Introduction of *tmie* Gene Can Recover the Hearing Impairment and Abnormal Behavior in the Circling Mouse

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ABSTRACT

The spontaneous mutant circling mouse (*cir/cir*) shows a circling behavior and hearing loss. We produced transgenic mice overexpressing transmembrane inner ear (*tmie*) gene, the causative gene, for the phenotypic rescue of the circling mouse. Through the continuous breeding with circling mice, the *cir/cir* homozygous mice carrying the transgene (*cir/cir*-tg) were produced. The rescued *cir/cir*-tg mice were able to swim in the water with proper orientation and did not show any circling behavior like wild type mice. Western blot and immunohistochemical analysis exhibited that the transgenic *tmie* was expressed in the inner ear. Inner and outer hair cells were recovered in the cochlea and spiral ganglion neurons were also recovered in the rescued mice. Auditory brainstem response (ABR) test demonstrated that the *cir/cir*-tg mice are able to respond to sound. This study demonstrates that *tmie* transgene can recover the hearing impairment and abnormal behavior in the circling mouse.

(Key words : Transmembrane inner ear, *tmie*, Transgenic animal, Hearing loss)

INTRODUCTION

Hearing loss is the most common sensory deficit in humans and it can be caused by environmental factors as well as genetic factors. Environmental causes include pre- and post-natal infection and ototoxic drug exposure. But it is estimated that about 50 to 75% of all childhood deafness is due to hereditary causes (Lalwani *et al.*, 1999). The most frequent cause is a degeneration of the organ of Corti within the cochlea of the inner ear. There is evidence that the cochlea in lower vertebrates and avians possesses a self-repair mechanism that can be activated following damage to the sensory epithelium (Corwin *et al.*, 1988; Ryals *et al.*, 1988; Woolley *et al.*, 2001). However, in mammals, the cochlear sensory epithelium and neural components do not regenerate, and there is currently no effective intervention for their repair. The auditory sensory epithelium is composed of sensory hair cells and several types of non-sensory supporting cells. All these cells are highly differentiated in their structure and function. Most types of epithelial tissues maintain a population of undifferentiated basal cells, which facilitate turnover and repair, but this is not the case for the organ of Corti in the cochlea (Ishimoto *et al.*, 2002; Kawamoto *et al.*, 2003; Minoda *et al.*, 2004). Therefore, when cochlear hair cells are lost, they cannot be replaced.

Generation of hair cells from a renewable source of progenitors that can be transplanted into damaged inner ears is a principal requirement for potential cell therapy in this organ. Gene therapy to grow new auditory hair cells was used in adult guinea pigs (Ishimoto *et al.*, 2002; Kawamoto *et al.*, 2003). 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.

VOT-E36 cells derived from the E10.5 otocyst of the H-2Kb-tsA58 transgenic mouse were induced to be converted into hair cells in the cochlea using overexpression of Math1 (Liu *et al.*, 2006). VOT-E36 cells with Math1 overexpression could respond to mechanical vibrations and active spiral ganglion neurons. The *shaker2* (*Myo15a*$^{sh2/sh2}$) mouse mutant is deaf and exhibits abnormal circling behavior (Snell *et al.*, 1939; Probst *et al.*, 1998). The cochlear phenotype of Myo15a mutants is consistent with profound deafness. The stereocilia on both inner and outer hair cells are short and stubby and hair cells eventually degenerate (Deol, 1952). As a

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gene therapeutic way, bacterial artificial chromosome (BAC) transgene correction with wild-type Myo15a corrected structure and function of the inner ear in Myo-15a mutant mice (Kanzaki *et al.*, 2006). The transgenesis was discovered to be sufficient for complete phenotypic rescue until adult age.

The spontaneous mutant circling mouse (*cir/cir*) becomes hyperactive at about postnatal 7 days, and then shows a circling behavior (Lee *et al.*, 2001; 2002). The most notable pathological phenotypes are the almost completely degenerated cochlea, and the remarkably reduced cellularity in the spiral ganglion neurons. The causative gene was *transmembrane inner ear* (*tmie*) gene of which the mutation is a 40-kilobase genomic deletion including *tmie* gene itself (Cho *et al.*, 2006). In this study, we established *tmie*-overexpression trasngenic mice. Individuals with germline transmission have been mated with circling homozygous mutant mice 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 which is originally missing in the mutant individual might compensate for the disease such as hearing loss, circling behavior, and swimming inability.

