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Case Study

tmie Is Required for Gentamicin Uptake by the Hair Cells of Mice

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The circling (*cir*/*cir*) mouse is a spontaneous model of deafness due to deletion of a 40-kb genomic region that includes the transmembrane inner ear (*tmie*) gene. In addition to being deaf, *cir*/*cir* mice exhibit abnormal behaviors including circling and hyperactivity. Here we investigated differences between 3-d-old (that is, before hair-cell degeneration) *cir*/*cir* and phenotypically normal (+/*cir*) mice and the reason underlying the degeneration of the inner ear structure of *cir*/*cir* mice. To this end, we used gentamicin, gentamicin–Texas red conjugate, and FM1-43 to investigate mechanotransducer channel activity in the hair cells of *cir*/*cir* mice; these compounds are presumed to enter hair cells through the mechanotransducer channel. Although the structure of the inner ear of +/*cir* mice was equivalent to that of *cir*/*cir* mice, the hair cells of *cir*/*cir* mice (unlike +/*cir*) did not take up gentamicin, gentamicin–Texas red conjugate, or FM1-43. These findings suggest that hair cells in *cir*/*cir* mice demonstrate abnormal maturation and mechanotransduction. In addition, our current results indicate that *tmie* is required for maturation and maintenance of hair cells.

Abbreviations: GTTR, gentamicin–Texas red conjugate; *tmie*, transmembrane inner ear gene; TRITC, tetramethyl rhodamine isothiocyanate.

The inner ear comprises the cochlea, which is the structure for hearing, and the vestibule, which is involved in maintaining the balance of body; these functions are possible due to the hair cells of the inner ear. Hair cells have mechanosensing organelles called stereocilia, the ends of which have a structure known as the 'tip-link.' The tip-link is attached to nonspecific ion channels (mechanotransducer channels) and moves in response to motion in the surrounding fluid. Accordingly, changes in fluid motion alter the tension of tip links, thus opening and closing these mechanotransducer channels. Abnormalites in mechanotransduction lead to problems associated with the inner ear.[18] Cells in the mammalian inner ear do not regenerate after damage or loss,[2,5,17] thereby eventually leading to hearing impairment.

Circling mice(*cir/cir*) have a spontaneous mutation of the inner ear that induces deafness, hyperactive behaviors including circling and head-tossing, and decreased body weight compared with that of wildtype mice. These behavioral abnormalities first appear at 7 d of age.[11] The underlying genetic abnormality is an autosomal recessive mutation involving the deletion of a 40-kb genomic region that includes the transmembrane inner ear (*tmie*) gene.[3,11] The spinner mouse is a model of human hearing loss that is linked to the DFNB6 locus; spinner mice share behavioral similarities with circling mice, including circling and head shaking.[4] Although different genomic regions are deleted in circling and spinner mice, both regions involve *tmie*.[3,15] Circling mice transgenic for *tmie* recovered from their inner ear defect and regained hearing and normal behavior.[19] Therefore *tmie* is assumed to be the causative gene for both mouse mutants, but the exact function of *tmie* in the inner ear is unknown. Simply put, *tmie* is associated with hearing loss linked to the DFNB6 locus.[8,15]

Although aminoglycosides are highly effective in the treatment of gram-negative bacterial infection, they have several negative side effects. When aminoglycosides enter the hair cells of the inner ear, they induce ototoxicity.[10] Studies have shown that aminoglycosides enter the sensory hair cells of the inner ear, either by permeating the mechanoelectrical transduction channels at the tips of the stereocilia or by apical endocytosis.[1,9] FM1-43, which has a divalent cationic head group and lipophilic tail, is a styryl pyridinium dye that stains live hair cells in cultured tissues.[6] Hair cells typically rapidly take up FM1-43 through the tips of the stereocilia; however, when the mechanotransduction channel is closed, FM1-43 cannot enter the hair cells.[14] Moreover, FM1-43 has been shown to reduce the ototoxic effects of aminoglycosides.[7] These observations suggest that FM1-43 enters hair cells through the mechanotransducer channel. The mechanisms by which hair cells take up aminoglycosides and FM1-43 have not yet been determined, but they are presumed to enter through similar pathways involving the mechanotransducer channel.

