In vitro and in vivo suppression of GJB2 expression by RNA interference

Yukihide Maeda¹, Kunihiro Fukushima², Kazunori Nishizaki² and Richard J.H. Smith¹,*

¹Molecular Otolaryngology Research Laboratory, Department of Otolaryngology—Head and Neck Surgery, Interdepartmental Ph.D. Genetics Program, The University of Iowa, 200 Hawkins Drive—21151 PFP, Iowa City, IA 52242, USA and ²Department of Otolaryngology—Head and Neck Surgery, Okayama University Graduate School of Medicine, Okayama, Okayama 700-8558, Japan

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Mutations in GJB2 (gap junction protein, beta-2) are the major cause of autosomal recessive non-syndromic hearing loss. A few allele variants of this gene also cause autosomal dominant non-syndromic hearing loss as a dominant-negative consequence of expression of the mutant protein. Allele-specific gene suppression by RNA interference (RNAi) is a potentially attractive strategy to prevent hearing loss caused by this mechanism. In this proof-of-principle study, we identified a potent GJB2-targeting short interfering RNA (siRNA) to post-transcriptionally silence the expression of the R75W allele variant of GJB2 in cultured mammalian cells. In a mouse model, this siRNA duplex selectively suppressed GJB2 R75W expression by >70% of control levels, thereby preventing hearing loss. The level of endogenous murine Gjb2 expression was not affected. Our data show that RNAi can be used with specificity and efficiency in vivo to protect against hearing loss caused as a dominant-negative consequence of mutant gene expression.

INTRODUCTION

GJB2 (Gap junction protein, beta2) is a gene of particular relevance in the field of auditory science. Its single coding exon (exon 2) encodes a 26 kD isoform of the transmembrane protein family of connexins called Connexin 26 (CX26). Connexins oligomerize to form hexameric units called connexons, the constituent components of gap junctions (1). In the murine cochlea, Gjb2 gap junctions are hypothesized to facilitate potassium recycling in the inner ear. After entry into hair cells during mechanosensory transduction, potassium is released by basolaterally expressed KCNQ4 potassium channels (2,3) and taken up by adjacent supporting cells (4). These cells are interconnected by Gjb2 gap junctions to form the epithelial gap junction pathway, which shunts potassium to the lower part of the spiral ligament. Potassium then passes through a connective tissue pathway of fibrocytes functionally linked by Gjb2 gap junctions to the stria vascularis and ultimately into the scala media (4).

GJB2 allele variants are found in 50% of the people with congenital severe-to-profound autosomal recessive non-syndromic hearing loss, making mutations in this gene the most common cause of recessive genetic deafness in different populations (5–8). Three particularly common deletions in the open reading frame of GJB2—35delG, 167delT and 235delC—lead to premature protein termination in northern European and Mediterranean (6–8), Ashkenazi Jewish (9) and Japanese (10,11) populations, with mutation-specific carrier frequencies ranging from 1 to 4% (12). A few mutant alleles of GJB2 also cause autosomal dominant non-syndromic hearing loss (ADNSHL) through a dominant-negative effect (13,14). The incidence of these allele variants is low and the associated deafness is commonly syndromic, as it segregates with various types of skin disorders. Among the dominant mutations, which include W44C (13,15), R75W (15,16) and D66H (15), R75W is a well characterized cause of deafness and palmoplantar keratoderma (16). R75W connexons inhibit intercellular coupling of paired Xenopus oocytes co-injected with wild-type GJB2 or GJA1 (15) and the dye-transfer capacity of GJB6-heteromeric connexons in HeLa cells (17).

RNA interference (RNAi) is a post-transcriptional process in which double-stranded RNA (dsRNA) triggers sequence-specific suppression of homologous genes. The first evidence that dsRNA leads to post-transcriptional gene silencing in animals came from work on Caenorhabditis elegans (18).

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As reconstructed in cell extract experiments in *Drosophila melanogaster* (19,20) and *Homo sapiens* (21,22), dsRNA is digested into 21–23 nucleotide (nt) fragments of small interfering RNA (siRNA) by Dicer, a member of the RNase III family of ATP-dependent, dsRNA-specific ribonucleases (23).

