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Spatiotemporal Expression of *tmie* in the Inner Ear of Rats during Postnatal Development

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The circling (*cir*/*cir*) mouse is a murine model for human nonsyndromic deafness DFNB6. Transmembrane inner ear (*tmie*) is the causative gene and its mutation through deletion of a 40-kilobase genomic region including *tmie* leads to deafness. The function of Tmie is unknown. To better understand the function of Tmie, we focused on the spatiotemporal expression of *tmie* in the rat cochlea by using a Tmie-specific antibody. Results showed that *tmie* expression was prominent in early postnatal rat cochleas in the stereocilia bundles of hair cells. The Tmie signal spread from the stereocilia to the hair cell body region and on to organ of Corti cells. No Tmie signal was observed in cell nuclei; Tmie was localized to the cytoplasm. Because Tmie is predicted to have 1 or 2 transmembrane domains, we postulate that it is localized to membrane-based organelles or the plasma membrane. Our results imply that Tmie exists in the cytoplasm and may have a key role in the maturation and structure of stereocilia bundles in developing hair cells. After hair cell maturation, Tmie is thought to be involved in the maintenance of organ of Corti cells.

Circling is often observed in mouse and rat deafness mutants and is commonly suggested to be a consequence of inner ear defects that impair vestibular systems.[3,12,14] The circling (*cir*/*cir*) mouse is a murine model for human nonsyndromic deafness DFNB6; these mice have abnormal circling behavior, suggesting a balance disorder, and profound deafness.[6,7] The most notable pathologic phenotypes of circling mice are the almost completely degenerated cochlea and remarkably reduced cellularity in spiral ganglion neurons. The causative gene for circling is *transmembrane inner ear* (*tmie*), with a 40-kilobase genomic deletion including *tmie*.[1] *tmie* is also the causative gene of the spinner (*sr*/*sr*) mouse, which has phenotypes similar to circling mice, although the mutation patterns are different.[8] Spinner mice also show circling behavior, hearing loss, imbalance, and swimming inability. In addition, spinner mice have 2 mutations in the *tmie* gene: the 40-kb genomic deletion including *tmie* and a point mutation that leads to a truncated protein.[8]

In humans, 7 different homozygous recessive mutations in TMIE currently are known to exist in affected members of consanguineous families segregating severe-to-profound prelingual deafness, consistent with linkage to DFNB6.[9,10] Although the functions of murine Tmie and human TMIE are unknown, this protein appears to be important for normal hearing and vestibular function.

In a previous study, we produced transgenic mice overexpressing *tmie* that resulted in phenotypic rescue of circling.[11] Normal expression of transgenic *tmie* induced phenotypic rescue in circling homozygous mutants, although some mice did not show amelioration of abnormal behavior, hearing ability, or tissue morphology in the inner ear. Therefore the Tmie protein is required for normal inner ear function in mouse.[11]

To better understand the function of Tmie, we focused on the spatiotemporal expression of *tmie*. Knowing when, where, and to what extent this protein is produced in the developing inner ear will provide important clues to protein function. In adult mouse and rat, *tmie* is expressed in various tissues.[2,13] Whether Tmie plays an important role in those tissues is uncertain, because circling mice that lack the entire *tmie* gene have no noteworthy problems in any tissues except those of the inner ear systems.[6]

In this study, we were interested in the postnatal stages before and after the onset of hearing (around postnatal day [P] 12) in rats; therefore, the postnatal period P0 to19 was studied. Although all the cells that form the mature cochlea are present at birth, important conformational changes occur during this period, including the formation of the tunnel of Corti and the establishment or retraction of neuronal connections. The expression pattern of *tmie* in the developing inner ear during early postnatal development has not been investigated previously. Here we document our use of a Tmie-specific antibody to elucidate the spatial and temporal expression of *tmie* in the rat inner ear during postnatal development.