Identification of differences in the inner ear function of circling and wildtype mice likely would facilitate elucidation of the func-

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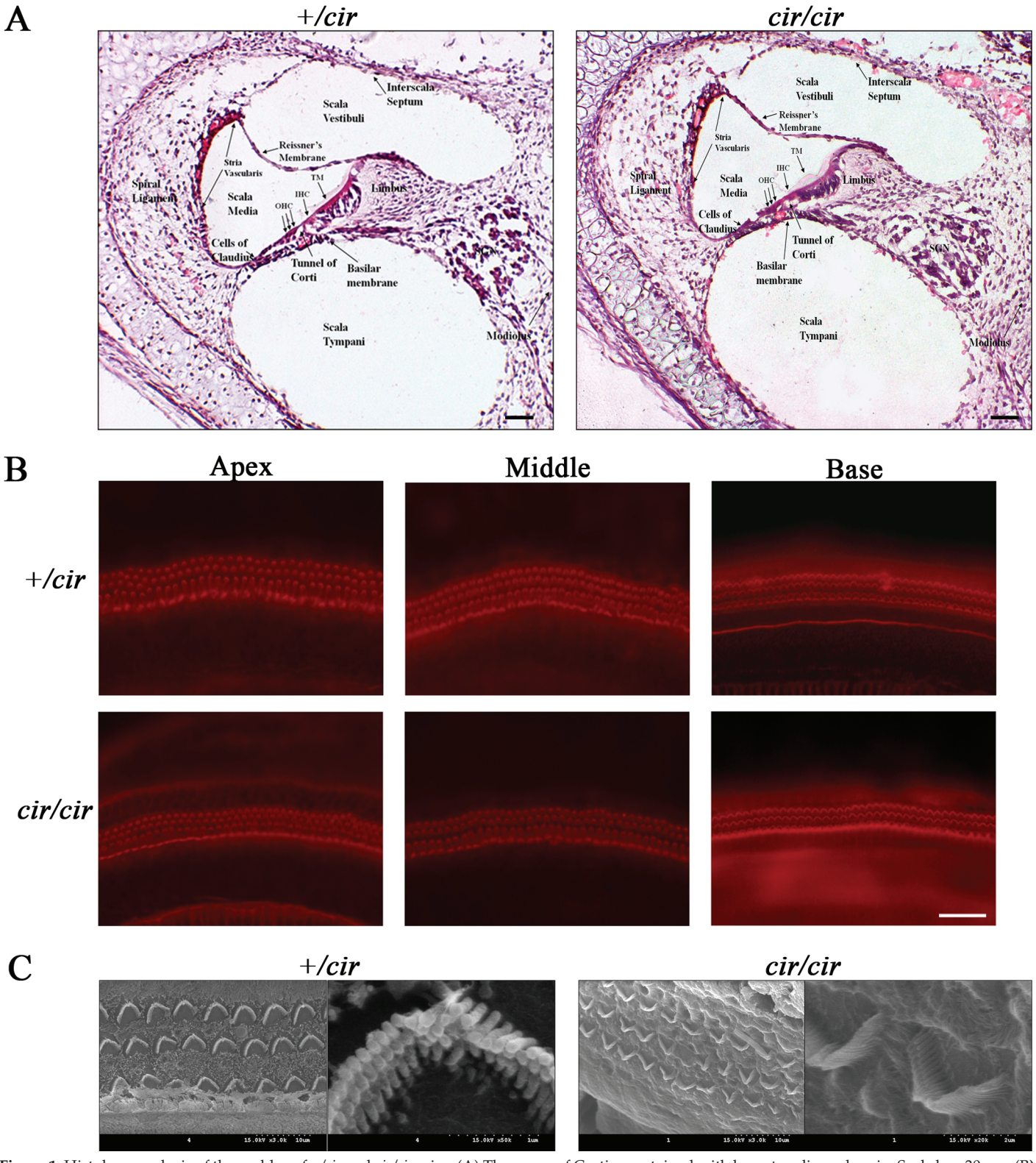

