. Ranum,2,3,8 Hideaki Moteki,2,4 Bifeng Pan,5 Alexander T. Goodwin,2 Shawn S. Goodman,6 Paul J. Abbas,6 Jeffrey R. Holt,5 and Richard J.H. Smith1,2,3,7,*

Hearing impairment is the most common sensory deficit. It is frequently caused by the expression of an allele carrying a single dominant missense mutation. Herein, we show that a single intracochlear injection of an artificial microRNA carried in a viral vector can slow progression of hearing loss for up to 35 weeks in the Beethoven mouse, a murine model of non-syndromic human deafness caused by a dominant gain-of-function mutation in Tmc1 (transmembrane channel-like 1). This outcome is noteworthy because it demonstrates the feasibility of RNA-interference-mediated suppression of an endogenous deafness-causing allele to slow progression of hearing loss. Given that most autosomal-dominant non-syndromic hearing loss in humans is caused by this mechanism of action, microRNA-based therapeutics might be broadly applicable as a therapy for this type of deafness.

Introduction

Hearing impairment is the most common sensory deficit. It affects more than 360 million people worldwide and broadly impacts their quality of life (see Web Resources).1 Not only does it limit the ability to interpret speech sounds (leading to delayed language acquisition in infancy), but in adulthood hearing impairment can lead to economic disadvantage, social isolation, and stigmatization. Current treatment options focus on hearing aids and cochlear implants to bypass the biologic deficit by amplifying sounds (hearing aids) or by encoding them as electrical impulses that are transmitted to the auditory nerve through an implanted electrode array (cochlear implants). Although these two habilitation options are effective, they do not restore "normal" hearing. As life expectancy improves and populations grow, the hearing-impaired population will increase, making the development of therapeutics to restore or prevent hearing loss important to enhancing quality of life.2

Over the past decade, we have focused on RNA interference (RNAi) as a means of selectively suppressing mutant alleles in animal models of deafness.3,4 Herein, we report on the use of an artificial microRNA (miRNA)-based approach to rescuing the progressive hearing-loss phenotype in the Beethoven (Bth) mutant mouse, a mouse model of human autosomal-dominant non-syndromic hearing loss (DFNA36 [MIM: 606706]). This mouse carries the semi-dominant Tmc1 c.1235T>A (p.Met412Lys) allele.5 The encoded protein, TMC1, is a transmembrane protein with six hydrophobic transmembrane domains (Figure 1A).6 TMC1 interacts with the tip-link proteins protocadherin 15 and cadherin 23 and, together with TMC2, is assumed to be a component of the mechanoelectrical transduction complex.8,9 Five mutations have been reported in the human homolog, TMCI (MIM: 606706), to cause autosomal-dominant non-syndromic hearing loss at the DFNA36 locus.10–15 One TMCI mutation, c.1253T>A (p.Met418Lys) (GenBank: NM_138691, NCBI build 36.3), is orthologous to the murine Bth mutation (Tmc1 c.1235T>A [p.Met412Lys]) and segregates in a large, 222 member Chinese family who suffers from progressive post-lingual sensorineural hearing loss (Figure 1B). In this kindred, age of onset varies from 5 to 25 years, potentially providing a window for therapeutic intervention to prevent the otherwise inevitable deterioration of hearing thresholds, which by 50 years of age are in the severe-to-profound range across all frequencies.15 This natural progression of hearing loss closely mimics the phenotype of the Bth-heterozygous mouse (Tmc1Bth+/+).

Herein, we report on the use of a single intracochlear injection of an artificial miRNA carried in an adeno-associated virus (AAV) vector to slow progression of hearing loss in the Tmc1Bth+/+ mutant mouse. In some animals so treated and otherwise expected to be profoundly deaf by 35 weeks, hearing thresholds were approximately 40 dB better than those of untreated Tmc1Bth+/+ control mice.

Material and Methods

Ethics Approval

The University of Iowa Institutional Biosafety Committee (rDNA Committee, rDNA approval notice 100024) and the University

1Department of Otolaryngology – Head and Neck Surgery, Carver College of Medicine, University of Iowa, Iowa City, IA 52242, USA; 2Molecular Otolaryngology and Renal Research Laboratories, Carver College of Medicine, University of Iowa, Iowa City, IA 52242, USA; 3Interdisciplinary Graduate Program in Molecular & Cellular Biology, Graduate College, University of Iowa, Iowa City, IA 52242, USA; 4Department of Otorhinolaryngology, Shinshu University School of Medicine, Matsumoto, Nagano 3908621, Japan; 5Department of Otolaryngology, F.M. Kirby Neurobiology Center, Boston Children’s Hospital and Harvard Medical School, Boston, MA 02115, USA; 6Department of Communication Sciences and Disorders, College of Liberal Arts and Sciences, University of Iowa, Iowa City, IA 52242, USA; 7Iowa Institute of Human Genetics, Carver College of Medicine, University of Iowa, Iowa City, IA 52242, USA

*These authors contributed equally to this work

*Correspondence: richard.smith@uiowa.edu
http://dx.doi.org/10.1016/j.ajhg.2016.03.028,
© 2016 The Authors. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
of Iowa Institutional Animal Care and Use Committee (protocol 0608169) approved all relevant procedures.

**Mice**

All procedures met NIH guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee at the University of Iowa. Mice were housed in a temperature-controlled environment on a 12 hr light-dark cycle. Food and water were provided ad libitum.

Isogenic Bth-heterozygous mice (Tmc1Bth/+; Tmc2Bth/+; Tmc2+/+) maintained on a C3HeB/FeJ (C3H) background were obtained as a gift from Dr. Karen Steel. Inbred wild-type C3H mice were obtained from the Jackson Laboratory. Crossbred homozygous Tmc1Bth/Bth mice were caged with wild-type C3H mice for the generation of heterozygous Tmc1Bth/+ animals. Genotyping was done on DNA from tail-clip biopsies extracted by a phenol-chloroform procedure and amplified with forward (5'-CTAATCATACCAAGGAAACAATGGAC-3') and reverse (5'-TAGACTCACCTTGTTAATCTCATC-3') primers in a 25 μl volume containing 150 ng DNA, 0.2 nM of each primer, and BioLase DNA polymerase (Bioline) for the generation of a 376 bp amplification product in Tmc1Bth/+ mice. Amplification conditions included an initial 2 min denaturation at 95°C followed by 35 step cycles of 30 s at 95°C, 30 s at 57°C, and 45 s at 72°C and a final elongation of 10 min at 72°C. PCR products were purified and sequenced on an automated sequencer (ABI PRISM model 3730XL, Applied Biosystems). For mechanotransduction experiments, two genotypes of Tmc1-mutant mice were used (Tmc1+/+; Tmc2+/+ and Tmc1Bth+; Tmc2+/+) and maintained on a C3HBL/6J background as previously described.