MATERIALS AND METHODS

Production of Transgenic Mice and Breeding Strategy

Human cytomegalovirus (CMV) immediate-early promoter was used in the transgene construction to achieve the systemic expression of mouse *tmie*. The 459 bp cDNA of *tmie* was amplified by PCR from total mouse brain RNA and subcloned into pGEM-T easy plasmid vector. The *tmie* coding region was cloned into the pcDNA3.0 vector. Hemagglutinin (HA) epitope tag was added to the end of *tmie* open reading frame (ORF) removing *tmie* stop codon. A diagram illustrating the DNA cassette used to generate transgenic mice is shown in Fig. 1C. The purified DNA cassette was microinjected into the pronuclei of fertilized one-cell embryos obtained from BDF1 females. Injected embryos were cultured for 20 h before being transferred to pseudopregnant ICR female mice. After screening of transgenic founder mice, we mated one founder mouse to *cir/cir* mice. Then the *+/cir* progeny carrying transgene were mated to *cir/cir* mice for the production of transgenic *cir/cir* mice. All animal experiments were carried out in accordance with the Guidelines for Animal Experimentation and under permission from the Institutional Animal Care and Use Committee (IACUC).

Genotyping and Transgenic Screening

To determine the genotypes of mice, genomic PCR was performed. Genomic DNA was extracted from a mouse tail biopsy. Sequences flanking deletion region was amplified for *cir* allele by PCR with primers a-a' (Fig. 1A; a-a'). Inside exon 1 of *tmie* was amplified for existence of wild type allele (+) and distinction itself from transgene (corresponding to *tmie* open reading frame) spanning exons three to six (Fig. 1B; b-b'). The transgenic mice were confirmed by PCR of the region from 3' end of hCMV to 3' end of *tmie* open ORF (Fig. 1C; c-c'). The primers used here were as follows: a, 5'-CTT GCT TAG CAC CTC AGT TT-3'; a', 5'-ACT GCC TCA GGT CTT TGT TA-3'; b, 5'-AAG CTG TAG CTC TGA AAT CT-3'; b', 5'-TCT GGC AGA ATG CAT GGA GGC T-3'; c, 5'-AAG CAG AGC TCT CTG GCT AA-3'; c', 5'-TCT ACC TTG ATA GCC ACT GTG T-3'. Each amplification condition depends on the set of primers. +/+ or *+/cir* heterozygous mice were used as wild type mice.

Clinical Observations

The circling and rearing (rising in the hind legs) 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 the swimming behavior.

tmie Antibody Production and Western Blot Analysis

tmie antibody was 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. The 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 (1:1,500). The membrane was washed extensively with PBS-T and then incubated with anti-rabbit IgG antibody (1:1,500, Amersham) for 1 h. After extensive washes, immunoreactive bands on the membrane were visualized using chemiluminescent reagents according to the manufacturer's protocol (Supersignal Substrate; Pierce, Rockford, IL).

Immunohistochemical Analysis of Organ of Corti

The removed temporal bone was fixed in 4% paraformaldehyde (PFA) for 16 h at 4℃, decalcified with