Figure 1. Histology analysis of the cochlea of *+/cir* and *cir/cir* mice. (A) The organ of Corti was stained with hematoxylin and eosin. Scale bar, 30 μm. (B) The stereocilia of hair cells in individual turns of the cochlea were stained with phalloidin–TRITC. Scale bar, 50 μm. (C) Scanning electron micrographs of the stereocilia of hair cells.

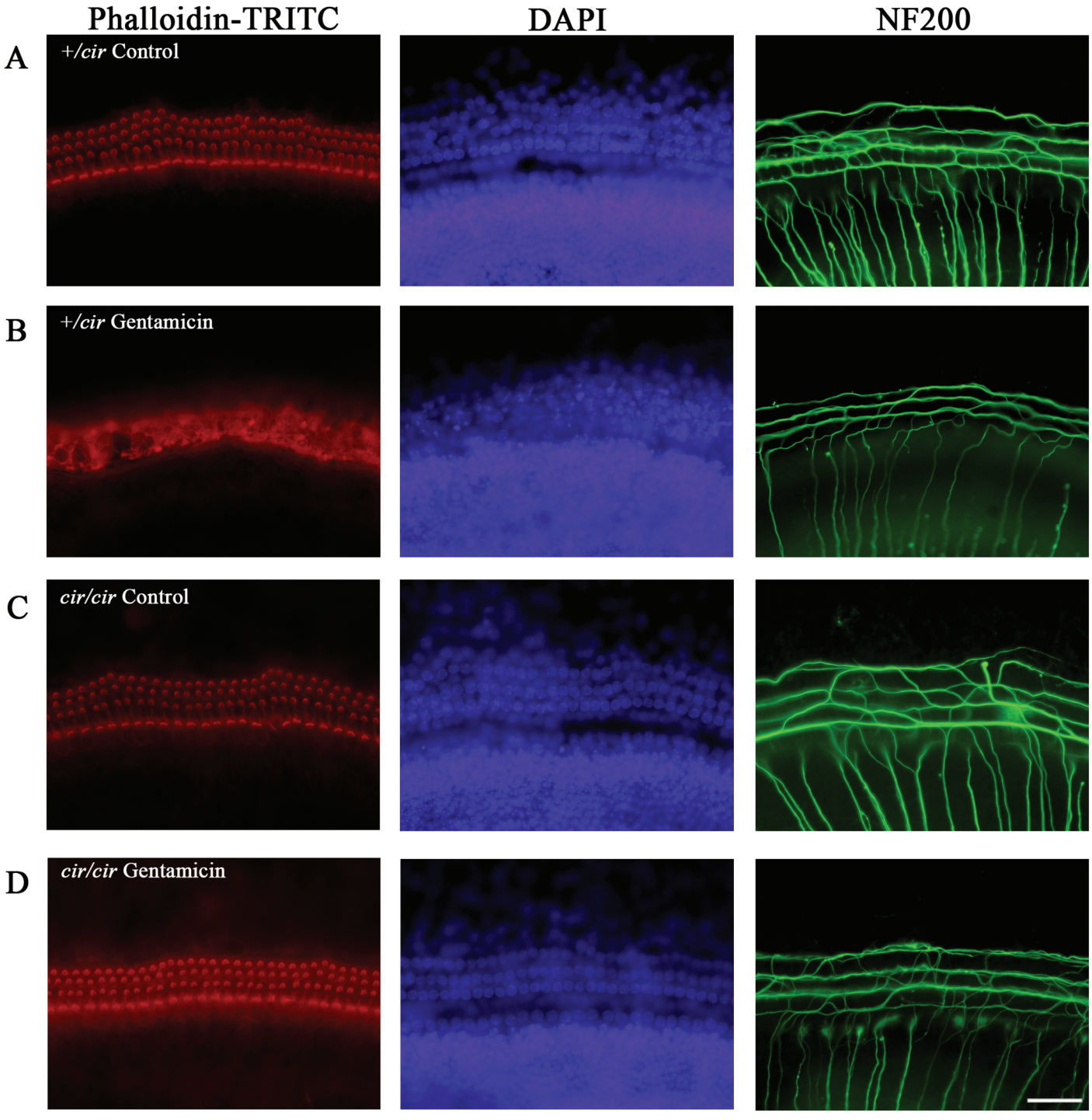

Figure 2. Immunofluorescence of the organs of Corti from *+/cir* and *cir/cir* mice after gentamicin treatment. Phalloidin–TRITC was used to detect F-actin in sterocilia bundles; the nuclei of immunostained cells were stained with DAPI, and spiral ganglion neurons were immunostained with an antineurofilament antibody. (A, C) Before gentamicin treatment, there were no differences between the organs of Corti of *+/cir* and *cir/cir* mice. (B, D) Gentamicin eliminated the hair cells of *+/cir* but not *cir/cir* mice. Spiral ganglion neurons showed no difference between *+/cir* and *cir/cir* mice before and after gentamicin treatment. Scale bar, 50 µm.