These siRNA duplexes bind to a nuclease complex to form the RNA-induced silencing complex (RISC), with the antisense strand serving as a cognate template for specific transcript recognition (24,25). RISC catalyzes cleavage of the RNA-induced silencing complex (RISC), with the antisense strand serving as a cognate template for specific transcript recognition (24,25). RISC catalyzes cleavage of specific mRNAs, which is followed by rapid degradation by cellular exonuclease activity. Longer dsRNAs (>50 bp) have a more widespread effect in mammalian somatic cells, inducing general arrest of protein synthesis through interferon response and protein kinase activation. Shorter siRNAs of 21–23 nt, in contrast, have a more targeted effect, inducing up to 90% depletion of specific mRNAs both *in vitro* and *in vivo* (26–28).

High suppression potency and sequence specificity of even single nucleotide resolution (29) have encouraged the development of RNAi-based therapeutic models for possible use in viral infections [i.e. HIV-1 (30), HBV (31), HCV (27)], respiratory viruses (32,33)] and cancer [i.e. K-ras (34), PI 3-kinase (35)] (36). This approach is also particularly attractive for dominant genetic diseases in which mutant proteins cause an inhibitory or toxic effect, as in spinocerebellar ataxia types 1 (37) and 3 (38), spinobulbar muscular atrophy (39) and slow channel congenital myasthenic syndrome (40).

In the present study, we transfected cochleae of adult mice with a plasmid vector expressing GJB2R75W-eGFP (41). As this allele variant of GJB2 has been shown to cause hearing loss by a dominant-negative effect *in vivo* in mice (42), we hypothesized that it may be possible to decrease expression of the mutant allele and modify the deafness phenotype by RNAi.

**RESULTS**

*In vitro* suppression of GJB2 expression

We conducted initial RNAi experiments measuring endogenous expression of GJB2 or Gjb2 in human HEK293 and mouse P19 cells, respectively, to identify potent duplexes that selectively suppress human GJB2 expression. Four siRNA-targeting sites of sequence type AA (N19) (N, any nucleotide) were selected from the coding region of human GJB2 mRNA (siRNA 1–4) according to published guidelines (29,43) (Fig. 1). siRNA2m was designed as a mouse Gjb2-targeting siRNA homologous to siRNA2.

Synthetic siRNAs were introduced into cultured cells using a polyanine transfection reagent (siPORTAmine, Ambion, Inc.). Cy3-labeled siRNA1 (Fig. 2A) and biotin-labeled siRNA2m (Fig. 3A) were clearly detected in the cytoplasm of transfected cells, with transfection efficiencies of 90.3 ± 3.1% (HEK293 cells, mean ± SD) and 78.6 ± 3.6% (P19 cells, mean ± SD). Reverse transcription polymerase chain reaction (RT–PCR) analysis showed that siRNA1 specifically suppressed GJB2 mRNA in HEK293 cells, as assessed by comparison with scrambled-sequence siRNAs or transfection reagent only (Fig. 2B–E). A GAPDH-targeting siRNA that successfully induced GAPDH mRNA suppression did not affect GJB2 expression, and siRNA1 exerted no remarkable effect on GAPDH expression (Fig. 2E).

To quantitate GJB2 suppression by GJB2-targeting siRNAs, RT–PCR of endogenous GJB2 expression was quantified by densitometry and expressed as a band intensity value. Logarithm of the band intensity value of GJB2 expression correlated linearly with a number of amplification cycles in the exponential ranges of 28–34 (r = 0.961, P < 0.01; n = 7), using 100 ng total RNA per PCR. When 12.5, 25, 50, 75, 100, 150 or 200 ng of RNA was used in RT per each PCR with GJB2 primers and 30 thermocycles, band intensity values correlated with the amount of RNA added per PCR (r = 0.808; P < 0.001; n = 6, for each RNA amount). Therefore, the band intensity values of GJB2 were determined in each sample following 30 thermocycles using 100 ng of total RNA per PCR. Levels of gene transcription were compared with GJB2 expression in control samples prepared with negative control siRNAs and expressed as percentage relative abundance of mRNA.