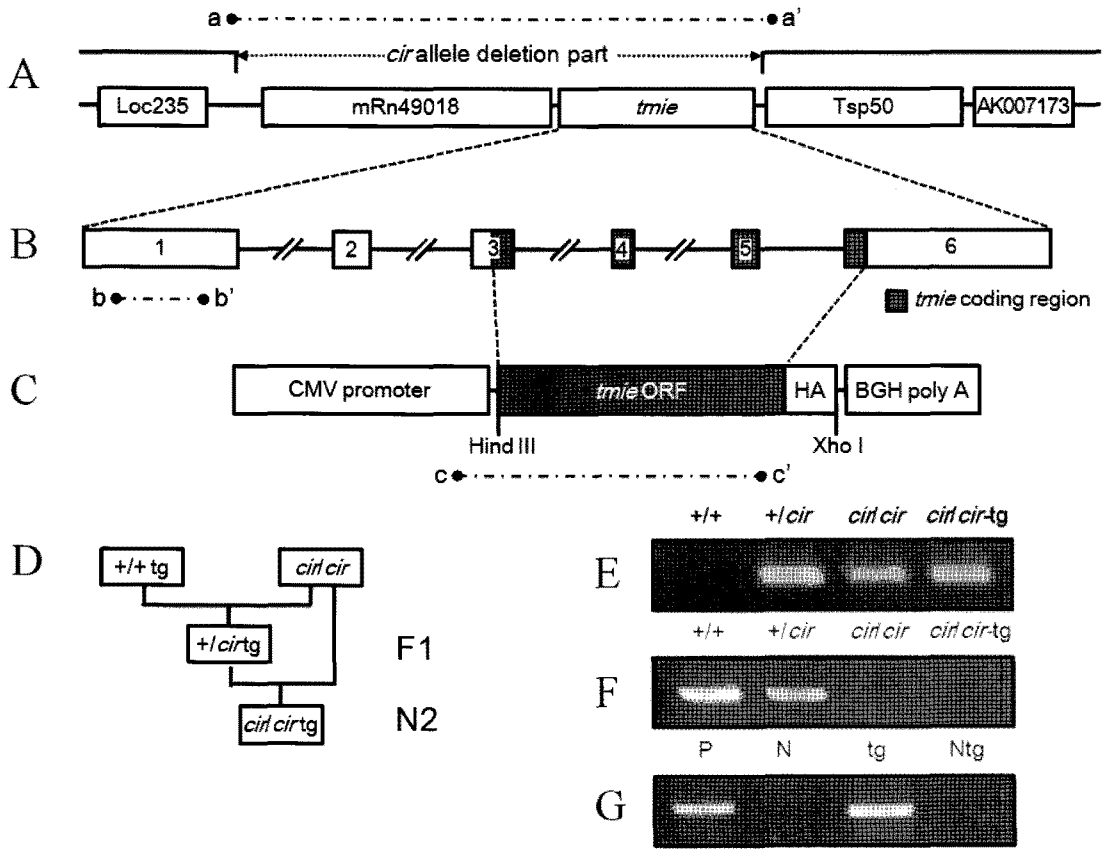

Fig. 1. Construction of *tmie* transgene, breeding strategy and genotyping of the N2 mice. (A) Diagram of the deleted region in the circling mouse. (B) The exon structure of *tmie* is indicated, with 459 bp open reading frame denoted by gray box. (C) Transgenic construct for overexpression of *tmie* gene. (D) Breeding strategy. (E) *cir* allele was amplified with primers flanking deletion region (a-a'). The PCR product is 406 bp. (F) Inside exon 1 of *tmie* was amplified for existence of wild type allele (+) (b-b'). Amplified products corresponds to 564 bp. (G) *tmie* transgenic mice were identified by genomic PCR (c-c'). The lanes were as follows: P, positive control (amplified from the injection DNA); N, negative control (normal mouse genomic DNA); tg, transgenic mouse; Ntg, non-transgenic mouse. Positive control corresponds to 527 bp.

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. After specimens were washed in PBS and nonspecific binding was blocked with 1% goat serum for 1 h. Then, primary antibody (*tmie*, 1:50; NF200 (Sigma), 1:100 diluted) 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.

Morphological Analysis of the Organ of Corti Surface

The organ of Corti and vestibular systems were prepared for histological analysis. The temporal bone was fixed in 4% PFA in 0.1M 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 0.1M 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).

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 pla-

tinum) 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 inch ACO Pacific condenser microphone (Belmont, USA) in a volume approximating the mouse external ear canal and expressed as dB SPL.

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

Rescued Mice Showed Normal Behavior

We produced *tmie*-overexpressing transgenic mouse model to induce the phenotypic rescue of the circling mouse. 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 (Mitchem *et al.*, 2002; Chung *et al.*, 2007). In order to achieve the systemic expression of mouse *tmie*, human cytomegalovirus (CMV) immediate-early promoter was used in the transgene construction (Fig. 1C). Through the continuous breeding experiments, we got the *cir/cir* homozygous mice carrying the transgene (Fig. 1D). Genotyping and transgenic confirmation were carried out by genomic PCR with indicated primer sets (Fig. 1E, F and G). The circling mice carrying *tmie* transgene did not show any circling behavior like wild type mice (Table 1). Rearing count of all the rescued mice (*cir/cir*-tg, 59.7±9.8) was similar to that of the wild type mice (54.3±6.4). The swimming test was conducted to test for vestibular function. The *cir/cir*-tg mice were also 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. As well, rescued mice did not exhibit head-shaking behavior. Circling mice were smaller in weight than their wild type littermates (Lee *et al.*, 2002). At P60, *cir/cir*-tgA mice also recovered in weight gain whereas *cir/cir* exhibited retarded growth (Table 1).

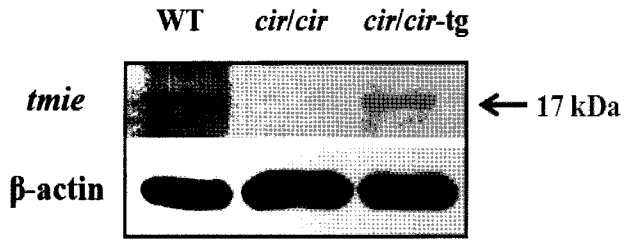

Fig. 2. Expression analysis of the *tmie* gene in the cochleas by Western blotting. *tmie* gene product was present in the wild type and *cir/cir*-tg mouse inner ear. The antibody demonstrates a single immunoreactive band of 17 kDa. β-actin was used as the internal control.