Case Report

Sprague–Dawley rats were used in this study. The animals were provided with a commercial diet and water ad libitum and housed at 22 ± 2 °C, relative humidity of 50% ± 5%, and with a 12:12-h light:dark cycle (lights on, 0730 to 1930). Rats were kept in a specific-pathogen–free conditioned animal care facility and were free of the following microorganisms: Sendai virus, *Mycoplasma pulmonis*, Tyzzer disease organism, *Pasteurella pneumotropica*,

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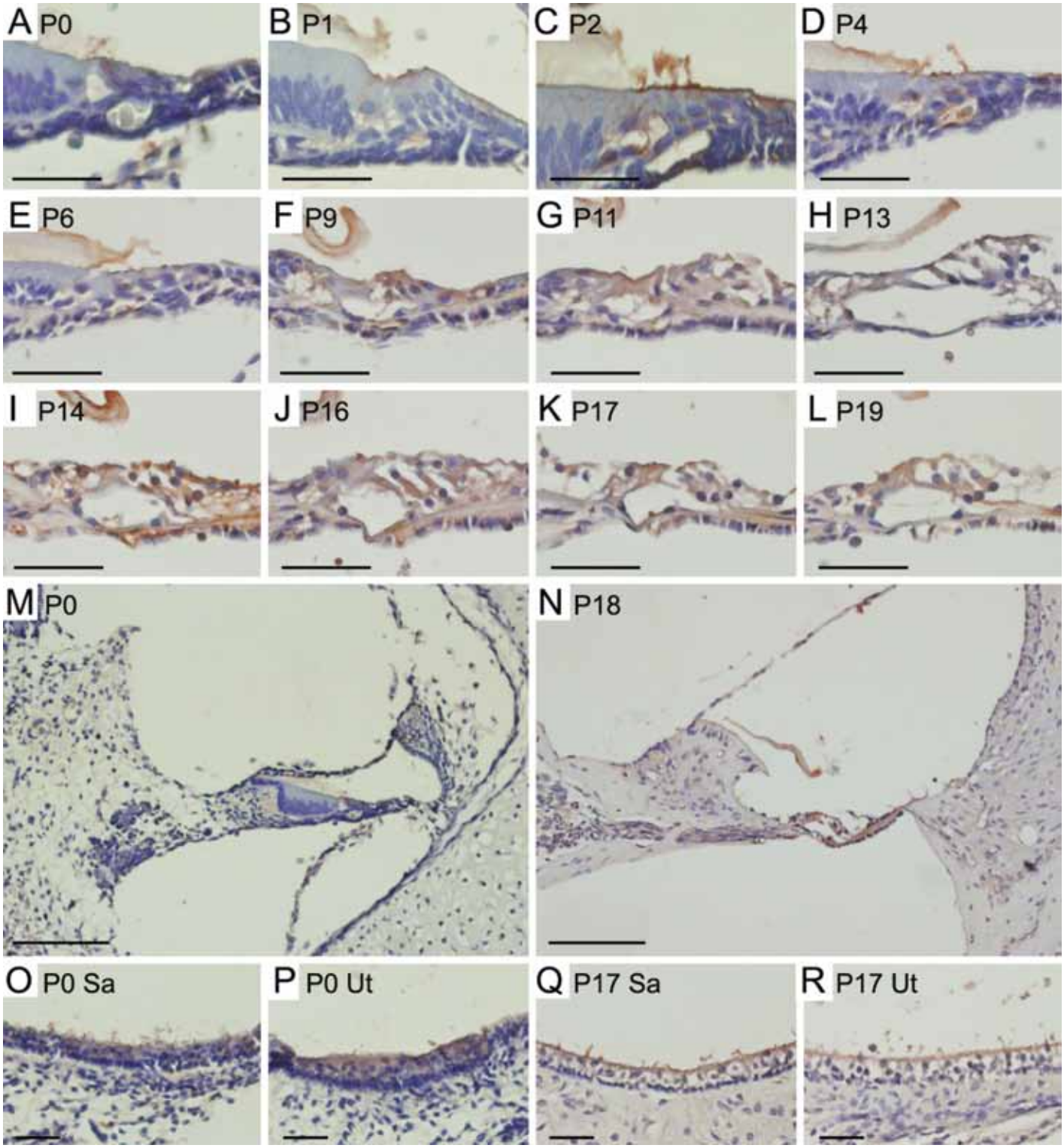