**RNAi Oligonucleotide Constructs**

The RNAi oligonucleotides and vector plasmids were designed by the Viral Vector Core at the University of Iowa. Fifteen small interfering RNA (siRNA) sequences, either hand designed or selected with siSPOTR (siRNA Seed Probability of Off-Target Reduction) software, were chosen for walking through the target sequence one base at a time (Table S1). Forward and reverse oligonucleotide primers, which included an overlap in the common loop of the miRNA, were purchased from Integrated DNA Technologies and used for creating artificial miRNAs. The artificial-miRNA expression cassette was generated by PCR, purified, and digested as previously described.17,18 Each artificial-miRNA expression cassette was cloned into a vector plasmid flanked by inverted terminial repeat sequences and containing the mouse U6 promoter (mU6) followed by a multiple-cloning site, cytomegalovirus (CMV)-promoter-driven eGFP, and a Pol II termination signal. miSafe, a sequence specifically selected for its validated low off-targeting potential, was used as a control.

**Virus Production**

AAV vectors were prepared by the Viral Vector Core at the University of Iowa by a standard triple-transfection method in 293FT cells and subsequent purification in a cesium chloride gradient as previously described.19 For vector selection, single-stranded recombinant AAV serotypes (rAAV2/1 and rAAV2/9) that carried CMV-driven eGFP (rAAV2/1eGFP and rAAV2/9eGFP) were tested. Viral titers used in trans-round-window-membrane (RWM) injections were rAAV2/1 at 3.09 × 1013 vg/ml and rAAV2/9 at 1.59 × 1013 vg/ml. The selected therapeutic was
single-stranded recombinant AAV serotype 2/9 (rAAV2/9) carrying a dual transgene cassette of mouse U6-driven miRNA #16, targeting the p.Met412Lys-mutating allele and downstream CMV-driven eGFP (rAAV2/9miTmc1k412.16eGFP [miTmc]). The control vector was mU6-driven miSafe and downstream CMV-driven GFP (rAAV2/9miSafeGFP [miSafe]). Vector titers were determined by real-time PCR and were miTmc at 1.69 × 10^13 and miSafe at 1.39 × 10^13 DNase-resistant particles per milliliter. Virus aliquots were stored at −80 °C before use.

Viral Inoculation

All mice were operated on postnatal days 0–2 (P0–P2) under hypothermic anesthesia, for which animals were placed in a container with crushed ice for 3–5 min. trans-RWM injections were performed under an operating microscope. First, a post-auricular incision exposed the cochlea bulb, which was opened with fine forceps. Anatomic landmarks included the RWM and stapedial artery, which were identified before injections (Figure S1). Then, for the trans-RWM injection, either miTmc or miSafe mixed in a 10:1 ratio with 2.5% fast green dye was loaded into a borosilicate glass pipette (1.5 mm outer diameter [OD] × 0.86 mm inner diameter [ID], Harvard Apparatus) pulled with a Sutter P-97 micro-pipette puller to a final OD of ~20 μm and affixed to an automated injection system pressured by compressed gas (Harvard Apparatus). Pipettes were manually controlled with a micropipette manipulator. A total volume of 0.5 μl was injected into the left ear of each mouse. After all procedures, mice were placed on a heating pad for recovery and rubbed with bedding before being returned to the mother. Recovery was closely monitored daily for at least 5 days post-operatively.

Auditory Testing

The hearing thresholds were recorded in the following groups: (1) wild-type littermates (C3HeB/FeJ inbred mice: n = 4 from 4 to 13 weeks; n = 5 from 26 to 35 weeks), (2) Tmc1Bth/− non-injected mice (n = 11), (3) Tmc1Bth/− + miSafe mice (n = 13), and (4) rescued Tmc1Bth/− + miTmc mice (n = 10).

Auditory testing was conducted in a sound-attenuating room. Stimulus presentation and recording were controlled with MATLAB software (MathWorks) running on a PC connected to a 24-bit external sound card (Motu UltraLite mk3) as previously described.20 Stimuli were delivered via an ER-10B+ external sound card (Tucker-Davis Technologies) connected to two MF1 Multi-Field Magnetic Speakers. Stimulus presentation and recording were controlled with MATLAB software (MathWorks) running on a PC connected to a 24-bit external sound card (Motu UltraLite mk3) as previously described.20 Stimuli were delivered via an ER-10B+ external sound card (Tucker-Davis Technologies). Specimens were mounted in ProLong Diamond mounting medium (Life Technologies).

z stack images of whole mounts were collected at 10×–40× on a Leica SP8 confocal microscope (Leica Microsystems). Maximum-intensity projections of z stacks were generated for each field of view, and composite images showing the whole cochlea were constructed in Adobe Photoshop CS6 in order to meet equal conditions and show the complete turns of the cochlea at high resolution. Distance from the apex was measured in 0.25 or 0.40 mm segments with imageJ (NIH Image). For the viral-vector-screening study, inner hair cells (IHCs) and outer hair cells (OHCs) with positive eGFP and overlapping MYO7A were counted with ImageJ Cell Counter. The total number of hair cells and GFP-positive hair cells were summed and converted to a percentage. For the miRNA study, IHCs and OHCs with positive MYO7A were counted with ImageJ Cell Counter; any segments that contained

Immunohistochemistry and Histology

All animals were sacrificed by CO2 inhalation. Temporal bones were removed, perfused with 4% paraformaldehyde, and incubated for 1 hr. Cochleae were then rinsed in PBS and stored at 4 °C in preparation for dissection and immunohistochemistry. Specimens were visualized with a dissecting microscope and dissected as previously described.20 Specimens were infiltrated with 0.3% Triton X-100 and blocked with 5% normal goat serum before tissues were incubated first in rabbit polyclonal Myosin-VIIA antibody (Proteus Biosciences) or mouse monoclonal antibody to GFP (Millipore) diluted at 1:1,000 in PBS for 1 hr and then in 1:1,000 dilution of the secondary antibody, fluorescence-labeled anti-rabbit IgG Alexa Fluor 568 or goat anti-mouse IgG Alexa Fluor 488 in a 1:2,000 dilution (Invitrogen, Molecular Probes) for 30 min. Filamentous actin was labeled by a 30 min incubation of phalloidin conjugated to Alexa Fluor 488 (Invitrogen, Molecular Probes). Specimens were mounted in ProLong Diamond mounting medium (Life Technologies).
dissection-related damage were omitted from the analysis. IHC and OHC survival was quantified with 20×–40× images of whole-mount cochleae compiled into cochleograms at 35 weeks as previously described.23