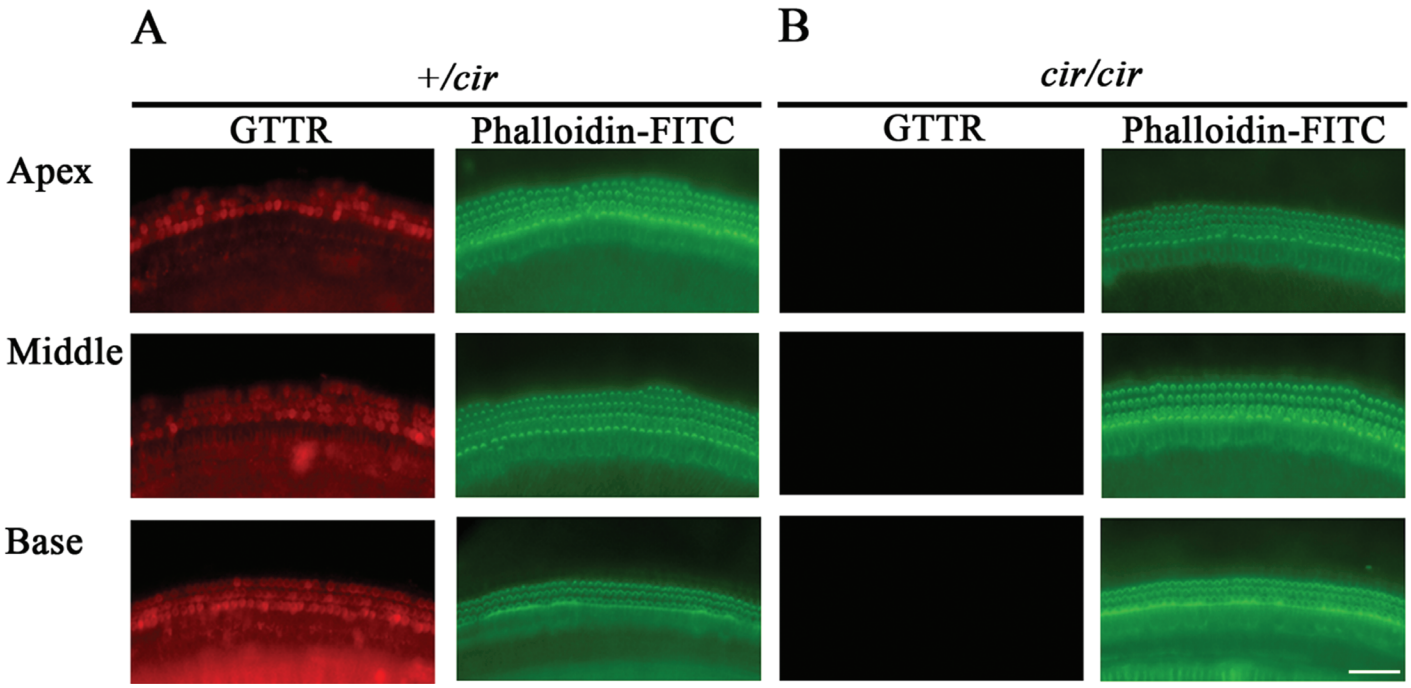

Figure 3. Fluorescence microscopic images of cochlear hair cells from *+/cir* and *cir/cir* mice exposed to GTTR. (A) Whereas GTTR was detected in the hair cells of *+/cir* mice, (B) no GTTR immunoreactivity was present in hair cells from *cir/cir* mice. As a positive control, hair cells also were stained with phalloidin–FITC, which detects F-actin in the stereocilia bundles. Scale bar, 50 µm.

tions of Tmie and other proteins. To this end, we studied 3-d-old normal (*+/cir*) and circling (*cir/cir*) mice; hair cells have not yet begun to degenerate in *cir/cir* mice of this age. However, treatment of cochlear explants from *cir/cir* mice with gentamicin and FM1-43 did not affect the hair cells of these mice. Therefore, circling mice show aberrations in mechanotrasduction of hair cells.

Materials and Methods

Animals. Circling mice (*cir/cir*) are spontaneous recessive mutants and have been kept as *cir/cir*-C57BL/6 for more than 20 generations by breeding between affected siblings. We used 3-day-old homozygous (*cir/cir*) mice for the mutant type and 3-day-old heterozygous (*+/cir*) mice as the wild type. During the experiments, mice were provided with a commercial diet and water ad libitum while housed at 22 ± 2 °C, a relative humidity of 50% ± 5%, and under a 12:12-h light:dark cycle (lights on, 0730 to 1930). All mice were housed under SPF conditions and known to be free of the following microorganisms: Sendai virus, *Mycoplasma pulmonis*, Tyzzer disease organism, *Pasteurella pneumotropica*, *Salmonella* spp., *Corynebacterium