Table 1. Clinical observation of the wild type, circling and rescued mice (N=5)

Phenotypes		Wild type	*cir/cir*	*cir/cir*-tg
Circling (counts)		0^a	225.6 ± 22.4^b	0^a
Rearing (counts)		54.3 ± 6.4^a	0^b	59.7 ± 9.8^a
Swimming		O.K.	No	O.K.
Head-shaking		None	Yes	None
Weight (g)*	Male	33.5 ± 5.2^a	21.8 ± 3.3^b	31.0 ± 4.2^a
	Female	25.5 ± 3.8^a	18.2 ± 2.6^b	24.9 ± 3.7^a

* Weight of mice was measured at P60.
Values in circling, rearing and weight expressed as mean± SEM. Figures in rows that share different letter superscripts are significantly different among the groups ($p<0.05$).

tmie Transgene was Expressed in the Cochlea

To assess expression of endogenous or transgenic *tmie* gene to the cochlea, total protein isolated from the inner ear was analyzed by Western blotting (Fig. 2). *tmie* protein was detected in the cochleas of wild type and rescued *cir/cir*-tg mice of which endogenous *tmie* expression is originally missing. This result was consistent with the immunohistological analysis. *tmie* protein level in the rescued mice cochlea was lower than that of wild type mice. β-actin was used as an internal standard.

Immunohistochemical staining with anti-*tmie* antibodies in the wild type cochlea showed *tmie*-positive cells in spiral limbus, basilar membrane and spiral ligament (Fig. 3A and B). In spiral ligament, type I, III, and IV fibrocytes were strongly labeled but *tmie*-positive cells in type II fibrocytes could not be found. Stria vascularis was not stained with anti-*tmie* antibody (Fig. 3B). The *cir/cir* mouse has the genomic deletion including entire *tmie* gene so that no stained parts were observed in the cochlea (Fig. 3C and D). Rescued *cir/cir*-tg mouse cochlea was also stained positively in the similar expression pattern to the wild type cochlea (Fig. 3E and F). *tmie* transgene product was