GJB2 expression was suppressed by >80% of control levels by siRNA2 (15.9 ± 11.5%, mean ± SD; n = 7; P < 0.01) and by ~50% of control levels by siRNA1 (48.2 ± 11.8%; n = 7; P < 0.01) and siRNA3 (59.1 ± 18.1%; n = 7; P < 0.01) at 72 h post-transfection; siRNA4 was ineffective at the same time point (69.4 ± 30.1%; n = 7; P = 0.116) (Fig. 2F). Suppression of GJB2 mRNA by siRNA2 was demonstrated at 48 h (19.7 ± 12.3%; n = 6; P < 0.05), 72 h (15.9 ± 11.1%; n = 6; P < 0.001) and 120 h (53.6 ± 14.4%; n = 6; P < 0.05) post-transfection but reversed to control levels by 168 h (125.2 ± 32.3%; n = 6; P = 0.337) post-transfection in HEK293 cells (Fig. 2G). At 72 h post-transfection (maximum GJB2 suppression), siRNA2 induced no significant change in the expression of GJB3 (73.9 ± 28.5%; n = 7; P = 0.116) or GJA1 (79.9 ± 28.5%; n = 16; P = 0.228) (Fig. 2H). GJB2 is not expressed constitutively in HEK293 cells and remained undetectable in the presence of siRNA2 reduction.

In accordance with the suppression of human GJB2 by siRNA2, siRNA2m induced significant reduction in endogenous Gjb2 expression in mouse P19 cells as compared to negative control siRNAs (37.3 ± 31.5%, n = 7 versus 100.0 ± 32.8%, n = 7; P < 0.05). siRNA4, with complete sequence identity between human and mouse, exerted no significant effect on Gjb2 expression levels (69.9 ± 24.8%, n = 7; P = 0.142) (Fig. 3E).

As a prerequisite step to studying the effect of RNAi *in vivo*, a CMV-driven mammalian expression vector conferring human GJB2R75W-eGFP (pGJB2R75W-eGFP) was introduced in mouse P19 cells using a cationic liposome reagent (LipofectamineTM2000, Invitrogen, Corp.) (Fig. 3B). pGJB2R75W-eGFP was derived originally from the expression plasmid pEGFP-N1 (Clontech, BD Biosciences, Inc.) (41). Co-transfection with siRNA2 or negative control siRNA confirmed efficient and specific silencing of GJB2R75W-eGFP expression (Fig. 3C and D). siRNA2 reduced transgene mRNA to <20% of levels seen using negative control siRNAs, (11.0 ± 4.3%, n = 7 versus 100.0 ± 24.8%, n = 7; P < 0.01) (data not shown), abolishing GJB2R75W-eGFP fluorescence (Fig. 3D). Endogenous Gjb2 mRNA was co-suppressed by siRNA2 but remained at ~60% of control
levels in cell cultures (60.3 ± 14.7%, n = 7; P < 0.05) (data not shown).

**In vivo GJB2<sub>R75W</sub>-eGFP transgene expression**

In vivo transfection of the mutant GJB2<sub>R75W</sub>-eGFP into adult mouse cochleae was achieved by complexing pGJB2<sub>R75W</sub>-eGFP (25 μg/100 μl of lipocomplex solution) with 4 mM DOTAP/cholesterol cationic liposome (44), following the manufacturer’s protocol (In vivo GeneSHUTTLE<sup>TM</sup>, Qbiogene, Inc.), and applying 1–2 μl of the lipocomplex to a small piece of gel foam, which was placed against the intact round window membrane (RWM). Exposure to the RWM was obtained through an anterior cervical approach to the middle ear (45), a relatively atraumatic procedure that permits post-operative assessment of hearing (46).