kutscheri*, *Pseudomonas aeruginosa*, and *Bordetella bronchiseptica*. Microbiologic monitoring for the aforementioned microorganisms is conducted quarterly. All experimental procedures were approved by the IACUC of Kyungpook National University (Daegu, Korea).

The cochlea explant culture. Mice (*+/cir* and *cir/cir*) were euthanized by CO_2 inhalation, and their temporal bones mice were isolated aseptically. Cochlea were placed in 6-cm dishes containing ice-cold PBS (pH 7.4), the cochlear capsule was peeled off, and the membranous labyrinth was exposed. The spiral ligament and stria vascularis were removed, and the organ of Corti was dissected into 3 parts (apex, middle, and base). These explants contained the organ of Corti, spiral limbus, spiral ganglion neurons, and modiolus. Each cochlear explant was maintained in 0.5 mL of culture medium consisting of high-glucose (4.5 g/L) DMEM with 10% FBS in a humidified environment containing 5% CO_2 at 37 °C.

Histologic examination and immunostaining. Temporal bones isolated from *+/cir* and *cir/cir* mice were fixed in 4% paraformaldehyde in PBS and decalcified in 10% EDTA. The specimens were cryoprotected in 30% sucrose solution, embedded in OCT compound, cut into sections on a cryostat, and stained with hematoxylin and eosin. For immunostaining, cochlear explants were fixed in 2% paraformaldehyde, rinsed in PBS, incubated in 0.25% Triton X100, immersed in the dye conjugate of phalloidin and tetramethyl rhodamine isothiocyanate (TRITC), and examined under a fluorescence microscope.