Molecular Studies for In Vitro and In Vivo Expression Analyses

Previously reported cDNAs p.AcGFPMtmC1ex1WT and p.GEMT-easyTmC1ex1Bth were provided by Dr. Andrew Griffith. p.GEMT-easyTmC1ex1Bth was PCR amplified with forward (5’-GTTCGGACGCAGATCCACCACAAAAAG-3’) and reverse (5’-ATGGATTCCACTGCGCCACCCAGCAGC-3’) primers containing restriction sites SalI and BamH1. cDNAs were inserted by 1% agarose gel electrophoresis and QIAquick Gel Extraction (QIAGEN) and subcloned and ligated into Sall- and BamH1-digested p.AcGFPl-N2 vector (catalog no. 632483, Takara Clontech). Successful cloning was verified with Sanger sequencing (ABI PRISM model 3730XL, Applied Biosystems).

For in vitro miRNA screening, the aforementioned miRNA expression plasmids were used. COS-7 cells, which do not contain native TMC1, were used in this study and grown in DMEM (Invitrogen, Thermo Fisher Scientific) with 10% fetal bovine serum at 37°C with 5% CO2. Prior to transfection, COS-7 cells were transferred and grown on a 24-well plate for 1 day. The transfection mix was made with Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific) according to the manufacturer’s protocol; miRNA expression plasmids were cotransfected with p.AcGFPMtmC1ex1Bth. RNA was extracted from cells with the use of TRIzol (Invitrogen, Thermo Fisher Scientific). Expression levels were assessed in triplicate by real-time PCR (StepOnePlus, Applied Biosystems) with intron-spanning TmC1 forward (5’-GTTCGGACGCAGATCCACCACAAAAAG-3’) and reverse (5’-GGATGGTTAATCTTCCAGTTCAGCA-3’) primer sets and One Step SYBR PrimeScript RT-PCR Kit II (Clontech), and results were normalized to β-actin (5’-GTTCGGTCGCTGCTGCTGCTG-3’) primer sets in triplicate for a total of nine reactions per sample. Expression levels were determined with Power SYBR Green Master Mix (Applied Biosystems). qRT-PCR was carried out with a StepOnePlus Real-Time PCR System. Each sample was amplified with three primer sets in triplicate for a total of nine reactions per sample. Primer sets included primers specific to the wild-type TmC1 allele, primers specific to the Bth TmC1 allele, and β-actin primers. Melting curves and gel electrophoresis confirmed that PCR products were primer specific. Results were normalized to β-actin with the ΔΔCt algorithm.

Mechanotransduction

Inner ears of Tmc1<sup>1<sub>Bth</sub>-<sub>/</sub>−;TmC2<sup>+/−</sup></sup> mice and littermate control TmC1<sup>+/−</sup>;TmC2<sup>+/−</sup> mice were injected with 0.5 μl miTmc at P0–P1 by a trans-RWM approach. Cochleae were harvested at P8–P10, and the organs of Corti were bathed in standard artificial perilymph containing 137 mM NaCl, 0.7 mM NaH2PO4, 5.8 mM KCl, 1.3 mM CaCl2, 0.9 mM MgCl2, 10 mM HEPES, and 5.6 mM D-glucose. Vitamins (1:50) and amino acids (1:100) were added to the solution from concentrates (Invitrogen, Thermo Fisher Scientific), and NaOH was used to adjust the final pH to 7.40 (310 mOsm/kg). Recordings pipettes (3–5 MΩ) were pulled from R6 capillary glass (King Precision Glass) and filled with intracellular solution containing 135 mM CsCl, 5 mM HEPES, 5 mM EGTA, 2.5 mM MgCl2, 2.5 mM Na2-ATP, and 0.1 mM CaCl2; CsOH was used to adjust the final pH to 7.40 (285 mOsm/kg). Whole-cell, tight-seal, voltage-clamp recordings were done at ~84 mV at room temperature (22°C–24°C) with an Axopatch 200B amplifier (Molecular Devices). Hair bundles were deflected with stiff glass probes fabricated from capillary glass with a fire polisher (World Precision Instruments) for creating a rounded probe tip of ~3–5 μm in diameter. Probes were mounted on a PICMA Chip piezo actuator (Physik Instrumente) and driven by a 400 mA ENV400 amplifier (Piezosystem). Sensory-transduction currents were recorded without knowledge of GFP expression from control and miTmc-treated hair cells. The data were filtered at 10 kHz with a low-pass Bessel filter and digitized at ≥20 kHz with a 16-bit acquisition board (Digidata 1440A, Molecular Devices) and pClamp 10 software (Molecular Devices). Data were stored for offline analysis with OriginPro 8 (OriginLab).

Statistical Analysis

Statistical analysis of ABR, DPOAE, and cell-counting data was completed in R with two-sample t tests for samples of equal variance. Samples with unequal variance were compared with Welch two-sample t tests. Sample variance was determined with F tests comparing two variances. All comparisons were made between the Tmc1<sup>1<sub>Bth</sub>-<sub>/</sub>−</sup> and TmC1<sup>+/−</sup> mice and littermate control TmC1<sup>+/−</sup>;TmC2<sup>+/−</sup> mice with an Axopatch 200B amplifier (Molecular Devices). Hair bundles were deflected with stiff glass probes fabricated from capillary glass with a fire polisher (World Precision Instruments) for creating a rounded probe tip of ~3–5 μm in diameter. Probes were mounted on a PICMA Chip piezo actuator (Physik Instrumente) and driven by a 400 mA ENV400 amplifier (Piezosystem). Sensory-transduction currents were recorded without knowledge of GFP expression from control and miTmc-treated hair cells. The data were filtered at 10 kHz with a low-pass Bessel filter and digitized at ≥20 kHz with a 16-bit acquisition board (Digidata 1440A, Molecular Devices) and pClamp 10 software (Molecular Devices). Data were stored for offline analysis with OriginPro 8 (OriginLab).