RT–PCR demonstrated GJB2<sub>R75W</sub>-eGFP mRNA expression in the operated ear 72 h after surgery (Fig. 4A). Transgene expression was not detectable in the contralateral ear or in control ears with vehicle-only application (Fig. 4A). Immunohistochemistry using an anti-eGFP monoclonal antibody (BD Biosciences, Inc.) showed pronounced expression in the endothelial cells of the basilar membrane, inner and outer pillar cells, outer hair cells and in the spiral limbus and spiral ligament (Fig. 4B–E).

Click-ABR (auditory brainstem responses) thresholds in the operated and contralateral ears were determined at the same time point using incremental steps of 5 dB from 0–100 sound pressure level (SPL) in a sound-proof booth as described previously (47). Hearing impairment was measured as a change in the intraaural differences of ABR thresholds for each mouse. pGJB2<sub>R75W</sub>-eGFP (25 μg/100 μl) induced a significant increase in ABR thresholds when compared with lipocomplexes with empty vector (25 μg/100 μl) (20.0 ± 10.4 dB, n = 8 versus 8.8 ± 5.8 dB, n = 8; P < 0.05) or vehicle-only application (5.6 ± 3.2 dB, n = 8; P < 0.01). There was no significant difference in hearing between empty vector and vehicle-only control animals (P = 0.194).

**Suppression of GJB2<sub>R75W</sub>-eGFP expression and its effect on phenotype**

In vivo RNAi was assessed in four study groups using liposome complexes composed of (1) 25 μg pGJB2<sub>R75W</sub>-eGFP; (2) 25 μg pGJB2<sub>R75W</sub>-eGFP and 25 μg empty vector; (3) 25 μg pGJB2<sub>R75W</sub>-eGFP and 25 μg negative control siRNAs and, (4) 25 μg pGJB2<sub>R75W</sub>-eGFP and 25 μg GJB2-targeting siRNA2, all in 100 μl of DOTAP/cholesterol lipocomplex. Semiquantitative RT–PCR analysis was performed with specific primers for human or mouse GJB2/Gjb2 (primer pairs c and d in Fig. 1). Typically, 10–20 ng RNA was PCR amplified in the exponential phase, measuring Gapdh levels as an internal control and expressing mRNA levels as percentage relative abundance. Gjb2 amplification correlated with the amount of RNA added per PCR (range of RNA 7–56 ng; r = 0.944; n = 36; P < 0.0001).

Among groups 1, 2 and 3, in cochlear tissues dissected at 72 h post-treatment, levels of GJB2<sub>R75W</sub>-eGFP mRNA in group 1 (138.2 ± 28.7%, n = 6) were higher than in groups 2 (85.2 ± 29.6%, n = 6; P < 0.05) and 3 (100.0 ± 17.7%,
Figure 2. GJB2 silencing in HEK293 cells. (A) Red indicates Cy3-GJB2siRNA1; green indicates cytoplasm (CellTracker™ Molecular Probes, Inc.) and blue indicates nuclei (DAPI). Scale bar, 5 μm. (B) Vehicle, (transfection reagent) only control. Scale bars in (B), (C) and (D), 20 μm. (C) Cy3-siRNA1 and (D) Cy3-negative control siRNA transfected HEK293 cells. Transfection efficiency was 90.3 ± 3.1%. (E) siRNA1 specifically suppressed endogenous GJB2 expression, as assessed by RT–PCR in the exponential phase. (F) Semiquantitative RT–PCR in HEK293 cells shows suppression of GJB2 expression by >80% of control levels with siRNA2 and by ~50% of control levels with siRNA1 and siRNA3; siRNA4 was ineffective (72 h post-transfection). PCR signals were quantified with 100 ng total RNA and thermocycles in exponential phase. GJB2 signals correlated with amount of total RNA added (range of RNA, 12.5–200 ng; r = 0.808; P < 0.001; n = 36). (G) GJB2 silencing by siRNA2 was maximum at 72 h post-transfection and returned to control level within 168 h. (H) siRNA2 did not affect expression levels of GJB3 and GJA1.
n = 6; \( P < 0.05 \) (Fig. 5A), although differences between groups 2 and 3 were not significantly different (\( P = 0.423 \)). We assume that the total amount of nucleic acid in group 1 affected the formulation of the lipocomplex (44), resulting in a difference in in vivo transfection.

