Biochemical and Biophysical Research Communications 374 (2008) 17–21

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Ectopic expression of *tmie* transgene induces various recovery levels of behavior and hearing ability in the circling mouse

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ARTICLE INFO

Article history:
Received 17 June 2008
Available online 27 June 2008

Keywords:
tmie
Circling mouse
Organ of Corti
Hearing loss
Transgenic animal

ABSTRACT

The circling (*cir*/*cir*) mouse is one of the murine models for human non-syndromic deafness DFNB6. The mice have abnormal circling behavior, suggesting a balanced disorder and profound deafness. The causative gene was *transmembrane inner ear* (*tmie*) gene of which the mutation is a 40-kb genomic deletion including *tmie* gene itself. In this study, *tmie*-overexpression trasngenic mice were established. Individuals with germline transmission have been mated with circling homozygous mutant mice (*cir*/*cir*) in order to produce the transgenic mutant mice (*cir*/*cir*-tg) as a gene therapy. After the genotyping, phenotypic analyses were performed so that the insertion of the new gene might compensate for the diseases such as hearing loss, circling behavior, or swimming inability. Some individuals exhibited complete recovery in their behavior and hearing but the others did not show any amelioration in behavior or hearing. Individual mice had very different levels of *tmie* transgene expression in the cochlea. These results clearly indicate that *tmie* protein plays an important role when the appropriate expression level of *tmie* was expressed in the inner ear. The protein levels were variable in each individual and these are thought to induce the differences in disease amelioration levels.

Sensorineural hearing loss (SNHL) comprises hearing disorders with diverse pathologies of the inner ear and auditory nerve. Commonly the congenital hearing loss is caused by mutations in one or more genes which are related to the normal development of the inner ear tissues. At present, at least 30 syndromic and over 120 non-syndromic loci have been discovered that are implicated in causing hereditary hearing loss [1]. The primary cause of hearing loss is damage or death of the sensory receptor hair cells in the inner ear. Current research efforts are focused on gene manipulation, gene therapy for repairing or replacing damaged mammalian cochlear hair cells, which could lead to therapies for treating deafness in humans. Gene therapy may provide a way to restore cochlear function to deaf patients. Gene therapy to grow new auditory hair cells was used in adult guinea pigs [2,3]. This was achieved by inserting Math1 gene into cells lining the inner ear. Non-sensory epithelial cells in adult guinea pig cochlea could generate new sensory hair cells following the expression of Math1. When Math1 was overexpressesd in the non-sensory cells of the mature cochlea, it caused them to transdifferentiate into hair cells.

The circling (*cir*) mouse is a spontaneous mutant in the inner ear from ICR out-bred strain [4,5]. The circling mouse becomes hyperactive at about 7 postnatal days, and then shows a circling behavior. The most notable pathological phenotypes are the almost completely degenerated cochlea, and the remarkably reduced cellularity in the spiral limbus. An auditory test demonstrated hearing loss of the *cir*/*cir* mouse [5]. The genetic mapping demonstrated that the *cir* gene was mapped to 60.1 cM on mouse Chr 9. The distal portion of mouse Chr 9 encompassing the *cir* region is homologous with human chromosome 3p21, which contains DFNB6 locus [6]. And through the deletion analysis by genomic PCR of the candidate causative genes, the *cir*/*cir* mouse has a 40-kb genomic deletion that includes the transmembrane inner ear (*tmie*) gene and mRn49018 [7]. The spinner (*sr*) mice have similar phenotypes as the circling mice. The mutant mouse shows behavioral dysfunction including bidirectional circling and head-shaking [8]. The auditory function in *sr*/*sr* mice was found to be reduced, based upon the lack of a startle reflex to sound at any age [9]. The *sr*/*sr* mouse was identified to have two types of mutations: a 40-kb genomic deletion including 4 genes (*K007173, Tsp50, tmie, and mRn49018*) and a nonsense mutation expected to truncate the C-terminal end of its

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doi:10.1016/j.bbrc.2008.06.064

product. The *cir*/*cir* and *sr*/*sr* mice have a common deleted region, including *tmie* gene [7]. Therefore, *tmie* is identified as the causative gene of two mutants. However, the functional roles of *tmie* in the cochlea remain unclear. The amount, distribution, and time course of *tmie* protein have not been defined. Only the expression patterns in the adult mouse and rat were analyzed [10,11].