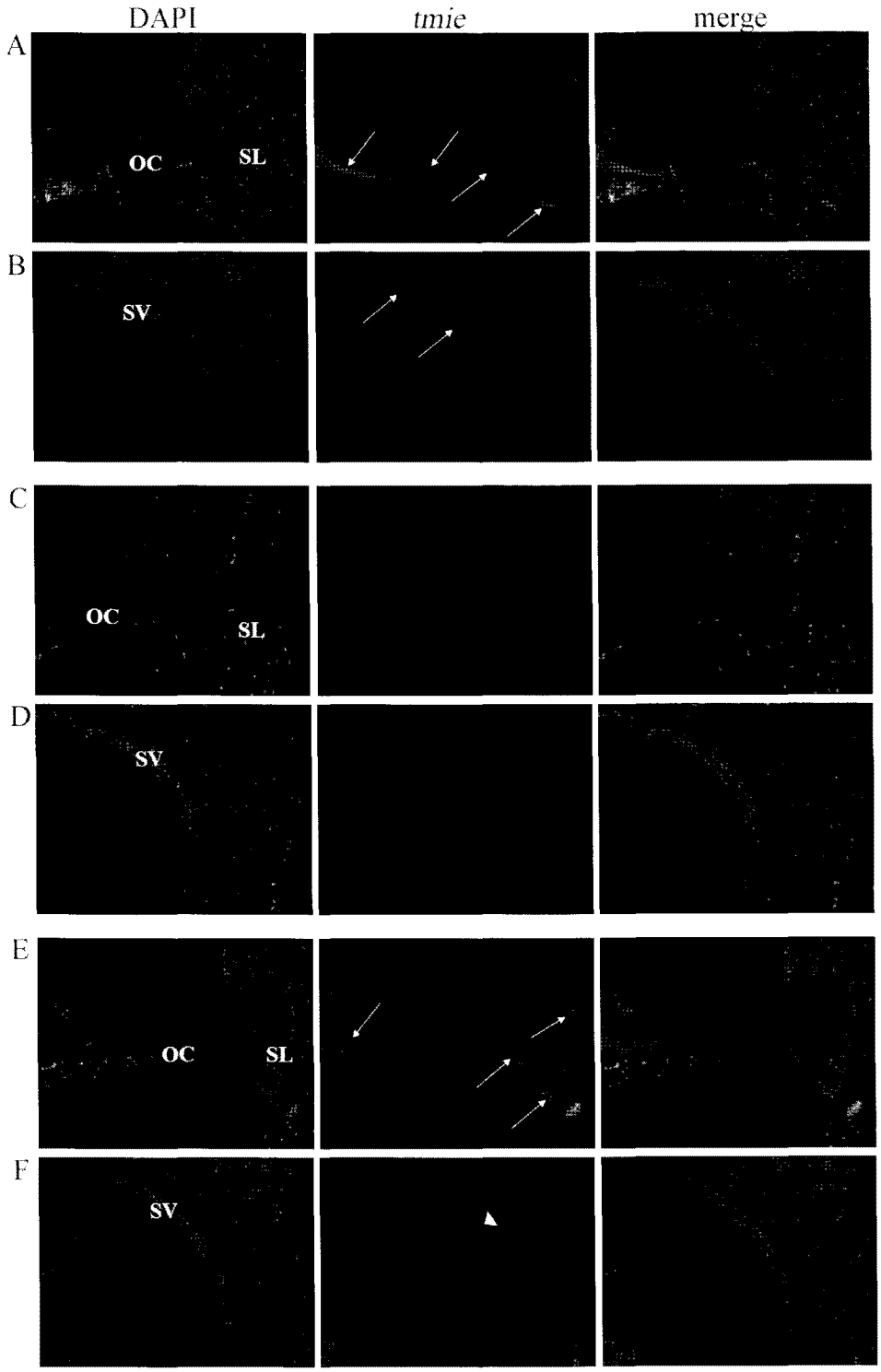

Fig. 3. Endogenous or transgenic *tmie* expression in the inner ears of the adult wild type (A and B), *cir/cir* (C and D) and *cir/cir*-tg mice (E and F). The surroundings of organ of Corti (OC) and stria vascularis (SV) were immunostained with anti-*tmie* antibodies (green). Nuclei are visualized with DAPI (blue). Right images are the merged images from the left (DAPI) and center (anti-*tmie*). OC, organ of Corti; SV; stria vascularis; SL, spiral ligament. *tmie* protein in the wild type cochlea was detected in spiral limbus, basilar membrane and spiral ligament (A, arrows). Upper parts of spiral ligament were positively stained but stria vascularis cells showed no detection of *tmie* protein (B, arrows). *tmie* protein was not observed in the circling (*cir/cir*) mouse inner ear (C and D). The inner ear of *cir/cir*-tg mouse showed the expression pattern of *tmie* similar to wild type (E and F). Cells in spiral limbus, spiral ligament, and stria vascularis were positively stained with anti-*tmie* antibodies (arrows in E and arrowhead in F).