Figure 1. Immunohistochemical analysis of *tmie* expression (brown) in the inner ear during postnatal development. For *tmie* expression analyses during the early postnatal period, cochlear samples were collected from postnatal day (P) 0 to P19. Positive signals were detected in stereocilia bundles in the inner and outer hair cells from P0; expression increased and expanded as the organ of Corti matured(A–N). Expression in vestibular systems showed no obvious change between P0 and P17 (O–R). The nuclei of immunostained cells were counterstained with Mayer hematoxylin. Each panel indicates the day that the sample was collected. Sa, saccule; Ut, utricle. Scale bars: 50 μm (A–L, O–R), 100 μm (M, N).

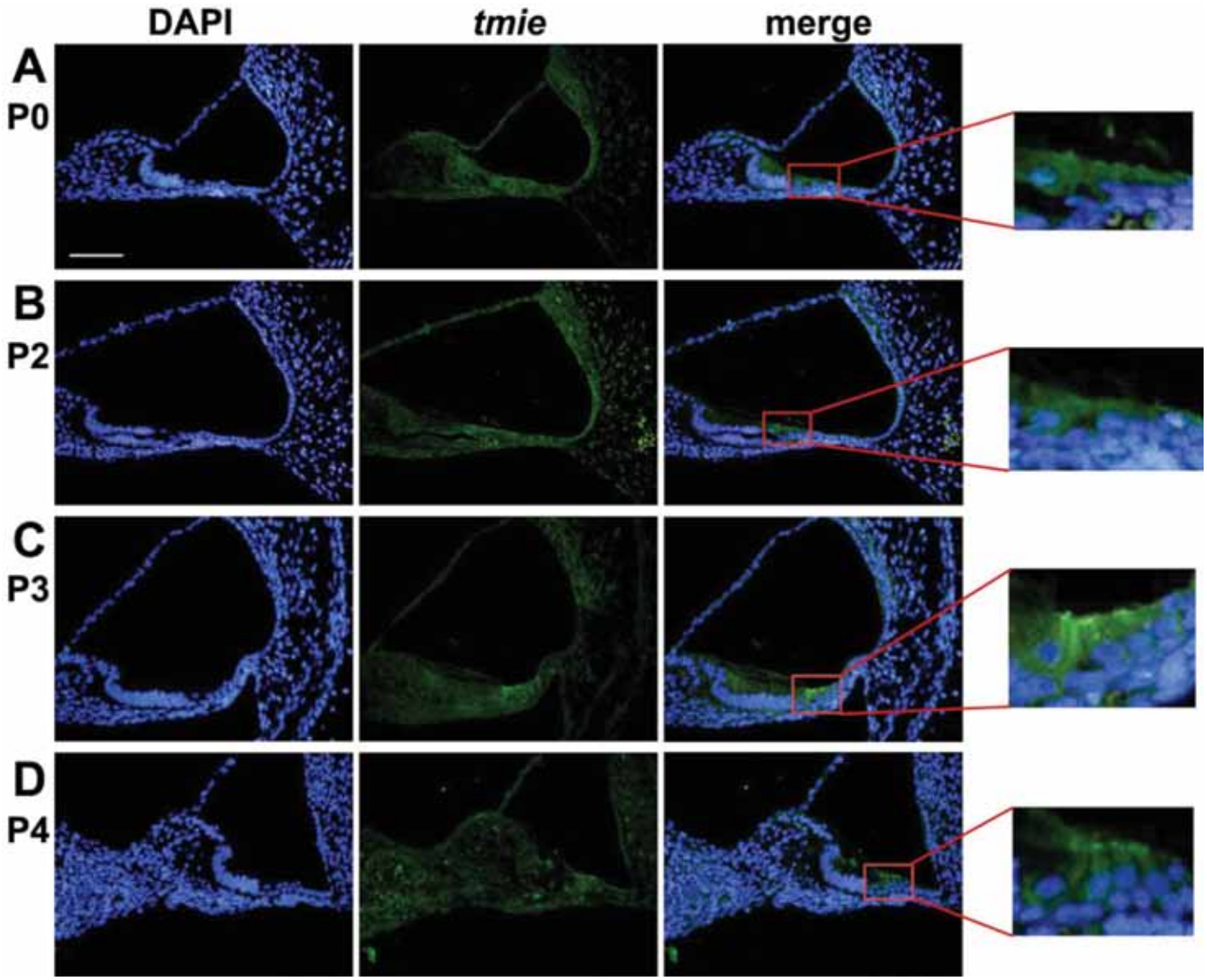

Figure 2. Immunofluorescence of Tmie antibody-positive signals (green) in the organ of Corti. The far right panels of each figure show magnifications of the areas boxed in the third panels. In the early postnatal period, Tmie signals were clearly detected in the stereocilia bundles of inner and outer hair cells. The nuclei of immunostained cells were counterstained with DAPI. Each figure indicates the day that the sample was collected. Scale bars, 70 μm.

Salmonella spp., *Corynebacterium kutscheri*, *Pseudomonas aeruginosa*, and *Bordetella bronchiseptica*. Microbiologic monitoring for the listed microorganisms was conducted quarterly. At least 3 subjects were used in each experiment. All experimental procedures were approved by the Institutional Animal Care and Use Committee at Kyungpook National University.