In group 4, siRNA2 significantly reduced \( GJB2_{R75W} \)-eGFP mRNA (27.6 ± 2.6%, \( n = 6 \)) to <30% of levels in control group 3 (\( P < 0.01 \)) (Fig. 5A and C) but did not affect levels of endogenous \( Gjb2 \) mRNA (116.1 ± 15.6%, \( n = 6 \)) as compared to either group 2 (113.4 ± 34.9%, \( n = 6 \)) or group 3 (100 ± 21.0%, \( n = 6 \); \( P = 0.523 \)) (Fig. 5B and C). The hearing loss seen with \( GJB2_{R75W} \)-eGFP expression was prevented by siRNA—intraaural differences in ABR thresholds in group 4 (14.5 ± 11.2 dB, \( n = 10 \)) were significantly lower than in group 3 (23.0 ± 8.6 dB, \( n = 10 \); \( P < 0.05 \)) and were not statistically different from the control animals with empty vector only (\( P = 0.287 \)) (Fig. 6).

**DISCUSSION**

We have shown that siRNAs can be used to suppress human \( GJB2 \) expression in vitro and in vivo. The most potent siRNA we tested selectively suppressed by \( >70\% \) mutant \( GJB2_{R75W} \)-eGFP expression in murine cochleae without significantly decreasing expression of endogenous \( Gjb2 \). Suppression of \( GJB2_{R75W} \)-eGFP expression by siRNA2 prevented hearing loss otherwise associated with the \( R75W \) dominant-negative allele variant of CX26.

The major post-transcriptional mechanism of eukaryotic RNAi involves incorporation of siRNAs into RISC. The sequence-specific endonuclease activity of RISC can be reconstituted in cytosolic extracts of HeLa cells (25). We have shown that in mammalian cultured cells, only some chemically synthesized siRNAs localize in the cytoplasm and specifically deplete \( GJB2 \) mRNAs. siRNA2 was identified as the most potent of the four \( GJB2 \)-targeting siRNAs we tested in this study: siRNA2 depleted expression of both endogenous \( GJB2 \) and plasmid-delivered \( GJB2_{R75W} \)-eGFP to \(<20\% \) of control levels in cultured cells. \( GJB2_{R75W} \)-eGFP fluorescence was abolished by siRNA2.

The 21 nt sequence of siRNA2 shares a 12 nt match in maximum alignment with \( GJB3 \) and \( GJA1 \) mRNA, the two other connexin isoforms expressed in HEK293 cells, but expression levels of these connexins did not change. siRNA2 also has sequence mismatches against mouse \( Gjb2 \) mRNA at the 9th and 15th nucleotide positions in its 21 nt target sequence. These 2 nt mismatches were sufficient to distinguish human \( GJB2_{R75W} \)-eGFP and endogenous murine \( Gjb2 \) alleles in vivo, with selective depletion of \( GJB2_{R75W} \)-eGFP mRNA to \(<30\% \) of control levels, while maintaining endogenous \( Gjb2 \) expression.

\( GJB2_{R75W} \)-eGFP inhibits the function of wild-type \( Gjb2 \) and permeability of gap junction hemichannels in vitro (16) and causes hearing loss in vivo (42). siRNA2 was potent enough to modify the phenotype caused by the expression of \( GJB2_{R75W} \)-eGFP, decreasing the expected ABR threshold shift. This observation raises the possibility of allele-specific RNAi as a therapeutic option to mitigate some types of sensorineural deafness caused by dominant-negative mechanisms. Other hereditary deafness of this type may include ADNSHL (hereditary hearing loss homepage, http://webhost.ua.ac.be/hhh).