In this study, *tmie*-overexpression trasngenic mice were established. Individuals with germline transmission have been mated with circling homozygous mutant mice in order to produce the transgenic mutant mice as a gene therapy. After the genotyping, phenotypic analyses were performed so that the insertion of the new gene might compensate for the diseases such as hearing loss, circling behavior, or swimming inability. Some individuals exhibited complete recovery in their behavior and hearing but the others did not show any amelioration in behavior or hearing. Therefore the behavioral differences and transgenic *tmie* expression patterns in *cir*/*cir* mice with transgene have been assessed.

Materials and methods

Animals. The circling (*cir*/*cir*) mice are the spontaneous mutants in ICR out-bred strain [4] and have been maintained in the *cir*/*cir*-C57BL/6. In order to induce the systemic expression of mouse *tmie*, human cytomegalovirus (CMV) immediate-early promoter was used in the transgene construction and the transgenic mice were produced by microinjection (Fig. 1A). Hemagglutinin (HA) epitope tag was added to the end of *tmie* open reading frame (ORF) removing *tmie* stop codon. The transgenic mice were confirmed by PCR of the region from 3′ end of hCMV to 3′ end of *tmie* ORF. After screening of transgenic founder mice, we mated one founder mouse to *cir*/*cir* mice. Then the F1 +/*cir* progeny carrying transgene were mated to *cir*/*cir* mice for the production of N2 transgenic *cir*/*cir* mice (Fig. 1B). +/+ or +/*cir* heterozygous mice were used as wild type mice.

Clinical observations. The circling behavior was observed using an open-field apparatus (75 × 75 × 30 cm), where vertical and horizontal lines were drawn every 15 cm. The circling counts were defined as the number of times the mouse ran in circles in the 5 min after being placed in an open field. Swimming test was done by placing mice into the water to observe their swimming behavior.

Auditory brainstem response tests. Mice were anesthetized with xylazine (4 mg/kg) and ketamine (40 mg/kg) by i.m. prior to measurement. The animals were placed in a sound-isolated, electrically shielded booth. Needle electrodes (Grass E2 platinum) were subcutaneously placed below the tested ear (reference electrode), in the vertex (active electrode), and below the contralateral ear (ground electrode). The sound stimulus consisted of 15/ms tone bursts (rise-fall time 1 ms) at 4, 8, 16, and 32 kHz and were generated by Tucker-Davis hardware. The sound stimuli were delivered into the ear canal from an encased, shielded Beyer earphone through a 13 mm tube. Response waveforms (1,000,000 gain, filtered from 0.3–3.0 kHz) were averaged (1024 epochs) using a Tucker-Davis data acquisition system. The response threshold was defined as the interpolated value between the last level at which no response was observed. The sound delivery system was calibrated with a 1/4 in. ACO Pacific condenser microphone (Belmont, USA) in a volume approximating the mouse external ear canal and expressed as dB SPL.

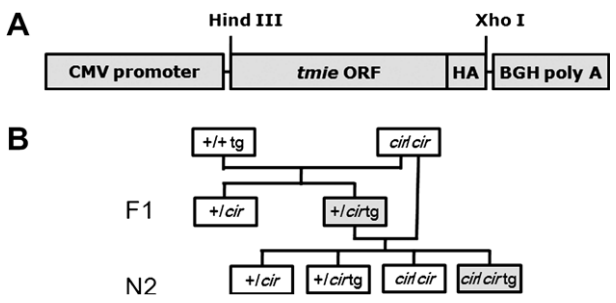

Fig. 1. Construction for the production of *tmie*-overexpressing mice (A) and breeding strategy (B). *cir*/*cir*-tg represents the *cir*/*cir* mice carrying *tmie* transgene.

tmie antibody production and Western blot analysis. *tmie* antibodies were generated by immunizing a rabbit (Peptron, Daejeon, Korea) with a synthetic peptide (aa118-133, GenBank Accession No. NP666372) of mouse *tmie*. Western blot was performed as follows. An equal volume of 1× SDS sample buffer was added and the samples were then boiled for 5 min. Sample (50 μg) was subjected to electrophoresis on 13% SDS–polyacrylamide gels for 2 h at 200 mA and then transferred onto nitrocellulose. The membrane was incubated for 1 h in 5% (w/v) skim milk in PBS containing 0.05% (v/v) Tween 20 (PBS-T), washed in PBS-T and then incubated for 2 h in the presence of primary antibody (1:1500). The membrane was washed extensively with PBS-T and then incubated with anti-rabbit IgG antibody (1:1500, Amersham) for 1 h. After extensive washing, immunoreactive bands on the membrane were visualized using chemiluminescent reagents according to the manufacturer's protocol (Supersignal Substrate; Pierce, Rockford, IL).