Results

RNAi Suppresses the TmC1 c.1235T->A Allele

We sought to prevent progression of hearing loss in Tmc1<sup>1<sub>Bth</sub>-<sub>/</sub>−</sup> mice by using an artificial miRNA to suppress the expression of the mutant TmC1 c.1235T->A allele. Of
15 miRNA constructs evaluated (Table S1), one miRNA, #16, was chosen for further study because it robustly and selectively suppressed the \( Tmc1 \) c.1235A allele without affecting the wild-type allele (Figures 1C and 1D). We chose to use rAAV2/9 as the delivery vector after we compared the transduction efficiency of rAAV2/1-eGFP and rAAV2/9-eGFP injection into the cochleae of wild-type mice at P0–P2 by a \textit{trans}-RWM approach. At 2 weeks, rAAV2/1-eGFP localized to a variety of cell types, including robust localization in supporting cells; rAAV2/9-eGFP, in comparison, predominantly localized to IHCs, which were transduced with 74% efficiency in the apical cochlear turn (Figures 2A and 2B). The corresponding transduction of OHCs was 7%. Because IHC dysfunction is primarily responsible for the deafness phenotype in \( Tmc1^{Bth/+} \) animals and OHC loss is subtle in the apical cochlear turns,\(^5,26\) we considered the observed levels of hair cell transduction acceptable for this study.

The miRNA #16 expression cassette was cloned into rAAV2/9 as a dual transgene cassette of mouse U6-driven miRNA targeting the p.Met412Lys-encoding transcript coupled upstream of CMV-driven eGFP (rAAV2/9miTmck412.16eGFP, miTmc). A control vector was designed to carry a U6-driven specific sequence selected for its validated low off-targeting potential and CMV-driven eGFP (miSafe)\(^6\) (Figure 3A). \textit{trans}-RWM inoculation surgery, whereby 0.5 µl of miTmc or miSafe was injected into only the left cochlea, was performed on \( Tmc1^{Bth/+} \) mice at P0–P2 (Figure S1).

We verified robust IHC eGFP localization in cochleae harvested from \( Tmc1^{Bth/+} \) P0–P2 miTmc-injected mice 2 weeks after surgery, consistent with the transduction pattern observed in ears injected with rAAV2/9-eGFP (Figure 3B; Figure S3). To assess in vivo allele-specific suppression in individual hair cells, 4 weeks after surgery we isolated GFP-positive hair cells from miTmc-injected cochleae and control hair cells from contralateral non-injected cochleae and completed real-time qPCR by using primers specific to the wild-type and \( Bth \ Tmc1 \) alleles (Movie S1). In non-injected ears, the relative expression...
of the Bth Tmc1 c.1235T>A allele and the wild-type allele was comparable (Figure 3C). In biological replicates from miTmc-injected ears, expression of the Bth Tmc1 c.1235T>A allele was suppressed by more than 88% in comparison to levels of Bth mRNA detected in the sample from the untreated contralateral ear (Figures 3C).

**Mechanotransduction**

TMC1 and its closely related ortholog, TMC2, are assumed to be components of the mechanotransduction channel. In an earlier report, the mutant TMC1 p.Met412Lys variant was found to reduce calcium permeability and single-channel currents in IHCs.16 To test the effect of RNAi and allele-specific suppression, we therefore measured transduction currents at P8–P10 after exposing IHCs to miTmc. To ensure that observed currents were only due to expression of Tmc1, we recorded from mice on a Tmc2−/− background and compared Tmc1Bth/− mice (carrying only one mutant Tmc1 allele) to Tmc1+/− mice (carrying only one wild-type Tmc1 allele) before and after miTmc exposure. Tmc1Bth/− mice had larger currents than Tmc1+/− mice, consistent with data reported by Pan and colleagues.16 miTmc had no effect on currents observed in Tmc1+/− mice. In contrast, exposure to miTmc reduced current amplitudes in Tmc1Bth/− cells (p < 0.02), consistent with reduced expression of the mutant allele (Figures 4A and 4B).

**miTmc Treatment Prevents Hearing Loss**

On the basis of the above results, we completed a longitudinal study to quantitate the effect of miTmc gene therapy on the hearing-loss phenotype in Tmc1Bth/− mice (Figure 5A). To determine how auditory function was
affected, we measured hearing thresholds as ABRs and DPOAEs in four groups of mice: (1) wild-type littermates, (2) Tmc1<sup>Bth/+</sup> non-injected mice, (3) Tmc1<sup>Bth/+</sup>+miSafe mice, and (4) Tmc1<sup>Bth/+</sup>+miTmc mice. ABR thresholds were assessed as a response to both clicks and tone bursts at 8–32 kHz, whereas DPOAEs were measured at half-octave intervals across the same frequency range.

Click ABRs cover a broad frequency and stimulus range (2–8 kHz and up to 90 dB SPL). We tested animals at 4 week intervals for 35 weeks and documented the expected deterioration of hearing thresholds in Tmc1<sup>Bth/+</sup> mice, which by 17–21 weeks of age had hearing in the severe-to-profound range (Figure S4). The rate and degree of hearing loss were consistent with those reported by Noguchi and colleagues and were also seen in the untreated contralateral right ears of Tmc1<sup>Bth/+</sup>+miTmc mice and in the injected left ears of Tmc1<sup>Bth/+</sup>+miSafe mice, demonstrating that neither the viral inoculation procedure nor the vector itself added to the decline in auditory function<sup>26</sup> (Figure 5B).

The progression of hearing loss was slower in the injected left ears of Tmc1<sup>Bth/+</sup>+miTmc mice. In all Tmc1<sup>Bth/+</sup>+miTmc mice, preservation of hearing was significant in comparison to hearing in controls for up to 21 weeks; however, by 30 weeks ABR thresholds in the Tmc1<sup>Bth/+</sup>+miTmc mice increased (Figure 5B). In the two best-performing animals, hearing thresholds at 8 kHz were preserved throughout the entire study period (dashed blue line); thresholds were stable and only mildly higher than those of wild-type littermate controls (solid black line) (Figure 5D, 4–35 weeks; Figure S5).

We also measured 8 kHz wave I amplitudes at 4 weeks as a measure of synaptic integrity. The treated left ears in Tmc1<sup>Bth/+</sup>+miTmc mice had smaller amplitudes overall than did those of wild-type littermate controls. Wave I amplitudes were even smaller in Tmc1<sup>Bth/+</sup>+miSafe mice and continued to dampen at 8 weeks. In the Tmc1<sup>Bth/+</sup>+miTmc mice, the wave I amplitudes remained stable (Figures S6A and S6B).