Immunohistochemical analysis of Tmie in the postnatal rat inner ear. Antibodies to Tmie were generated by immunizing a rabbit (Peptron, Daejeon, Korea) with a synthetic peptide (corresponding to aa118–133, Refseq no. NP_666372) from mouse *tmie*, which is identical to that of rat. For *tmie* expression analysis, cochlear samples were collected from P0 to P19. The inner ear tissues of rats were fixed by cardiac perfusion with 2.5% glutaraldehyde and 4% paraformaldehyde in PBS. After 3 to 4 d of fixation, the removed temporal bone was fixed in 4% paraformaldehyde for 16 h, decalcified with 10% EDTA in PBS for 2 wk, dehydrated, and embedded in paraffin wax. Sections of 4 μm were deparaffinized in xylene and rehydrated through graded concentrations of ethanol. For the immunohistochemical study, LSAB-kit Universal K680 (DAKO, Carpinteria, CA) was used according to the manufacturer's instructions. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 5 min at room temperature. Sections were washed in PBS, and nonspecific binding was blocked with 1% bovine serum albumin for 1 h. Primary antibody (antiTmie, 1:50 dilution) was added to the section and incubated for 1 h. After repeated washes with PBS, the section was incubated with a biotinylated secondary antibody for 1 h and then covered for 15 min with streptavidin peroxidase. Finally, after repeated washes with PBS, the section was stained in a freshly prepared substrate solution (3 mg 3-amino-9-ethylcarbazole in 10 mL 3 M sodium