Immunohistochemical analysis of the organ of Corti. The removed temporal bone was fixed in 4% paraformaldehyde for 16 h at 4 °C, decalcified with 10% EDTA in PBS for 1 week, dehydrated, and embedded in paraffin wax. Sections of 4 μm were deparaffinized in xylene and rehydrated through graded concentrations of ethanol. For immunohistochemical study, the LSAB-kit Universal K680 (DAKO, Carpinteria, CA, USA) was used and all the procedures were carried out according to the manufacturer's instructions. The endogenous peroxidase was blocked with 3% hydrogen peroxide for 5 min at room temperature. Afterward, specimens were washed in PBS and non-specific binding was blocked with 1% goat serum for 1 h. Then, primary antibody (anti-*tmie*, 1:50) was added to the slides, and incubation proceeded for 2 h. After repeated washes with PBS, the section was incubated with Alexa Fluor® 488-conjugated goat anti-rabbit IgG (Invitrogen) at 1:150 dilution in 1% goat serum in PBS for 2 h at room temperature, followed by three washes with PBS. In the final step, the nuclei of immunostained cells were counterstained with DAPI.

Surface preparation of the cochlea. The organ of Corti and vestibular systems were prepared for histological analysis. The temporal bone was fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) overnight at 4 °C. Following fixation, the otic capsule was removed and the cochlea was microdissected into individual turns. The specimens were rinsed in 0.1 M PBS, then incubated in 0.25% Triton X-100 for 2 min and immersed in TRITC-labeled phalloidin (Sigma P1951, 1:4000) in PBS for 20 min. After three washes with PBS, the specimen was examined under fluorescence microscope with appropriate filters for TRITC (excitation: 510–550 nm, emission: 590 nm).

Statistics. Data were analyzed by chi-square analysis to determine the differences between groups. A value of $P < 0.05$ was considered to be statistically significant.

Results

Clinical observation and hearing tests

According to the previous studies about the expression pattern of mouse *tmie*, *tmie* gene is expressed in many kinds of organs as well as the inner ear [9,10]. We produced *tmie*-overexpressing transgenic mouse model using CMV promoter for the phenotypic rescue of the circling (*cir*/*cir*) mouse. Through the continuous

breeding experiments, we got the *cir*/*cir* homozygous mice carrying *tmie* transgene (*cir*/*cir*-tg, Fig. 1B). Above all, the clinical observation was performed to test the phenotypic rescue in N2 *cir*/*cir*-tg mice (Table 1). Some individuals showed a complete recovery in the behavior and hearing ability but others incomplete or no phenotypic recovery. The *cir*/*cir*-tg mice showing normalcy were classified by general activity, swimming ability, and hearing tests into group I ($n = 14$, 31.8%). They were able to swim in the water with proper orientation of the mouse with respect to the water surface and showed almost the same swimming ability as wild type mice (+/*cir*). The other N2 *cir*/*cir*-tg mice were grouped into II-V ($n = 30$, 68.2%) based on the incomplete recovery levels of the behavior and hearing ability. The wild type and *cir*/*cir*-tg group I mice did not show circling behavior like wild type mice. The average number of rotations of mice in the group IV and V was 73 ± 35, which is lower than that in the *cir*/*cir* mice (231 ± 21, data not shown). Auditory brainstem responses (ABR) were analyzed to determine the sound pressure level at which the characteristic ABR waveform could be detected. As shown in Fig. 2A, mice in groups I and II exhibited slightly elevated ABR thresholds (~40–70 dB) for each of the four sound stimuli but the average thresholds were not significantly different from the wild type mice. The circling and group V mice exhibited no responses in the ABR test, which means complete hearing loss (Fig. 2B). The rescued group I mice were able to respond to each stimulus and the threshold was almost the same as the wild type mice.