DPOAEs are an objective measure of OHC function and were recorded at the same time points as the ABRs. Consistent with prior reports, we found DPOAE recordings to be comparable between Tmc1<sup>Bth/+</sup> mice and wild-type controls, reflecting a high degree of OHC preservation in the cochlear apex<sup>26</sup> (Figure 5E, 4–35 weeks). At ultrahigh frequencies (22–32 kHz), we did identify a DPOAE decline in Tmc1<sup>Bth/+</sup> mice (Figure 5E, 4–35 weeks). miTmc treatment did not affect DPOAEs in the first few weeks
**Figure 5. miTmc Gene Therapy Slows Progression of Hearing Loss in Tmc1Bth/+ Mice**

(A) Experimental timeline catalogs the experimental procedures in Tmc1Bth/+ mice and controls from the time of artificial miRNA injection to the time of tissue collection.

(B) Click ABR thresholds in wild-type, Tmc1Bth/+ miTmc contralateral, Tmc1Bth/+ miSafe, and Tmc1Bth/+ miTmc animals. The two best-performing and two worst-performing Tmc1Bth/+ miTmc-treated animals are shown as dashed and dotted blue lines, respectively, to illustrate variability in performance within the treated cohort.

(C) Representative 8 kHz ABR traces recorded from the wild-type, non-injected Tmc1Bth/+ miTmc contralateral, and Tmc1Bth/+ miTmc 13-week-old mice.

(D and E) Tone-burst ABR thresholds (D) and DPOAE amplitudes and noise floors (E) in wild-type, Tmc1Bth/+, Tmc1Bth/+ miSafe, and Tmc1Bth/+ miTmc animals at 4, 8, 13, 26, and 35 weeks. The dotted black line indicates the average noise floor for each group of DPOAEs. Black arrows indicate no response at equipment limits. *p < 0.05, **p < 0.005. See also Figures S4 and S5.
of the study, and at 4 weeks, we observed no differences between \(Tmc1^{Bth/+}\)-miTmc and \(Tmc1^{Bth/+}\)-miSafe mice. By 8 weeks, however, whereas \(Tmc1^{Bth/+}\)-miTmc mice showed a sharp decline in DPOAEs in the ultra-high-frequency range, DPOAEs in \(Tmc1^{Bth/+}\)-miTmc mice were maintained (Figures 6E, 8 weeks). This difference persisted, consistent with some preservation of OHCs in the basal turn of the cochlea in \(Tmc1^{Bth/+}\)-miTmc mice (Figure 6E, 4–35 weeks).

In summary, a single injection of miTmc significantly slowed progression of hearing loss in \(Tmc1^{Bth/+}\) mice for approximately 21 weeks. At the end of the 35-week study period, the two best-performing \(Tmc1^{Bth/+}\)-miTmc mice had hearing thresholds that were only 15–20 dB above thresholds for wild-type C3HeB/FeJ littermate controls. These thresholds were approximately 40 dB better than expected in the absence of treatment.

**miTmc Improves Hair Cell Survival**

The histological correlate of auditory function in \(Tmc1^{Bth/+}\) mice is hair cell survival. To quantitate the effect of miTmc on hair cell survival, we counted hair cells in 0.25 mm segments from cochlear whole mounts in four groups: (1) wild-type littermates, (2) \(Tmc1^{Bth/+}\)-miTmc mice, (3) \(Tmc1^{Bth/+}\)-miSafe mice, and (4) \(Tmc1^{Bth/+}\) non-injected mice.

Consistent with reported data, in \(Tmc1^{Bth/+}\) mice IHC loss was more pronounced than OHC loss and occurred in a base-to-apex gradient. By 35 weeks, we found complete IHC loss in the basal turn and 40%–50% IHC loss in the apical turn (Figures 6A and 6B, \(Tmc1^{Bth/+}\)). As expected, there was no IHC or OHC loss in 35-week-old wild-type control animals (Figures 6A and 6B, wild-type).

Hair cell survival in all \(Tmc1^{Bth/+}\)-miSafe-treated left ears was indistinguishable from hair survival in \(Tmc1^{Bth/+}\) mice (Figures 6A and 6B, \(Tmc1^{Bth/+}\)-miSafe and \(Tmc1^{Bth/+}\)). In contrast, \(Tmc1^{Bth/+}\)-miTmc mice showed markedly improved hair cell survival (Figures 6A and 6B, \(Tmc1^{Bth/+}\)-miTmc). IHC counts in \(Tmc1^{Bth/+}\)-miTmc mice were greatest in the apical region of the cochlea (10%–20%), and visible gaps occurring in the mid-modiolar region (40%–50%) showed inter-animal variability. In the lower mid-modiolar to basal regions of the cochlea (60%–70%), the pattern of IHC loss was indistinguishable between \(Tmc1^{Bth/+}\)-miTmc mice and \(Tmc1^{Bth/+}\) and \(Tmc1^{Bth/+}\)-miSafe mice. When we compared the entire \(Tmc1^{Bth/+}\)-miTmc cohort to the two best performers, we observed only a slight difference in mean IHC survival in the apical to mid-modiolar regions of the cochlea (10%–50%; Figure 6C). Compared to \(Tmc1^{Bth/+}\)-miSafe animals, \(Tmc1^{Bth/+}\)-miTmc mice showed minimal improvement in OHC survival in the apical 10% of the cochlea and in the lower mid-modiolar to basal regions of the cochlea, although these differences were not significant. OHC survival was otherwise comparable between \(Tmc1^{Bth/+}\)-miTmc and \(Tmc1^{Bth/+}\) animals (Figure 6D).

We also examined stereocilia-bundle morphology in surviving hair cells in both the best-performing and worst-performing \(Tmc1^{Bth/+}\)-miTmc mice alongside \(Tmc1^{Bth/+}\) and wild-type controls at 35 weeks (Figure S7). In the \(Tmc1^{Bth/+}\)-miTmc mice, stereocilia bundles appeared to be thinner but nevertheless well organized and comparable to those of wild-type controls in the apical regions; in the basal region, any remaining stereocilia bundles were sparse and distorted. This apex-to-base gradient of stereocilia-bundle degeneration is consistent with earlier observations in \(Tmc1^{Bth/+}\) mice. In \(Tmc1^{Bth/+}\) mice, fewer surviving hair cells remained, and in those cells, the stereocilia bundles were distorted or absent (Figure S7, \(Tmc1^{Bth/+}\)). We observed no difference between the two best performers and the entire cohort treated with \(Tmc1^{Bth/+}\)-miTmc (Figure S7).