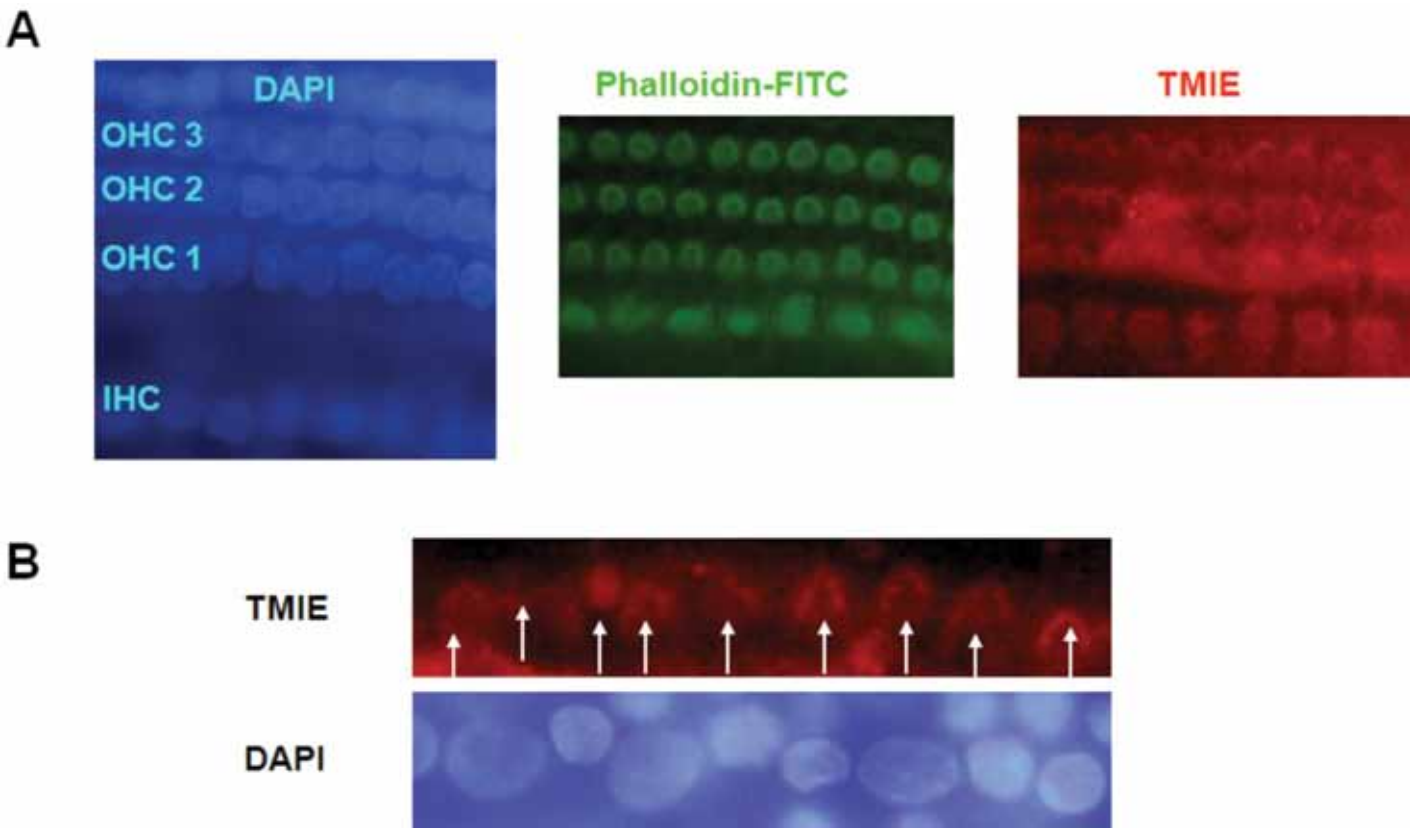

Figure 3. Detection of Tmie (red) in the hair cell stereocilia of cochlear explants from P3 rat inner ear. Phalloidin–FITC was used to detect F-actin in V-shaped stereocilia bundles (green). The nuclei of immunostained cells were counterstained with DAPI. (A) Positive signals were detected exclusively in the V-shaped stereocilia region (B) but not in round-shaped nuclei stained with DAPI. IHC, inner hair cell; OHC, outer hair cell.

acetate buffer [pH 4.9], 500 µL dimethylformamide, 0.03% hydrogen peroxidase) for 10 min. The nuclei of immunostained cells were counterstained with Mayer hematoxylin (Sigma-Aldrich, St Louis, MO).