Table 1
Different behavior and hearing recovery levels in the circling mouse carrying transgene

Group	Phenotypes			No. of mice born (%)
	General activity	Swimming	Hearing	
I	○	○	○	14 (31.8)
II	△	○	○	8 (18.2)
III	△	○	×	4 (9.1)
IV	×	×	○	1 (2.3)
V	×	×	×	17 (38.6)

Mice were grouped into I or II–V based on the complete or incomplete recovery levels of the behavior and hearing ability, respectively.
General activity ○: no circling, no head-shaking, almost normal; △: no circling, but mild head-shaking, slightly active; ×: circling, head-shaking, hyperactive. Hearing ○: responding to stimuli in ABR tests; ×: no response to each stimulus in ABR tests.

tmie expression analysis in the cochlea

With exactly the same genotypes, an individual *cir*/*cir*-tg mouse showed different levels of recovery in the phenotypic characteristics. To understand the reasons of these results, *tmie* expression pattern in the protein level was analyzed by Western blotting and immunohistochemistry in the cochleas with anti-*tmie* antibody (Fig. 3). β-Actin was used as an internal standard. In Western blotting analysis (Fig. 3A and B), lanes 1–4 mice showed more *tmie* protein expression in the inner ear than control +/*cir* mouse. Those mice were from group I with complete recovery in both behavior and hearing ability (Table 1). Two mice in lanes 5 and 6 were from group II and they showed similar phenotypic rescue levels but totally different transgene expression levels. Lanes 7–9 mice were from group V with no ameliorations in either behavior or hearing. They showed much lower transgene expression levels in the inner ear than the wild type mouse. Although there is an exceptional sample, the hearing and behavior correction levels have a tendency to be dependent on the transgenic *tmie* expression levels in the inner ear. These results were consistent with *tmie* immunostaining analysis (Fig. 3C). Immunohistochemical staining with anti-*tmie* antibodies in the wild type cochlea showed *tmie*-positive cells in spiral limbus, basilar membrane and spiral ligament (Fig. 3C, panel a). The *cir*/*cir* mouse has the genomic deletion including the entire *tmie* gene so that no stained parts were observed in the cochlea (Fig. 3C, panel b). Mouse cochleas from group I with complete rescue were also stained positively in the similar expression pattern to wild type cochlea (Fig. 3C, panel c). The organ of Corti of group V mouse was also slightly immunostained in the spiral ligament but the intensity was very low compared with wild type and group I mice (Fig. 3C, panel d).