**Discussion**

This report demonstrates that using RNAi to suppress expression of an endogenous deafness-causing allele can slow progression of hearing loss. We selectively suppressed the \(Tmc1\) c.123ST>A (p.Met412Lys) dominant gain-of-function allele in \(Tmc1^{Bth/+}\) mice at an early developmental stage. We were able to prevent profound hearing loss from developing for over 35 weeks (the duration of the study) in some animals otherwise destined to have severe to profound levels of deafness across all frequencies by 17–21 weeks. Treatment with miTmc also appeared to have a particularly striking protective effect on hair cell survival. Compared to \(Tmc1^{Bth/+}\)-miSafe and \(Tmc1^{Bth/+}\)-non-injected mice, \(Tmc1^{Bth/+}\)-miTmc mice showed significantly enhanced IHC survival in the apical turn of the cochlea. Although the reasons for enhanced IHC survival remain to be determined, these observations are consistent with both the pattern of rAAV2/9 transduction and audiometric data.

In the majority of \(Tmc1^{Bth/+}\)-miTmc mice, a single injection of miTmc maintained hearing acuity for ~26 weeks. Thereafter, the protective effect of miTmc on hearing was variable, a result consistent with well-documented differences in the efficiency and longevity of viral transduction. For example, Akil et al. used trans-RWM injection of AAV1-Vglut3 to restore hearing in neonatal Vglut3+/− mice and observed variable degrees of hearing loss 7 weeks after treatment.

There are also reports of successful restoration of normal inner-ear morphology but failed restoration of auditory function after inner-ear gene therapy employing AAV vectors. Chien et al. attempted to restore hearing in deaf whirler (\(wtr\)) mice with trans-RWM injections of AAV8-\(Wtr\), and although they demonstrated restored morphology of the stereocilia, hearing sensitivity was not rescued. Similarly, attempts using AAV1-Gjb2 to restore
Figure 6. miTmc Gene Therapy Improves Hair Cell Survival
Wild-type, Tmc1Bth/++, Tmc1Bth/+++miSafe, and Tmc1Bth/+++miTmc animals sacrificed 35 weeks after treatment. Ears were fixed, dissected, and stained as cochlear whole mounts.
(A) 10× images of representative whole-mount apical turns from wild-type, Tmc1Bth/+++, Tmc1Bth/++miSafe, and Tmc1Bth/++miTmc animals. Samples were stained with MYO7A (red) and phalloidin (green) for labeling hair cells and filamentous actin, respectively. Arrowheads show the apical tip and 8 and 16 kHz regions along the apical turn of the cochlea. Note IHC preservation in the Tmc1Bth/++miTmc animals. The white cross shows the area devoid of IHCs. Scale bars represent 150 μm.
(B) 40× magnification at the indicated position in relation to the cochlear apex. The three rows of OHCs (1–3), pillar cells (P), and IHCs are shown. Areas with dark hallows illustrate OHC or IHC loss. The white cross shows the area devoid of IHCs. Scale bars represent 50 μm.
(C and D) IHC (C) and OHC (D) survival was quantified with 20–40× images of whole-mount cochlea compiled into cochleograms at 35 weeks. Hair cells were counted in 0.25 mm segments and plotted against the distance (%) from the apex. Tmc1Bth/++miSafe, Tmc1Bth/++miTmc, and Tmc1Bth/++miTmc best performers (n = 2) are shown. *p < 0.05, **p < 0.005.

The American Journal of Human Genetics 98, 1101–1113, June 2, 2016
hearing in cCX26 knockout mice by Yu et al. demonstrated restoration of gap-junction function in supporting cells without successful hearing restoration.32 Explanations for this dichotomy have included (1) injection methods, (2) injection timing, (3) promoter types, (4) vector serotypes, and (5) transduction efficiency.

The low rate of viral transduction appears to be the most common conclusion for unsuccessful restoration of hearing sensitivity and could be the reason for the variability observed in our study as well. Indeed, although we achieved high viral transduction in the apical turn and observed hearing preservation at 8 kHz, transduction in the lower turns was very low, and at 32 kHz, the progression of hearing loss was not affected. These observations, however, do not explain the hearing deterioration we documented in the majority of treated mice after 26 weeks.

The longevity of rAAV therapeutics is believed to maintain stable transgene expression for a year or longer, as noted in studies of human gene therapy with AAV2-hRPE65v2 in Leber’s congenital amaurosis.33 One explanation for the time-limited hearing preservation we observed could be the natural progression of OHC loss that occurs in Tmc1Bth+/− mice in a base-to-apex gradient over time, although this loss is minimal in the apical region at 20 weeks.26 An alternate explanation could be the variability of RNAi in Tmc1Bth+/− miTmc mice at the molecular level, which leads to early loss of the RNAi-mediated effect. Side effects of RNAi include miRNA off-target effects, saturation of miRNA endogenous machinery, and immune stimulation via siRNA.34 A combination of these side effects could possibly lead to molecular changes at the hair cell level, although we did not observe structural damage, significant IHC loss, or signs of inflammation even in treated animals that were profoundly deaf at the end of the study, suggesting that miTmc gene therapy did not cause ototoxicity at the cellular level. Further studies will be needed to improve OHC transduction and to investigate any long-term consequences of RNAi-mediated gene therapy at the molecular level.

Missense mutations underlie 85% of all human autosomal-dominant non-syndromic hearing loss, raising the possibility that an RNAi-based therapeutic strategy could be broadly applicable to this type of hearing loss.35 Targeting autosomal-dominant non-syndromic hearing loss would be attractive because this type of loss is postlingual and progressive, thus providing a large window of opportunity for surgical intervention with a miRNA-based gene-silencing strategy. Prior to any clinical trial, however, several challenges must be addressed. First, although this and other studies attest to the value of mouse models for testing different forms of gene therapy directed at hearing preservation or restoration,28,36 because functional maturation of the cochlea occurs during the second postnatal week in mice and during the third trimester in humans, direct translation of these studies across species is not possible. Additional studies in rodents must focus on the age at injection, which remains a potential challenge given that transduction efficiency of inner-ear structures appears to be inversely related to the postnatal time of exposure. Target tissue must also be addressed because although HCs express one-third of all genes known to be associated with deafness37—which makes them excellent transduction candidates—efforts to effectively and selectively target other cells must be established.

Route of injection and vector choice must be optimized. Because of the small volume of the inner ear, direct-injection techniques will limit the total number of viral-genome-containing particles per milliliter that can be delivered, suggesting that alternative approaches should be explored to permit higher total injection loads. These experiments will require testing a variety of vectors to define the injection route, injection time, and inner-ear cell specificity for each. Finally, the possibility that therapeutic intervention after hearing loss begins could improve some genetic types of deafness if irreversible damage has not occurred should be explored.