As a potential therapeutic option for this type of deafness, RNAi must be potent enough to prevent the expression of the mutant allele but permissive enough to preserve expression of the wild-type transcript at levels sufficient to permit normal inner ear function. We believe that it should be possible to rescue a deafness phenotype by means of allele-specific RNAi targeting mutant \( GJB2 \) alleles. Haploinsufficiency of
GJB2 does not cause hearing loss in human; in mouse mutants heterozygous for a targeted deletion of Gjb2 (Gjb2 +/−) hearing loss does not develop despite wild-type Gjb2 mRNA level that are 50% of normal (46.5 ± 20.0%, n = 6 versus 100.0 ± 17.6%, n = 6; data not shown). It would be beneficial to know the level of Gjb2 expression necessary to preserve normal hearing level, a question that can be addressed by expressing Gjb2 in vivo. siRNA preparation

Target sequences of siRNAs 1, 2, 3 and 4, respectively, correspond to c.c. 184–204, 313–333, 562–582 and 120–140 of mouse Gjb2. siRNA2m and siRNA4m correspond to c.c. 313–333 and 120–140 of mouse Gjb2. As siRNA4m and siRNA4m are identical, only the designation siRNA4 is used. Specificity was tested using the BLAST search engine (www.ncbi.nlm.gov/BLAST), and excluding orthologous identity, no siRNA had matches of >13 nt to known human or murine mRNAs or expressed sequence tags. siRNA-targeting GAPDH and scrambled siRNA without significant homology to known human and murine genes (negative control siRNAs) were obtained from Ambion, Inc. siRNA with the same nucleotide composition as siRNA2 but without significant homology to known genes was designed as a negative control for experiments with siRNA2 (siRNA Scrambler, https://www.genscript.com/ssl-bin/app/scramble, GenScript Corp.).

RNAs were chemically synthesized and PAGE purified by Ambion, Inc. 2'-deoxythymidine residues were used as the 3' overhangs in the both sense and antisense strands so that 19 nt RNA duplexes with symmetric 2 nt overhangs were formed. An aliquot of 20 μM sense and antisense RNAs was heated at 90°C in 50 μl of annealing buffer (Ambion, Inc) for 1 min and annealed at 37°C for 60 min; aliquots were stored at −20°C. Fluorescence-labeling of siRNA1 and negative control siRNA was performed using a Cy3-siRNA labeling kit (Ambion, Inc). The 3' ends of both strands of siRNA2m were bionylinated and PAGE purified by.
Dharmacon, Inc. Annealing and labeling of siRNAs were verified by 20% PAGE.

Cell culture and transfection
Human HEK293 cells and mouse P19 cells were maintained, respectively, in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco Cell Culture Systems, Invitrogen Corp.) with 10% fetal bovine serum (FBS) (HyClone Corp.) and α-Minimum Essential Medium (Gibco Cell Culture Systems, Invitrogen Corp.) with 7.5% bovine calf serum (HyClone Corp.) and 2.5% FBS, in a humidified incubator at 37°C and 5% CO2 and regularly passaged at sub-confluence. Twenty-four hours before transfection, cultured cells were trypsinized, diluted in fresh medium without antibiotics (HEK293 cells, 5.0 × 10^6 cells ml^-1; P19 cells, 4.0 × 10^5 cells ml^-1) and transferred to 12-well plates (400 µl per well). Cell confluency was 50–70% at the time of transfection.

siRNA transfection of HEK293 cells was carried out with siPORT amine transfection reagent (Ambion, Inc) using 100 nM siRNA duplex (1.33 µg/ml) in a final volume of 500 µl per well. Growth media (2.5 ml/well) was added 12 h later and cells were harvested 72 h post-transfection. For P19 cells, transfection was carried out in opti-MEM1 reduced serum medium (Gibco Cell Culture Systems, Invitrogen Corp.), with siPORT amine using 200 nM siRNA (2.66 µg/ml); growth media were added 12 h later and cells were harvested at 42 h post-transfection. pGJB2R75W-eGFP was provided by Prof. David Kelsell (Centre for Cutaneous Research, London) (41). pGJB2R75W-eGFP (1.3 µg/ml) and 200 µM GJB2-targeting siRNA (GJB2siRNA2) or 200 µM negative control siRNA were introduced to mouse P19 cells using Lipofectamine 2000 (Invitrogen, Corp.). Co-transfection was performed at a cell confluency of 1.5 × 10^5 cells ml^-1 and final transfection volume of 1.2 ml.