At P0 to 1, weak *tmie* expression was detected in the stereocilia of hair cells in the cochlea (Figure 1 A, B). Stria vascularis, spiral limbus, spiral ligament, and spiral ganglion cells had very weak or no Tmie antibody staining. At P2, expression was also seen in the cell body region of inner and outer hair cells (Figure 1 C). Expression in the stereocilia region was stronger. This pattern continued to P6, and the signal increased in the cell body from P9 to P13 (Figure 1 E–H). At P14, *tmie* was highly expressed in organ of Corti cells (Figure 1 I). Strong immunoreactivity for Tmie was detected in inner and outer hair cells, as well as in supporting cells. After P14, *tmie* expression remained strong through P19 (Figure 1 J–L). Expression in the organ of Corti at P18 was clearly much stronger than that at P0 (Figure 1 M, N). *tmie* was expressed at relatively low levels at P0 and gradually increased during development. In the vestibular systems, Tmie was detected in the hair cells of the saccule and utricle at P0 (Figure 1 O, P). We also observed immunoreactivity in these tissues at P17; the extent of *tmie* expression was not notably different from that at P0 (Figure 1 Q, R).

Immunofluorescence of Tmie antibody-positive signals in the rat organ of Corti. We also performed immunofluorescent staining in the organ of Corti from P0 to P4 (Figure 2). For immunofluorescence, sections treated with the primary antibody were washed 3 times with PBS and incubated with Alexa Fluor488-conjugated goat antirabbit IgG at 1:150 dilution in 1% bovine serum albumin in PBS for 1 h at room temperature, followed by 3 washes with PBS. In the final step, the nuclei of immunostained cells were counterstained with DAPI.

The Tmie signal in the cochlea at this early time point was weak, but it was clearly much stronger in the stereocilia of hair cells than in the cell body region. Consistent with the results shown in Figure 1, Tmie immunoreactivity was weak at P0 to 2 (Figure 2 A, B) and became much stronger at P3 to 4 (Figure 2 C, D). We did not detect *tmie* expression in the nucleus.

Tmie in the hair cell stereocilia of rat cochlear explants. The organ of Corti was prepared for histologic analysis. The temporal bone was fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) overnight at 4 °C. After fixation, the otic capsule was removed, and the cochlea was microdissected into individual turns. The specimens were rinsed in 0.1× PBS, incubated in 0.25% Triton X100 for 2 min, and immersed in a rhodamine (TRITC)-labeled phalloidin (catalog no. P1951, Sigma; 1:4000) in PBS for 20 min. After 3 washes with PBS, the specimen was examined under a fluorescence microscope with appropriate filters (excitation, 510 to 550 nm; emission, 590 nm).

To ensure that *tmie* expression is restricted to the stereocilia bundles within each hair cell during the early postnatal period, we performed immunostaining in cochlear explants. Phalloidin–FITC was used to detect F-actin in stereocilia bundles. A Tmie signal was clear in the V-shaped stereocilia (Figure 3). We observed