Many of these questions will be addressed with murine models of hearing loss, but ultimately proof-of-concept studies in non-human primates will be needed to confirm vector tropism for specific types of inner-ear cells in the absence of off-target effects. Clinical trials will then be possible. Their design might require the recruitment of persons with gene-specific or even mutation-specific types of hearing loss, a selection process that will be facilitated through genetic databases that many laboratories are maintaining on persons who receive comprehensive genetic testing as part of their clinical diagnostic evaluation for hearing impairment.

We believe that preventing hearing loss in an organ destined to fail presents fewer challenges than restoring function in a cochlea that has already undergone substantial degenerative changes. Thus, although using gene therapy to replace lost hair cells or regrow inner-ear architecture is likely to be exceedingly difficult, its use for replacing defective genes, suppressing a deafness-causing allele, or correcting abnormal splicing before cochlear damage is rampant might be more feasible. Because substantial challenges remain in using gene therapy to restore the functional integrity of this unique and complex organ, we believe that efforts to prevent autosomal-dominant non-syndromic hearing loss warrant a concerted effort by the research community as an example of “low-hanging fruit.”
constructs. We thank Maria Scheel and Susan Stammnes (University of Iowa Viral Vector Core) for assistance and providing the viral vectors. We thank and acknowledge Drs. Hela Azaiez, Yuzhou Zhang, Yukihide Maeda, Samuel P. Gubbel, Michael S. Hildebrand, A. Eliot Shearer, Abraham M. Sheffield, Mark A. Behlke, and Scott D. Rose for earlier work on using RNA interference to suppress hearing loss. We thank Drs. Beverly Davidson and Andrew Griffith for their valuable critiques of the paper. The current work was supported in part by NIH National Institute on Deafness and Other Communication Disorders grants RO1 DC003544 (to R.J.H.S.) and T32 DC000040-17 (to S.B.S.), a Resident Research Award from the American Academy of Otolaryngology – Head and Neck Surgery Foundation (to S.B.S.), and the Bertarelli Program in Translational Neuroscience and Neuroengineering (J.R.H.).

Received: February 3, 2016
Accepted: March 30, 2016
Published: May 26, 2016

Web Resources
OMIM, http://omim.org/
RefSeq, http://www.ncbi.nlm.nih.gov/refseq/
World Health Organization, http://www.who.int/pbd/deafness/
estimates/en/

References
1. Smith, R.J., Bale, J.F., Jr., and White, K.R. (2005). Sensorineural hearing loss in children. Lancet 365, 879–890.
2. Géleoc, G.S., and Holt, J.R. (2014). Sound strategies for hearing restoration. Science 344, 1241062.
3. Maeda, Y., Fukushima, K., Nishizaki, K., and Smith, R.J. (2005). In vitro and in vivo suppression of GJB2 expression by RNA interference. Hum. Mol. Genet. 14, 1641–1650.
4. Maeda, Y., Fukushima, K., Kawasiku, A., Nishizaki, K., and Smith, R.J. (2007). Cochlear expression of a dominant-negative GJB2R87SW construct delivered through the round window membrane in mice. Neurosci. Res. 58, 250–254.
5. Vreugde, S., Erven, A., Kris, C.J., Marcotti, W., Fuchs, H., Kurima, K., Wilcox, E.R., Friedman, T.B., Griffith, A.J., Balling, R., et al. (2002). Beethoven, a mouse model for dominant, progressive hearing loss DFNA36. Nat. Genet. 30, 257–258.
6. Boudreau, R.L., Spengler, R.M., and Davidson, B.L. (2011). Rational design of therapeutic siRNAs: minimizing off-targeting potential to improve the safety of RNAi therapy for Huntington’s disease. Mol. Ther. 19, 2169–2177.
7. Labay, V., Weichert, R.M., Makishima, T., and Griffith, A.J. (2010). Topology of transmembrane channel-like gene 1 protein. Biochemistry 49, 8592–8598.
8. Kawashima, Y., Géleoc, G.S., Kurima, K., Labay, V., Lelli, A., Asai, Y., Makishima, T., Wu, D.K., Della Santina, C.C., Holt, J.R., and Griffith, A.J. (2011). Mechanotransduction in mouse inner ear hair cells requires transmembrane channel-like genes. J. Clin. Invest. 121, 4796–4809.
9. Kurima, K., Ebrahim, S., Pan, B., Sediack, M., Sengupta, P., Millis, B.A., Cui, R., Nakanishi, H., Fujikawa, T., Kawashima, Y., et al. (2015). TMGC1 and TMGC2 Locate the Site of Mechanotransduction in Mammalian Inner Hair Cell Stereocilia. Cell Rep. 12, 1606–1617.
10. Bakhchane, A., Charoute, H., Nahilli, H., Roky, R., Rouba, H., Charif, M., Lenaers, G., and Barakat, A. (2015). A novel mutation in the TMC1 gene causes non-syndromic hearing loss in a Moroccan family. Gene 574, 28–33.
11. Hilgert, N., Monahan, K., Kurima, K., Li, C., Friedman, R.A., Griffith, A.J., and Van Camp, G. (2009). Amino acid 572 in TMC1: hot spot or critical functional residue for dominant mutations causing hearing impairment. J. Hum. Genet. 54, 188–190.
12. Kitajiri, S., Makishima, T., Friedman, T.B., and Griffith, A.J. (2007). A novel mutation at the DFNA36 hearing loss locus reveals a critical function and potential genotype-phenotype correlation for amino acid-572 of TMC1. Clin. Genet. 71, 148–152.
13. Kurima, K., Peters, L.M., Yang, Y., Riazuddin, S., Ahmed, Z.M., Naz, S., Arnaud, D., Drury, S., Mo, J., Makishima, T., et al. (2002). Dominant and recessive deafness caused by mutations of a novel gene, TMGC1, required for cochlear hair-cell function. Nat. Genet. 30, 277–284.
14. Yang, T., Kahrizi, K., Bazazzadeghan, N., Meyer, N., Najmabadi, H., and Smith, R.J. (2010). A novel mutation adjacent to the Bth mouse mutation in the TMC1 gene makes this mouse an excellent model of human deafness at the DFNA36 locus. Clin. Genet. 77, 395–398.
15. Zhao, Y., Wang, D., Zong, L., Zhao, F., Guan, L., Zhang, P., Shi, W., Lan, L., Wang, H., Li, Q., et al. (2014). A novel DFNA36 mutation in TMC1 orthologous to the Beethoven (Bth) mouse associated with autosomal dominant hearing loss in a Chinese family. PLoS ONE 9, e97064.
16. Pan, B., Géleoc, G.S., Asai, Y., Horwitz, G.C., Kurima, K., Ishikawa, K., Kawashima, Y., Griffith, A.J., and Holt, J.R. (2013). TMC1 and TMC2 are components of the mechanotransduction channel in hair cells of the mammalian inner ear. Neuron 79, 504–515.
17. Boudreau, R.L., and Davidson, B.L. (2012). Generation of hairpin-based RNAi vectors for biological and therapeutic application. Methods Enzymol. 507, 275–296.
18. McBride, J.L., Boudreau, R.L., Harper, S.Q., Staber, P.D., Mon- teys, A.M., Martins, I., Gilmore, B.L., Burstein, H., Peluso, R.W., Polisky, B., et al. (2008). Artificial miRNAs mitigate shRNA-mediated toxicity in the brain: implications for the therapeutic development of RNAi. Proc. Natl. Acad. Sci. USA 105, 5868–5873.
19. Yang, G.S., Schmidt, M., Yan, Z., Lindblom, J.D., Harding, T.C., Donahue, B.A., Engelhardt, J.F., Kotin, R., and Davidson, B.L. (2002). Virus-mediated transduction of murine retina with adeno-associated virus: effects of viral capsid and genome size. J. Virol. 76, 7651–7660.
20. Soken, H., Robinson, B.K., Goodman, S.S., Abbas, P.J., Hansen, M.R., and Kopelovich, J.C. (2013). Mouse cochleostomy: a minimally invasive dorsal approach for modeling cochlear implantation. Laryngoscope 123, E109–E115.
21. Zheng, Q.Y., Johnson, K.R., and Erway, L.C. (1999). Assessment of hearing in 80 inbred strains of mice by ABR threshold analyses. Hear. Res. 130, 94–107.
22. Shibata, S.B., Di Pasquale, G., Cortez, S.R., Chiorini, J.A., and Raphael, Y. (2009). Gene transfer using bovine adeno-associated virus with adeno-associated virus: effects of viral capsid and genome size. J. Virol. 76, 7651–7660.
23. Vibeeg, A., and Canlon, B. (2004). The guide to plotting a cochleogram. Hear. Res. 197, 1–10.
24. Liu, H., Pecka, J.L., Zhang, Q., Soukup, G.A., Beisel, K.W., and He, D.Z. (2014). Characterization of transcriptomes of cochlear inner and outer hair cells. J. Neurosci. 34, 11085–11095.
25. Picelli, S., Faridani, O.R., Björklund, A.K., Winberg, G., Sagasser, S., and Sandberg, R. (2014). Full-length RNA-seq from single cells using Smart-seq2. Nat. Protoc. 9, 171–181.