Scanning electron microscopy. Cochlea obtained from *+/cir* and *cir/cir* mice and fixed in 2% glutaraldehyde and postfixed in 1% OsO_4. Cochlea were dehydrated through an ethanol series and dried by critical-point drying. Samples were analyzed by scanning electron microscopy (Hitachi, Tokyo, Japan).

Uptake of gentamicin and gentamicin–Texas red conjugate. Cochlear explants were treated with gentamicin (0.5 mM) in high-glucose DMEM containing 10% FBS at37°C and 5% CO_2 for 24 h; a control sample was cultured in parallel in DMEM without getamicin. For immunofluorescence, explants were fixed with 2% paraformaldehyde, and incubated in 0.25% Triton X100. Explants were treated with phalloidin–TRITC, which was used to detect F-actin in stereocilia bundles, and antibody to neurofilament 200 (Sigma-Aldrich, St Louis, MO) for detection of spiral ganglion neuron. Explants then were washed with PBS and incubated with Alex Fluor 488-conjugated goat antirabbit IgG as a secondary antibody. Finally, we counterstained the nuclei of immunostained cells by using DAPI.

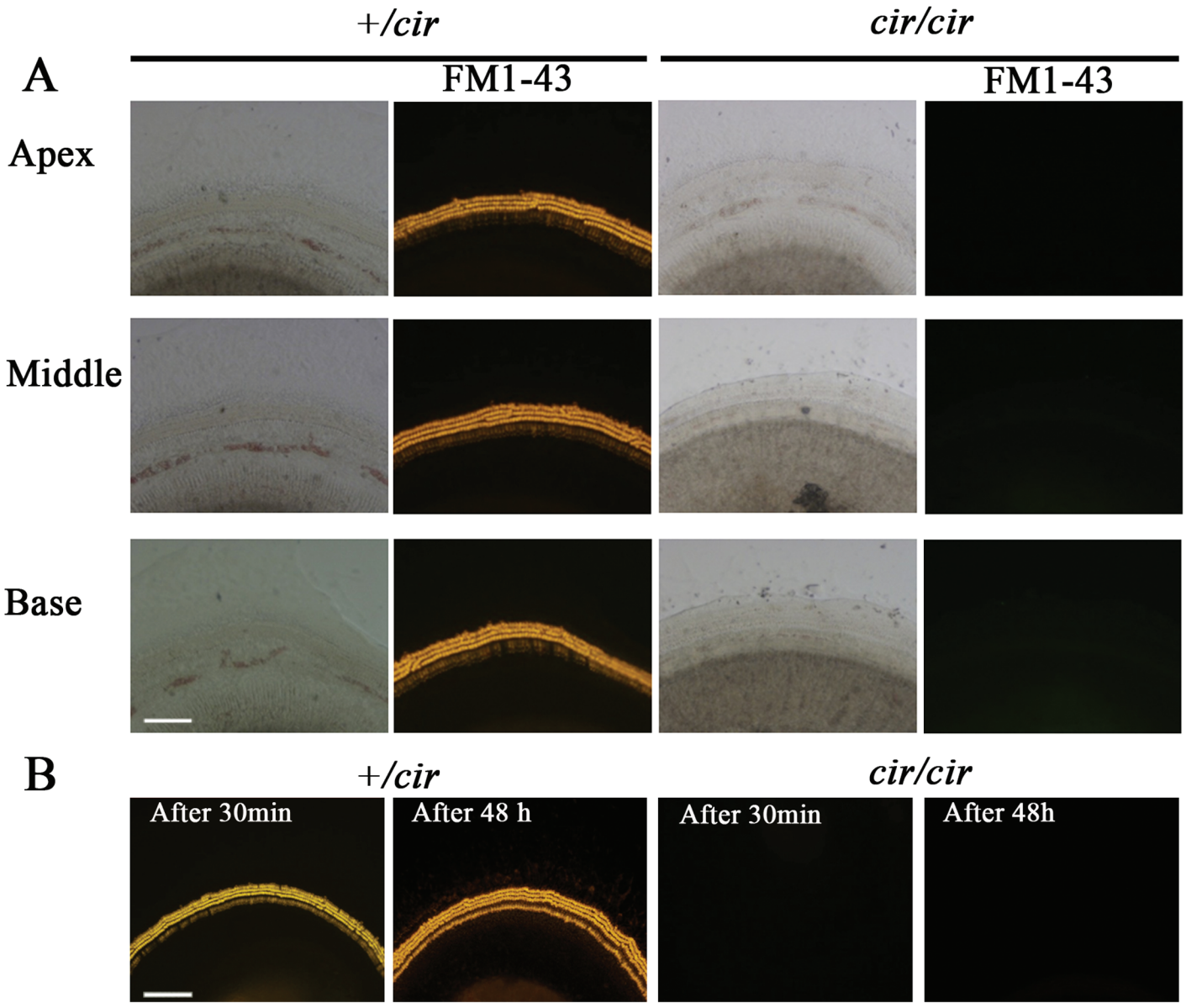

Figure 4. Fluorescence microscopic images of the cochlear hair cells from *+/cir* and *cir/cir* mice exposed to FM1-43 for 30 min. (A) Hair cells from *+/cir* mice took up FM1-43, but those from *cir/cir* mice did not. (B) Observation at 48 h after exposure to FM1-43 confirmed that the positive signal was detected only in *+/cir* mice. Scale bar, 100 µm.

Treatment with gentamicin–Texas-red conjugate (GTTR) was done as described previously.[17] We exposed the apical, middle, and basal turns of cochlear explants from *cir/cir* and *+/cir* mice to DMEM containing 0.5 mM GTTR for 3 h. Explants then were incubated with phalloidin–FITC. Stained samples were observed under a fluorescent microscope equipped with a digital camera, and fluorescent images were capture by using appropriate filters.