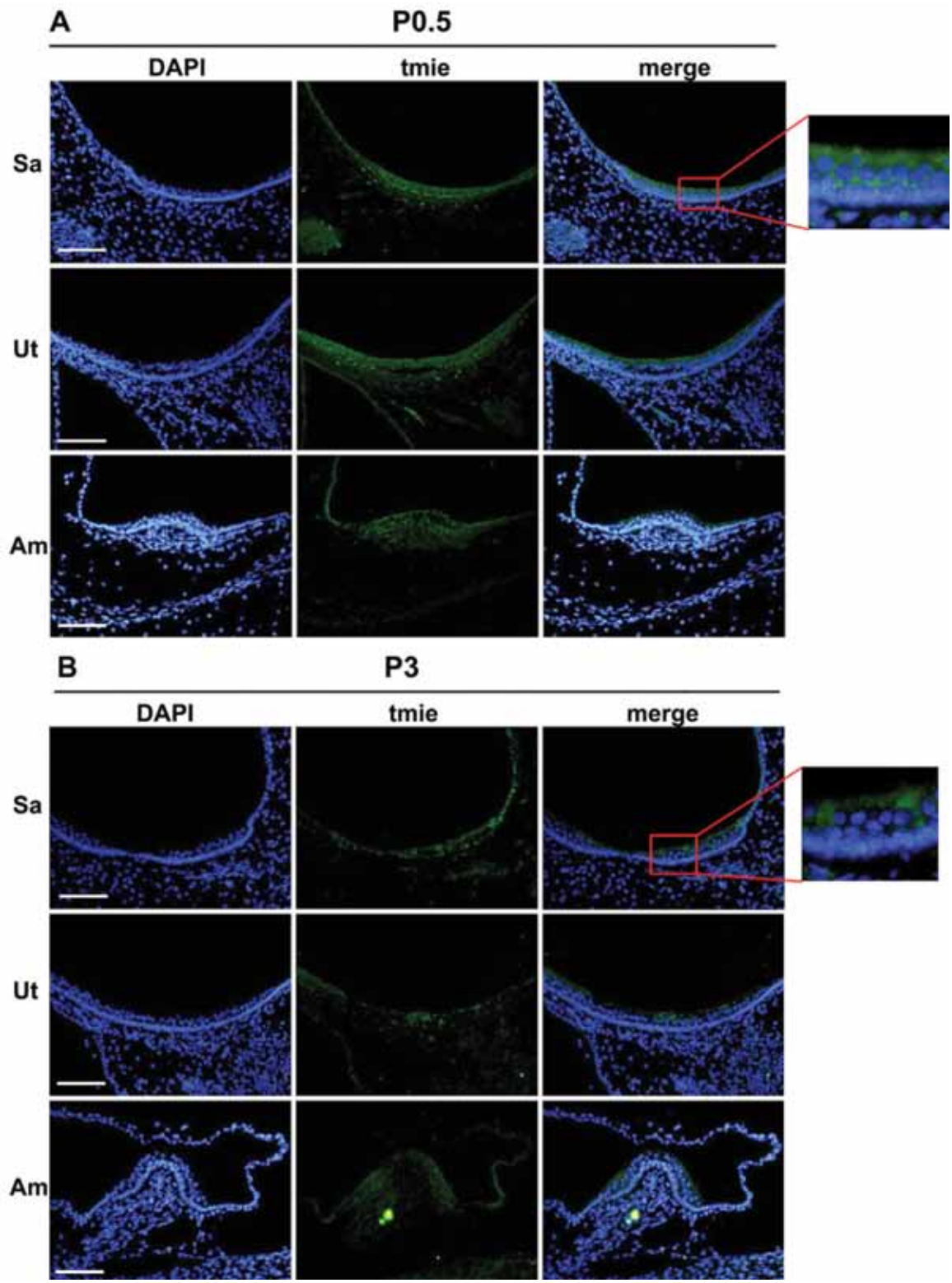

Figure 4. Immunofluorescence of Tmie in the vestibular system in the early postnatal period. Immunofluorescent staining was performed in the hair cells of vestibular systems from P0.5 and P3 rats. The nuclei of immunostained cells were counterstained with DAPI. The far right panels show magnifications of the areas boxed in the third panels. No positive signals were detected in nuclei (magnified images). Sa, saccule; Ut, utricle; Am, ampulla. Scale bars, 100 μm.

no Tmie antibody signals in cell nuclei (Figure 3 B). These results clearly suggest that Tmie localizes to the hair cell stereocilia bundles of the neonate rat cochlea.