26. Noguchi, Y., Kurima, K., Makishima, T., de Angelis, M.H., Fuchs, H., Frolenkov, G., Kitamura, K., and Griffith, A.J. (2006). Multiple quantitative trait loci modify cochlear hair cell degeneration in the Beethoven (Tmc1Bth) mouse model of progressive hearing loss DFNA36. Genetics 173, 2111–2119.

27. Marcotti, W., Erven, A., Johnson, S.L., Steel, K.P., and Kros, C.J. (2006). Tmc1 is necessary for normal functional maturation and survival of inner and outer hair cells in the mouse cochlea. J. Physiol. 574, 677–698.

28. Akil, O., Seal, R.P., Burke, K., Wang, C., Alemi, A., During, M., Edwards, R.H., and Lustig, L.R. (2012). Restoration of hearing in the VGLUT3 knockout mouse using virally mediated gene therapy. Neuron 75, 283–293.

29. Askew, C., Rochat, C., Pan, B., Asai, Y., Ahmed, H., Child, E., Schneider, B.L., Aebischer, P., and Holt, J.R. (2015). Tmc gene therapy restores auditory function in deaf mice. Sci. Transl. Med. 7, 295ra108.

30. Kim, M.A., Cho, H.J., Bae, S.H., Lee, B., Oh, S.K., Kwon, T.J., Ryoo, Z.Y., Kim, H.Y., Cho, J.H., Kim, U.K., and Lee, K.Y. (2016). Methionine Sulfoxide Reductase B3-Targeted In Utero Gene Therapy Rescues Hearing Function in a Mouse Model of Congenital Sensorineural Hearing Loss. Antioxid. Redox Signal. 24, 590–602.

31. Chien, W.W., Isgrig, K., Roy, S., Belyantseva, I.A., Drummond, M.C., May, L.A., Fitzgerald, T.S., Friedman, T.B., and Cunningham, L.L. (2016). Gene Therapy Restores Hair Cell Stereocilia Morphology in Inner Ears of Deaf Whirler Mice. Mol. Ther. 24, 17–25.

32. Yu, Q., Wang, Y., Chang, Q., Wang, J., Gong, S., Li, H., and Lin, X. (2014). Virally expressed connexin26 restores gap junction function in the cochlea of conditional Gjb2 knockout mice. Gene Ther. 21, 71–80.

33. Testa, F., Maguire, A.M., Rossi, S., Pierce, E.A., Melillo, P., Marshall, K., Banfi, S., Surace, E.M., Sun, J., Acerra, C., et al. (2013). Three-year follow-up after unilateral subretinal delivery of adeno-associated virus in patients with Leber congenital Amaurosis type 2. Ophthalmology 130, 1283–1291.

34. Jackson, A.L., and Linsley, P.S. (2010). Recognizing and avoiding siRNA off-target effects for target identification and therapeutic application. Nat. Rev. Drug Discov. 9, 57–67.

35. Sloan-Heggen, C.M., Bierer, A.O., Shearer, A.E., Kolbe, D.L., Nishimura, C.J., Frees, K.L., Ephraim, S.S., Shibata, S.B., Booth, K.T., Campbell, C.A., et al. (2016). Comprehensive genetic testing in the clinical evaluation of 1119 patients with hearing loss. Hum Genet. 135, 441–450.

36. Lentz, J.J., Jodelka, E.M., Hinrich, A.J., McCaffrey, K.E., Farris, H.E., Spalitta, M.J., Bazan, N.G., Duelli, D.M., Rigo, F., and Hastings, M.L. (2013). Rescue of hearing and vestibular function by antisense oligonucleotides in a mouse model of human deafness. Nat. Med. 19, 345–350.

37. Scheffer, D.L., Shen, J., Corey, D.P., and Chen, Z.Y. (2015). Gene Expression by Mouse Inner Ear Hair Cells during Development. J. Neurosci. 35, 6366–6380.