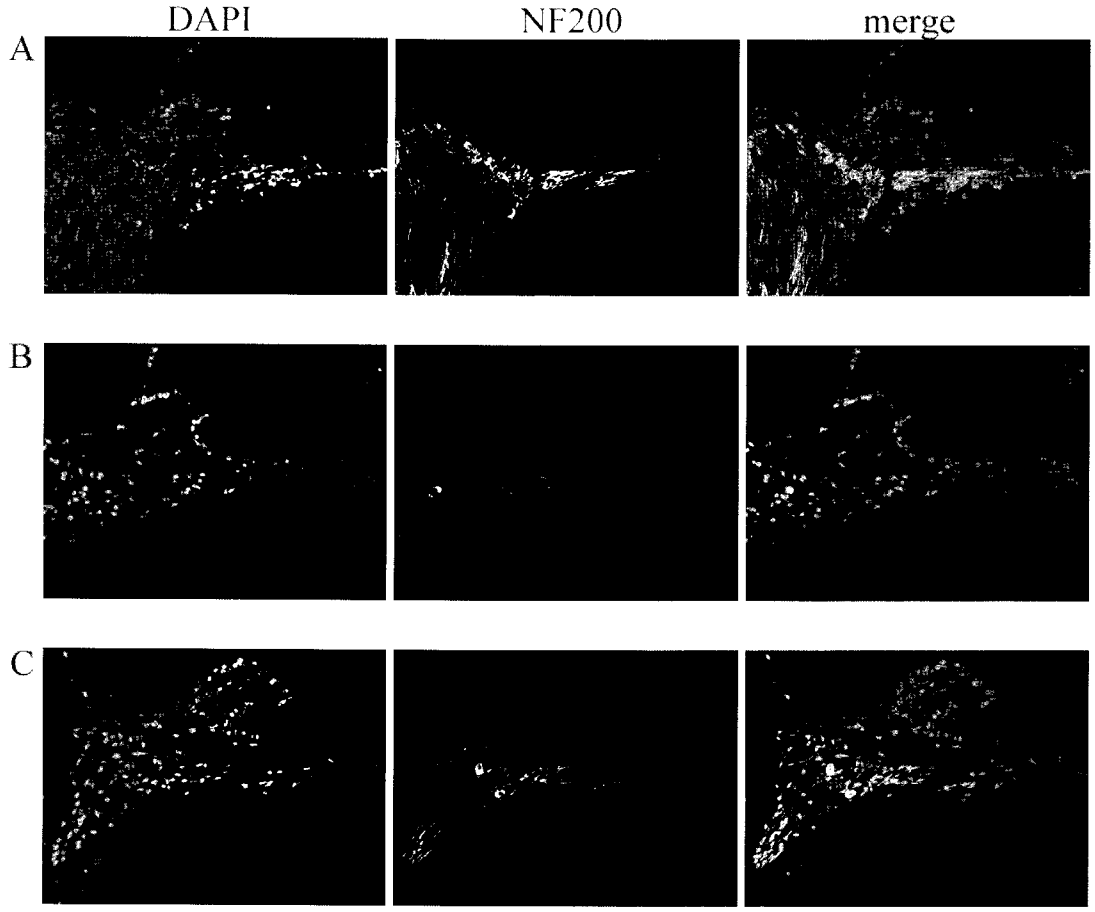

Fig. 4. Analysis of cellularity in the spiral ganglion neurons (SGNs) in the cochleas of the adult wild type (A), *cir/cir* (B), and *cir/cir*-tg mice (C). The SGNs were immunostained with anti-neurofilament 200 (NF200) antibodies (green). Nuclei are visualized with DAPI (blue). Right images are the merged images from the left (DAPI) and center (anti-NF200). Reduced SGN cellularity and the neurite formation were shown in the circling mouse compared with the wild type (B). The *cir/cir*-tg mouse revealed the recovery in the SGN cellularity and the formation of neurites (C).

observed in spiral limbus and spiral ligament. Positive staining was observed in the type I, III, and IV fibrocytes but not in the type II fibrocytes and these results are similar to the wild type. However, stria vascularis in rescued *cir/cir*-tg cochlea was also positively labeled (Fig. 3F, arrowhead).

Spiral Ganglion Neurons were also Recovered in the Rescued Mice

The circling mouse has degenerated inner and outer hair cells and reduced cell densities in the spiral ganglion neurons leading to hearing loss (Lee *et al.*, 2001). Immunostaining was performed with neurofilament 200 (NF200) antibodies to observe whether the rescued mice also have recovery in the spiral ganglion neurons. The circling mice exhibited reduced cellularity and less neurite formation in the spiral ganglion neurons compared to the wild type mouse (Fig. 4B). In contrast to homozygous mutant mice, the DAPI-stained nuclei showed that the *cir/cir*-tg mice (Fig. 4C) had recovered celluarity in the spiral ganglion neurons and the number of cells almost looked same as the normal wild type mouse (Fig. 4A). The neurite formation of *cir/cir*-tg spiral ganglion neurons also appeared normal.

The Rescued Mice Exhibited Normal Surface Appearance of The Organ of Corti

We investigated the surface structures of the inner and outer hair cells in three groups (Fig. 5). Norma wild type mouse showed inner and outer hair cells in lines without loss of cells (Fig. 5A) but, in the circling mouse cochlea, it was observed that lots of cells were missing (Fig. 5B). The neurite formation of spiral ganglion neurons in the circling mouse also exhibited abnormalities compared to the wild type mouse. The rescued *cir/cir*-tg mice revealed the recovery of the inner and outer hair cells in the cochlea (Fig. 5C). The spiral ganglion neurons were also normal and the result was consistent with Fig. 4C.