RNA extraction and reverse transcription
Cultured cells were lysed in a buffer containing β-mercaptoethanol, processed for RNA extraction using an RNAeasy column (Qiagen, Inc.) and treated with DNasel (Qiagen, Inc.). Amounts of RNA were quantitated by measuring absorbency at 260/280 nm; usually 3.5–5 and 2–3.5 µg of total RNA were purified from each well of HEK293 and P19 cells, respectively. Typically, 500 ng of total RNA (100 ng per RT–PCR) was subjected to reverse transcription in a 20 µl reaction mixture containing reaction buffer, 0.5 mM of each dNTP, 1 mM oligo dT primers, 20 units RNase inhibitor and 200 units MMLV reverse transcriptase, according to the manufacturer (Advantage RT for PCR kit, Clontech, Inc.). An aliquot of 4 µl of the RT mixture was added to a 50 µl PCR mixture.

For RNA purification from mouse cochleae, the animals were euthanized with CO2, blood was removed by transcardiac perfusion of phosphate buffered saline (PBS), and cochlear tissue was dissected and frozen in liquid nitrogen. The tissue was homogenized in the same buffer and subjected to total RNA extraction and DNaseI treatment. An aliquot of 240–480 ng of total RNA was usually obtained from six cochleae, 100–200 ng RNA (10–20 ng per RT–PCR) was used for RT in a 20 µl reaction mixture and 2 µl of RT mixture was added per PCR.

PCR and data analysis
Primer pairs for GJB2 (Fig. 1: primer pair a, c.c. −37 to −19, sense; c.c. 147–166, antisense), GJA1 (c.c. −24 to −5; c.c. 165–185), GJB3 (c.c. 104–123; c.c. 392–411) and GJB6 (c.c. −30 to −11; 163–183), respectively, amplified 203, 209, 308 and 213 bp fragments of human connexin cDNAs; human GAPDH primers (c.c. −21 to −3; c.c. 159–178) amplified a 199 bp fragment. Primers for mouse Gjb2 (Fig. 1: primer pair b, c.c. −36 to −17; c.c. 371–391) and Gapdh (c.c. 650–669; c.c. 976–995) amplified 427 and 346 bp fragments.
of murine cDNAs. Specific primers for human GJB2 (Fig. 1: primer pair c, c.c.149–168; c.c. 357–376) and mouse Gjb2 (Fig. 1: primer pair d, c.c.149–168; c.c. 357–376) were designed utilizing inter-species difference at the 3’ ends of both primers and used to amplify 228 bp products from cochlear tissue of mice.

Each PCR reaction mixture (50 μl) contained the reaction buffer, 1.5 mM MgCl₂, 0.2 mM of each dNTP, 0.5 μM each of the appropriate primers, 2.5 U DNA polymerase (Bioline USA Inc., Randolph, MA, USA) and RT reaction mix. Nineteen to thirty-seven cycles of PCR amplification of human cDNA were completed at 94°C for 30 s, 60°C for 30 s and 72°C for 30 s in a GeneMate Genius PCR machine (ISC BioExpress, Inc.). For amplification of cDNA from cochlear tissue, PCR was performed at 95°C for 9 min followed by 27–45 cycles at 94°C for 30 s and 60°C for 30 s in a reaction mix that contained the previously listed ingredients, substituting AmpliTaq Gold DNA polymerase (Applied Biosystems, Inc.) for DNA polymerase.

For semiquantitative RT–PCR, EtBr-stained PCR products were resolved on 3% agarose gels, imaged using a CCD camera (RT-Slider, Diagnostic Instruments, Inc.) and quantified by densitometry using GelExpert software (NucleoVision, Nuleotech, Inc.). After subtraction of background values as determined in an adjacent band-free area, the signal intensity was expressed as band intensity values. Differences between groups were examined non-parametrically using the Kruskal–Wallis test and Mann–Whitney U-test. Statistical significance was assigned to P-values of <0.05.