Uptake of FM1-43. A stock solution (10 mM) of FM1-43 was prepared in PBS and kept at 4 °C. Immediately before the experiment, the stock solution was diluted in HBSS to a final concentration of 10 µM. Cochlear explants were incubated in FM1-43 dye solution for 30 min and then washed once with HBSS. Samples then were placed in fresh medium without FM1-43 and observed directly under a fluorescence microscope immediately after staining and then 48 h later.

Results

Morphology of cochlea from *+/cir* and *cir/cir* mice. We noted no differences between the organs of Corti of 3-d-old *+/cir* and *cir/cir* mice (Figure 1 A). Likewise, the morphology of hair cells in the 3 regions of the cochlea (the basal, middle, and apical turns) were equivalent in both genotypes. Furthermore, scanning electron microscopy revealed no noteworthy differences in the stereocilia of *+/cir* and *cir/cir* mice (Figure 1 B and C). These results suggest that the structure of the inner ear of *cir/cir* mice is complete before it begins to degenerate soon after birth.

Effect of gentamicin in the cochlea explants of *cir/cir* mice. To confirm the effects of gentamicin on the inner ear of *cir/cir* mice, we cultured cochlear explants in media containing gentamicin. Before gentamicin treatment, hair cells and spiral ganglion neurons showed no differences in appearance between +/*cir* and *cir/cir* mice (Figure 2 A and C). Treatment with 0.5 mM gentamicin for 24 h led to differences between +/*cir* and *cir/cir* mice. Specifically, gentamicin treatment almost completely eliminated the hair cells of +/*cir* mice (Figure 2 B) but had no effect on those of *cir/cir* mice (Figure 2 D). The spiral ganglion neurons in both +/*cir* and *cir/cir* mice were unaffected by gentamicin.