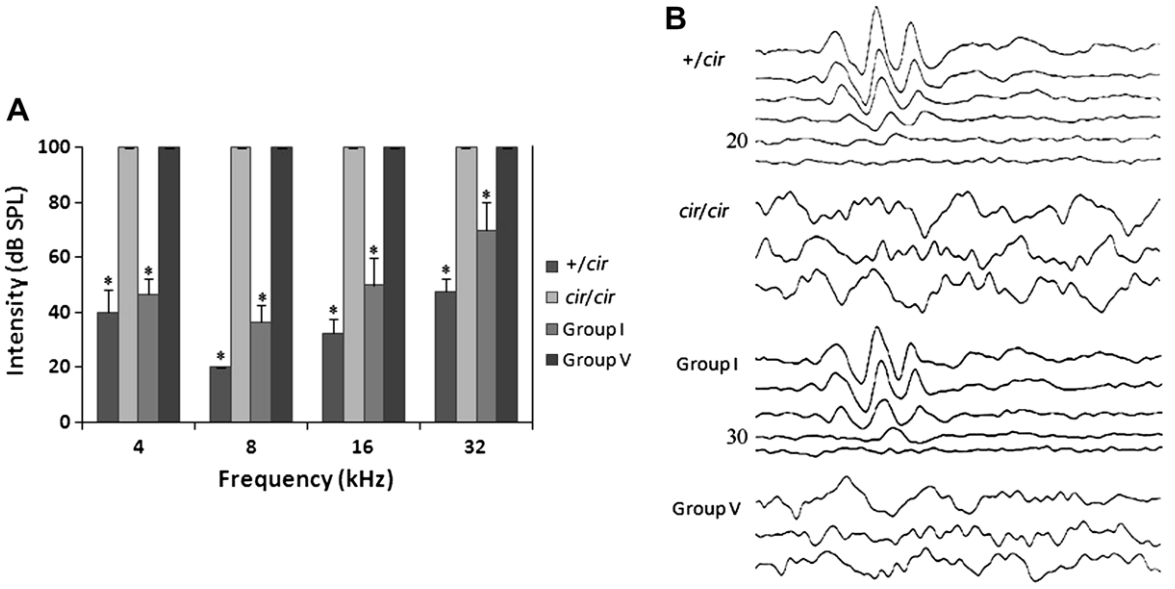

Fig. 2. ABR responses in +/*cir*, *cir*/*cir*, and *cir*/*cir*-transgenic mice group I, II, and V. (A) Average ABR thresholds in decibels referenced to sound pressure level (dB SPL) are plotted against frequency. Frequencies tested were 4, 8, 16, and 32 kHz. Hearing threshold with no response to at given stimuli was referred to 100 dB SPL. Standard errors of the mean ranges are plotted for each data point. Group differences that were statistically significant are indicated by asterisks ($P < 0.05$). (B) Representative recordings from 8 kHz stimuli at multiple intensities are shown. The thresholds of wild type and group I mice were 20 and 30 dB SPL, respectively; no waveforms were obtained at any given stimuli.