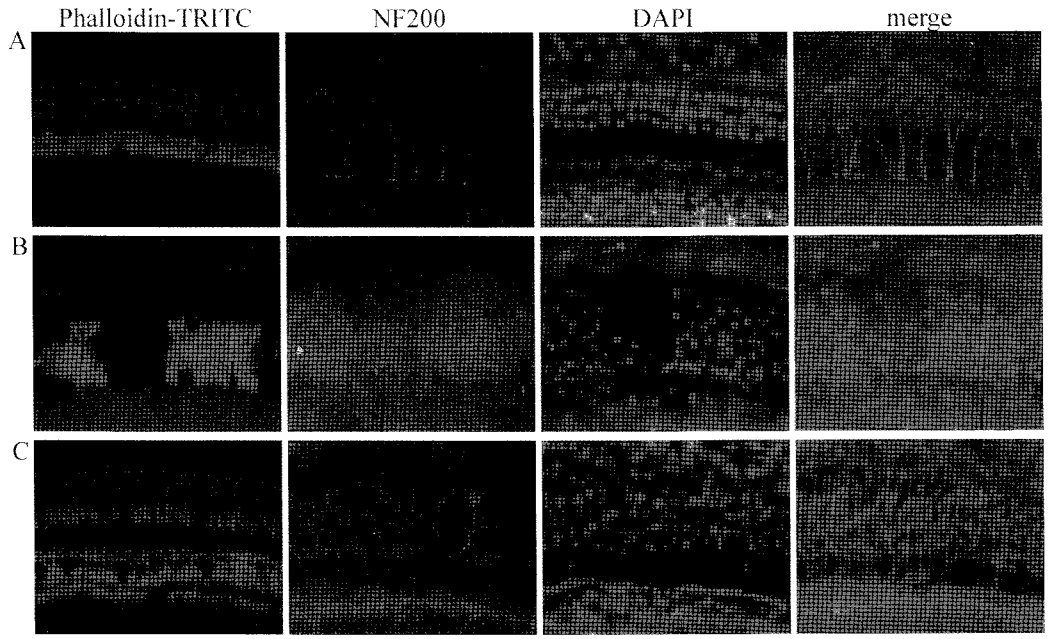

Fig. 5. Surface preparation of the wild-type (A), *cir/cir* (B) and *cir/cir*-tg (C) organs of Corti. Tissue was obtained from the basal turn of the cochlea, stained with TRITC-Phalloidin (red) to label F-actin in the stereocilia and NF200 (green) to label the spiral ganglion neurons. Far right images are merged images of second (NF200) and third (DAPI). Note that the *cir/cir* mouse hair cells and spiral ganglion neurons are missing (B) and *cir/cir*-tg mouse shows recovery in the hair cells and neurites of spiral ganglion neurons in the cochlea.

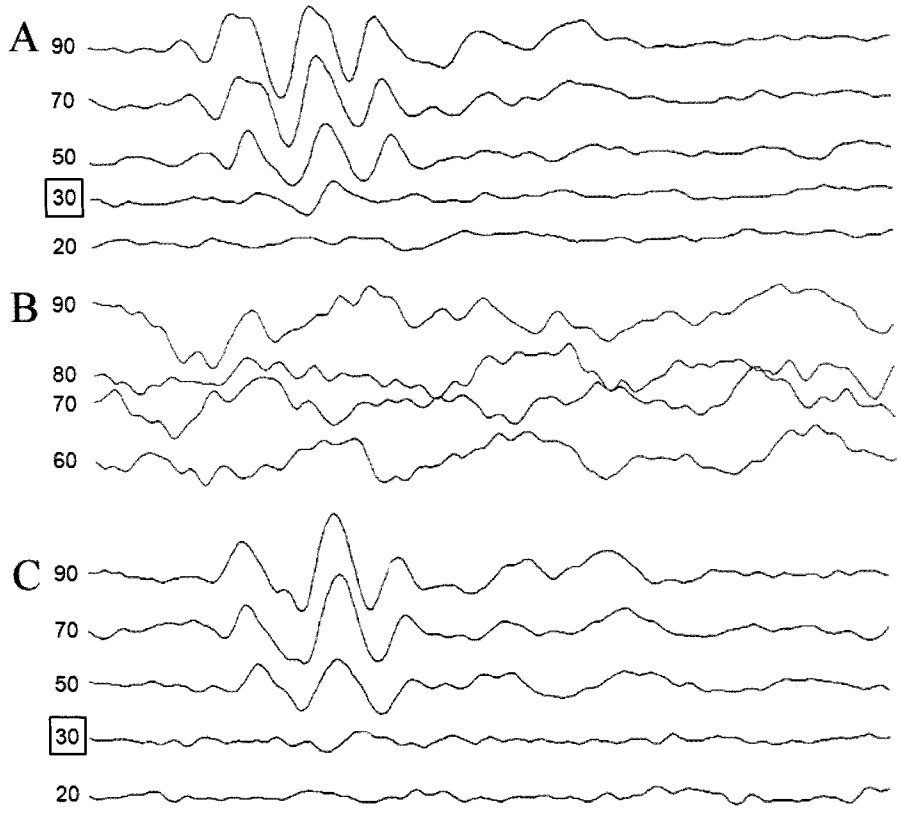

Fig. 6. Examples of auditory brainstem responses (ABR) from wild-type (A), *cir/cir* (B), and *cir/cir*-tg mice(C) at 8 weeks of age. Representative recordings from 16 kHz stimuli at multiple intensities (in dB SPL) are shown. Numbers in squares mean the threshold at given stimuli. Wild type and *cir/cir*-tg mice show normal ABR waveforms at 30 dB SPL or more, respectively (A, C), whereas *cir/cir* mice were profoundly deaf; no waveforms were obtained even at 90 dB SPL (B).