Analysis of GTTR uptake in hair cells of *cir/cir* mice. We then investigated why gentamicin failed to induce ototoxicity in the hair cells of *cir/cir* mice. To assess whether gentamicin entered the hair cells of *cir/cir* mice, we exposed the apical, middle, and basal turns of cochlea explants from *cir/cir* and +/*cir* mice to DMEM containing 0.5 mM GTTR. GTTR was detected only in the hair cells of +/*cir* mice (Figure 3 A); hair cells from *cir/cir* mice lacked GTTR uptake (Figure 3 B). These data demonstrate that gentamicin did not induce ototoxicity in *cir/cir* mice because their hair cells did not take up the drug.

Analysis of FM1-43 uptake in cochlea of *cir/cir* mice. Many studies suggest that aminoglycosides and FM1-43 use the same route to enter hair cells.[1,7,12-14,22] Therefore, we investigated whether hair cells of *cir/cir* mice take up FM1-43. Whereas cochlear hair cells from +/*cir* mouse became strongly fluorescent due to FM 1-43 uptake (Figure 4 B), those from *cir/cir* mice did not (Figure 4 A), even at 48 h after exposure to the compound.

Discussion

Due to a genomic deletion that includes the *tmie* gene, *cir/cir* mice show remarkable defects in the inner ear. Specifically, *cir/cir* mice are deaf and exhibit abnormal behaviors because of degeneration of the inner ear structure. However, the reasons underlying the abnormalities of *cir/cir* mice have not yet been elucidated but may be revealed once the function of the tmie protein is determined. To this end, we compared 3-d-old *cir/cir* (that is, before this genotype begins to show degeneration of the hair cells) and phenotypically normal +/*cir* mice of the same age. Our results revealed that the morphology of cochlear hair cells from *cir/cir* mice was normal. However, unlike findings from +/*cir* mice, gentamicin was not ototoxic to *cir/cir* hair cells, nor did they take up GTTR or FM1-43. In addition, gentamicin, GTTR, and FM1-43 showed similar lack of effect in vestibular hair cells from *cir/cir* mice (data not shown).

One possible explanation for our results is that *tmie* is required for the normal structure or function of the mechanotransducer channel and that deletion of *tmie* prevents mechanotransduction in the hair cells of *cir/cir* mice. In zebrafish, reduction of *tmie* expression is associated with the lack of tip links in their stereocilia.[8] Many studies have suggested[1,7,12-14,22] that aminoglycosides and FM1-43 enter the hair cells of the inner ear via the mechanoelectrical transduction channel. FM1-43 was shown to enter hair cells in *Xenopus* via the putative mechanosensitive cation channel in the plasma membrane.[16] In addition, the main route for the entry of aminoglycosides and GTTR into cochlear hair cells is through mechanotransducer channels.[1,13,22] Perhaps *cir/cir* mice have a defect in mechanotransduction and perhaps *tmie* is required for this process, thereby explaining our current findings.

An alternative explanation is that *cir/cir* mice lack mature sensory cells. During the early postnatal period, the expression of *tmie* spreads from the stereocilia to cells throughout the organ of Corti.[20] These results suggest that the tmie protein plays a role in the maturation of the inner ear and therefore in the maintenance of inner ear cells.[20]

Although the function of the tmie protein has not been elucidated conclusively, our current results suggest that it is important for mechanotransduction in the hair cells of the inner ear. To determine the role of tmie in the inner ear, it is necessary to investigate whether the hair cells of *cir/cir* mice function normally before the structure of the inner ear is degenerated. In addition, the expression patterns of *tmie* from embryonic developmental stages to the adult stage and in various tissues should be explored. Finally, long-term observation of the behavior of *cir/cir* mice may facilitate elucidation of the function of tmie and other aspects of the development and mechanotransduction of the inner ear.

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