Fluorescence microscopy

HEK293 cells were grown on coverslips in 12-well plates. Transfection with the Cy3-labeled siRNA was performed under the same conditions as described for siRNA transfection. After incubation for 72 h, cells were washed in DMEM without serum and incubated in 5% CO₂ with 5 μM chloromethylfluorescein diacetate in DMEM for 45 min at 37°C, and then maintained in DMEM with 10% FBS for 30 min at 37°C, in order to allow the cytosolic esterase and glutathione transferase to transform the chloromethylfluorescein to a cell impermeant–fluorescent dye to visualize the cytoplasm of living cells (Cell Tracker™, Molecular Probes, Inc.). After washing in PBS, cells were fixed with 4% formaldehyde in PBS for 15 min. Coverslips were washed in PBS, mounted in Vectashield medium with DAPI (Vector Laboratories, Inc.) and examined by fluorescence microscopy (BX-51, Olympus, Inc.). Data were recorded with a CCD camera (RT-Slider, Diagnostic Instruments, Inc.) and processed by image analysis software (SPOT software, Diagnostic Instruments; Photoshop, Adobe Systems, Inc.).

Transfection efficiency was determined in HEK293 and P19 cells 12 h after transfection by counting the number of cells with Cy3-siRNA signal and the total number of cells in the microscopic field; this process was repeated three times. Biotin-labeled siRNA2m was tested for transfection and localization in P19 cells. Cells transfected by biotin-siRNA2m were washed in PBS, fixed with 4% formaldehyde for 7 min, incubated with a peroxidase block reagent (DAKO) for 5 min and permeabilized by 0.1% TritonX-100 in PBS for 7 min. The biotinylated siRNA was visualized by the ABC–DAB method (Vectastain ABC kit, Vector Laboratories, Inc.).

Animal model

Female C57Bl6 mice (post-natal day 42–45) were anesthetized with intraperitoneal ketamine (100 mg/kg) and xylazine (9 mg/kg). Procedures were performed on the right ear via a ventral, paramedian incision in the neck. As described by Jero et al. (45,46), a small opening in tympanic bulla was made to provide direct visualization of the RWM. One to five microliter volume of the plasmid and/or siRNA-liposome complex was placed in the round window niche using a microsyringe equipped with a fine polyimide cannula (I.D. = 0.12 mm, O.D. = 0.16 mm, MicroLumen, Tampa, FL, USA) (51). Excess solution was gently aspirated and a small piece of Gelfoam soaked with the lipocomplex was placed in direct contact with the RWM. For transgene detection by RT–PCR and immunohistochemistry, the round window niche was entirely covered with the gelfoam. Post-operatively, the incision was sutured and chloramphenicol (30 mg/kg) was administered intramuscularly.
ABR testing

Animals were anesthetized using the same anesthetics and click sounds were unilaterally presented via a microphone inserted into the external auditory canal. Response thresholds were determined by decreasing the stimulus level by 5 dB decrements from 0 to 100 SPL. The threshold response in the right ear was determined relative to the response in the left ear, to minimize variation between mice. Differences between experimental groups were compared with the Kruskal–Wallis test and Mann–Whitney U-test. Significance was assigned to P-values of <0.05.

Immunohistochemistry

Animals were intracardially perfused with PBS and then 4% formaldehyde. Cochleae were dissected and after making a hole at the apex, immersion fixed at 4°C for 12 h. They were decalcified in 0.2 M EDTA for 48 h, dehydrated through graded alcohol and xylene and embedded in paraffin. Paraffin sections of 6 μM thickness were dewaxed and incubated with a mouse monoclonal antibody against eGFP (1:200 dilution; BD Biosciences, Inc.) at 4°C overnight. After washing, bound antibody was reacted with Alexa 488-antimouse IgG antibody (Molecular Probes, Inc.) for 4°C for 4 h. Specimens were mounted in Vectashield medium with DAPI (Vector Laboratories, Inc.) and examined by fluorescence microscopy.

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