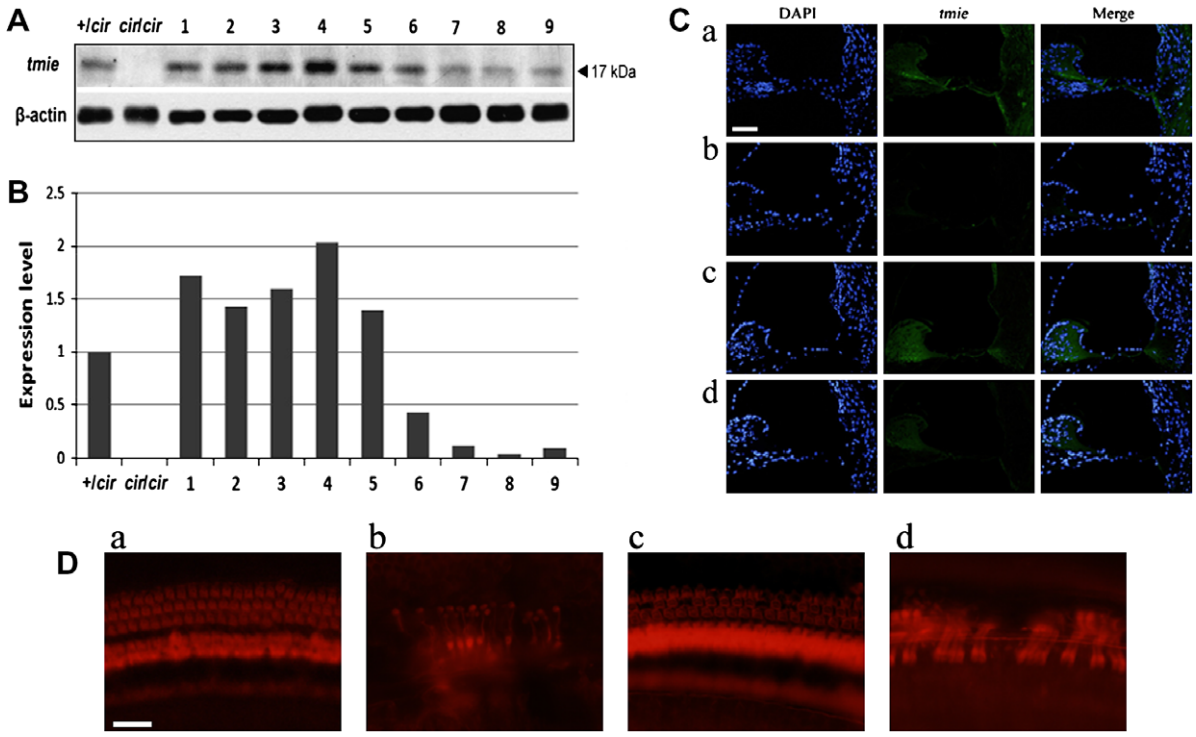

Fig. 3. *tmie* expression analysis in the cochlea by Western blotting and immunohistochemistry. (A) Western blot analysis is the +/*cir*, *cir*/*cir*, and some of the *cir*/*cir*-tg mice. Lanes 1–4, mice in group I; lanes 5 and 6, mice in group II; lanes 7–9, mice in group V. The antibody demonstrates a single immunoreactive band of 17 kDa. β-Actin was used as the internal control. (B) Densitometry data presented in band intensity relative to wild type control following normalization to actin. The intensity of the bands for *tmie* Western blot was quantitated using Image J program. (C) *tmie* immunostaining in the organ of Corti of the +/*cir* (a), *cir*/*cir* (b), group I (c), and group V (d) mice. Nuclei are visualized with DAPI. Right images are the merged images from the left and center. (D) Cochlear surface was stained with phalloidin-TRITC to detect hair cell stereocilia. a, +/*cir*; b, *cir*/*cir*; c, group I; d, group V. Scale bars: 50 μm in C (panels a–d); 20 μm in D (panels a–d).

Hair cells in cochlear explants

Cochlear hair cells were observed by phalloidin-staining of stereocilia F-actin bundles (Fig. 3D). The wild type mouse showed inner and outer hair cells in lines without loss of cells (Fig. 3D, panel a) but, in the circling mouse cochlea, it was observed that a significant amount of cells were missing (Fig. 3D, panel b). The rescued group I mice showed the recovery of the inner and outer hair cells in the cochlea (Fig. 3D, panel c). Group V mice showed the degenerated hair cells similar to homozygous *cir*/*cir* mice (Fig. 3D, panel d).

Discussion

We produced transgenic *cir*/*cir* mice by introduction of *tmie* transgene and they showed variable recovery levels in hearing and behavior. These results clearly indicate that *tmie* protein plays an important role when the appropriate expression level of *tmie* was expressed in the inner ear. The protein levels were variable in each individual and these are thought to induce the differences in disease amelioration levels. The circling mouse originally has degenerated inner and outer hair cells and reduced cell densities in the spiral ganglion neurons leading to hearing loss [4]. In fact, the correction levels in cochlear hair cells were dependant on the *tmie* protein expression levels. The spiral ganglion neurons also showed transgene expression level-dependent recovery (data not shown).

Transgene expression level can vary significantly as a function of the copy number of the integrated transgene and/or the influence of the surrounding DNA. Microinjected DNA usually integrates at only one site, or a very limited number of different sites, in individual embryos. It is possible that individual mice had different copy numbers so that they showed various recovery levels. We did not observe the copy numbers of transgene in the individual *cir*/*cir*-tg mice. Therefore, this possibility cannot be ruled out in the present study. As well, gene therapy could produce more *tmie* protein than the wild type allele. In some cases, excess dosage of the normal gene can be deleterious, and cause defects that are as profound as those produced by protein deficiency [12–14]. In introduction of bacterial artificial chromosome (BAC) containing Myo15a gene into *shaker2* mice, the excess Myo15a expression has no physiologically significant protective or deleterious effects on the hearing of normal mice, suggesting that the dosage of Myo15a may not be problematic for gene therapy [15].

In Western blot analysis of *tmie* expression levels in inner ear of +/+ mouse was almost two fold compared to +/*cir* mice (data not shown). These results indicate that excess *tmie* does not induce defects and its necessary amounts are required for normal hearing and behavior.

In humans, the loss of function of *TMIE* causes hearing loss [16,17]. Human *TMIE* encodes a protein with 156 amino acids that shares 92% sequence identity with the mouse homolog (153 amino acids) and exhibits no significant nucleotide or deduced amino acid sequence similarity to any other gene [16]. Although the function of mouse *tmie* and human *TMIE* is unknown, it appears to be important for normal hearing and vestibular function.

On the basis of conserved synteny and similar phenotypes, the circling mouse (with mutations in *tmie* gene) or rescued circling mouse with *tmie* transgene may provide ways to understand the mechanism of hearing loss and to further develop gene-based therapies for human inner ear disease.

Acknowledgments

This work was supported by the Korea Science and Engineering Foundation (KOSEF) grant funded by the Korea government (MEST)

(R01-2007-000-10672-0) and the Ministry of Science and Technology/Korea Science and Engineering Foundation through the Vestibulocochlear Research Center at Wonkwang University in 2008 (R13-2002-055-00000-0).

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