The *cir/cir*-tg Mice are able to Respond to Sound

Auditory brainstem response (ABR) test was performed to ascertain the rescue of the hearing ability of the mice which got a phenotypic rescue in the behavior test. The rescued *cir/cir*-tg mice showed a gradually normal ABR threshold. At 8 weeks, the rescued mice revealed normal threshold while *cir/cir* mutant mice showed no responses to all stimuli (Fig. 6).

DISCUSSION

Transgenic animal technology is a powerful tool to introduce new genes into animals. In this study, the *cir/cir* mice have a genomic deletion including entire *tmie* gene so that transgenic *tmie* gene was to be introduced for the phenotypic rescue.

The circling mouse introduced *tmie* transgene showed normal behavior and swimming ability. They did not show the circling behavior like normal mice. ABR tests demonstrate those rescued mice also have hearing recovery with a normal threshold to indicated stimuli. The organ of Corti of *cir/cir*-tg mice showed that *tmie* transgene was expressed in the inner ear structures such as stria vascularis, spiral limbus, and spiral ligaments so that they had similar histological morphology to the wild type mice. These results assure *tmie* gene is required for the normal behavior, balance maintaining and hearing function in the mouse.

CMV promoter is one of the most commonly used promoter to drive the transgene to be systemically expressed. In the rescued mouse organs of Corti, the expression pattern of *tmie* was very similar compared to the wild type. But *tmie* protein was detected in the stria vascularis in which was negatively stained in the wild type mouse and this may be caused by the effect of CMV promoter.

Spiral ganglion neurons (SGNs) depend on neurotrophic factors supplied by hair cells (HCs) and other targets for their development and continued survival. SGNs critically depend on contact with their presynaptic and postsynaptic partners for survival. Loss of HCs or sectioning of the central axons results in SGN death (Spoendlin, 1971, 1975; Webster *et al.*, 1981). The circling mouse also revealed a reduced cellularity in spiral ganglion neurons following inner hair cell (IHC) and outer hair cell (OHC) loss. If it is assumed that the main role of *tmie* protein is maintaining survival of inner and outer hair cells in the mouse cochlea, SGN loss in the circling mouse would be reasonable. But it cannot be ruled out the potential that *tmie* gene is necessary for survival of SGN itself regardless of IHCs or OHCs.

There have been trials to predict the exact structure of *tmie* through many kinds of protein prediction programs. Analysis of human *TMIE* with TMHMM predicts an intracellular amino terminus, two transmembrane domains, and an intracellular carboxy terminus (Krogh *et al.*, 2001). MemO and LOCATE methods predict that mouse *tmie* has a transmembrane domain (aa57~79), two non-transmembrane domain regions (aa1-56, 80~153) and signals TGN-endosome sorting (aa91~96, 115~120, 133~138).

The predicted results depend on the methods but the common result is helpful to research the function of *tmie*. In the adult mouse and rat, *tmie* is expressed in the various tissues (Chung *et al.*, 2007; Su *et al.*, 2008). The functional research of *tmie* needs to be assessed in light of those predicted results, in order to know the exact functions of *tmie* in the various tissues. Now it has been proven that *tmie* protein is required for the normal functions of the inner ear in the mouse. But its role in the other organs or tissues is still unknown. Because the addition of *tmie* was also able to induce the recovery of abnormal behavior of the *cir/cir* mouse, organs regulating the balance might be recovered in the rescued mice. However the gross appearance of the epithelia of the vestibular organs appeared not to be different among the wild type, *cir/cir* or rescued *cir/cir*-tg mice. Therefore it is necessary to investigate the main cause of the organs of abnormal behavior and swimming inability of the circling mouse and check whether the recovery of those organs got rescued morphology. Vestibular organs are mainly related to the regulation of the balance but the cerebellum is also responsible for the regulation and coordination of complex voluntary muscular movement as well as the maintenance of posture and balance. We are not sure whether *tmie* expression in the cerebellum induces the behavioral recovery in the rescued mice.

The circling (*ci2*) rat also displays circling behavior and hearing loss and had not revealed any gross abnormalities in the vestibular hair cells. But in the cochlear nuclei of the brainstem of mutant rats, neurons exhibited an abnormal shape, reduced size and increased density compared to controls (Kaiser *et al.*, 2001). There have not been researches about brainstem morphology in the circling mouse so that it is necessary to test the abnormalities in the brainstem of the circling mouse as well as the rescued mice.

Further studies should be directed to the researches about the functions of *tmie* in the other organs or tissues as well as in the inner ear in detail. Transgenesis by brain- or inner ear-specific promoters might be useful for the tissue-specific functions of *tmie*.

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