Detection of Tmie signals in vestibular hair cells during postnatal rat inner ear development. We also performed immunofluorescent staining in the hair cells of vestibular systems from P0.5 and P3 rats (Figure 4). In immunofluorescent staining of vestibular systems at P0.5, Tmie was detected in stereocilia at the surface of the saccule and utricle (Figure 4 A). No changes in the extent of *tmie* expression were observed between P0.5 and P3 (Figure 4). Similar to the immunostaining of the cochlea region, nuclei were negative for Tmie.

Discussion

The circling mouse lacks an intact *tmie* gene due to genomic deletion of a region in that gene. However, circling mice show no malformations or functional problems other than those associated with the inner ear. Therefore we evaluated the pattern of *tmie* gene expression in the inner ear. Tmie was detected in hair cell stereocilia bundles of both the cochlea and vestibular systems in the rat inner ear from P0. Tmie signal increased and expanded as the hair cells matured.

During late embryonic days in rat, microvilli of the organ of Corti begin to elongate. As stereocilia thicken and continue to elongate, a staircase pattern emerges around P1, with morphologic development complete around P5 to 7. In the present study, *tmie* expression in these early postnatal days was prominent in the stereocilia bundles of hair cells. Tmie signal spread from the stereocilia to the hair cell body and further to the organ of Corti cells. These results imply that Tmie may have a key role in the maturation and structure of stereocilia bundles in developing hair cells. After hair cell maturation, we hypothesize that Tmie is involved in the maintenance of organ of Corti cells.

Cadherin 23 participation in stereocilia links is restricted to developing hair bundles, and expression ceases in maturing hair cells at P16, when the staircase architecture of stereocilia bundles develops and some of the transient links disappear.[5] With expression patterns different from those of cadherin 23 during inner ear development, Tmie was present in stereocilia bundles in early postnatal days, but expression spread to the cell body and later to the whole organ of Corti. As mentioned earlier, the presence of Tmie protein in mature stereocilia bundles may indicate that it has broad functions, from early development to adulthood.

Tmie is required for normal postnatal maturation of sensory hair cells in the cochlea, including correct development of stereocilia bundles.[8] Therefore, the presence of Tmie in the stereocilia suggests that the protein plays a role in maintaining the structure or function of these specialized microvilli.

The function of Tmie was previously unknown. The exact structure of Tmie has been predicted by using many protein prediction programs.[4] Analysis of human *TMIE* with the program TMHMM predicts an intracellular amino terminus, 2 transmembrane domains, and an intracellular carboxy terminus.[4] The MemO and LOCATE algorithms predict that mouse Tmie has a transmembrane domain (aa57 to 79) and 2 nontransmembrane domain regions (aa1 to 56, 80 to 153), and signals for TGN-endosome sorting (aa91 to 96, 115 to 120, 133 to 138). The actual predictions depend on the method, but their similarities are helpful to researching Tmie function.

In the present study, we observed no Tmie signal in cell nuclei. Tmie is localized to the cytoplasm, but we did not confirm the exact places that Tmie exists. We postulate that Tmie is localized to membrane-based subcellular organelles or the plasma membrane because the protein is predicted to have 1 or 2 transmembrane domains. To determine the subcellular distribution of Tmie, it is helpful to predict the role of Tmie.

In adult mouse and rat, *tmie* is expressed in various tissues.[2,3] Functional research on Tmie needs to be assessed in light of its predicted structure to determine the exact functions of Tmie in various tissues. Tmie is required for normal function of the inner ear in mouse and rat. Given that *tmie* is expressed in various tissues as well as the inner ear, Tmie might have more ubiquitous functions than are appreciated currently. However, its role in other organs or tissues is unknown. To determine whether Tmie has a role in the structural formation of the cochlea, it is necessary to investigate its expression in embryonic developmental stages.

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