Statistical analysis

Data were expressed as mean±SEM. Input/output function data of the amplitudes were analyzed via a non-repeated measures analysis of variance (ANOVA). The significance of DPOAE amplitudes was analyzed further by post hoc multiple comparison tests using the Bonferroni procedure. The statistical difference of DPOAE threshold was determined by a two-sided Mann–Whitney's *U*-test. $P<0.05$ was accepted as the level of significance.

RESULTS

Distortion product otoacoustic emission

DPOAE responses were examined during postnatal development. Non-transgenic mice started to show a measurable response of DPOAE from P12–14 followed by gradual increase of amplitude (Fig. 1A, B, C). Significant differ-

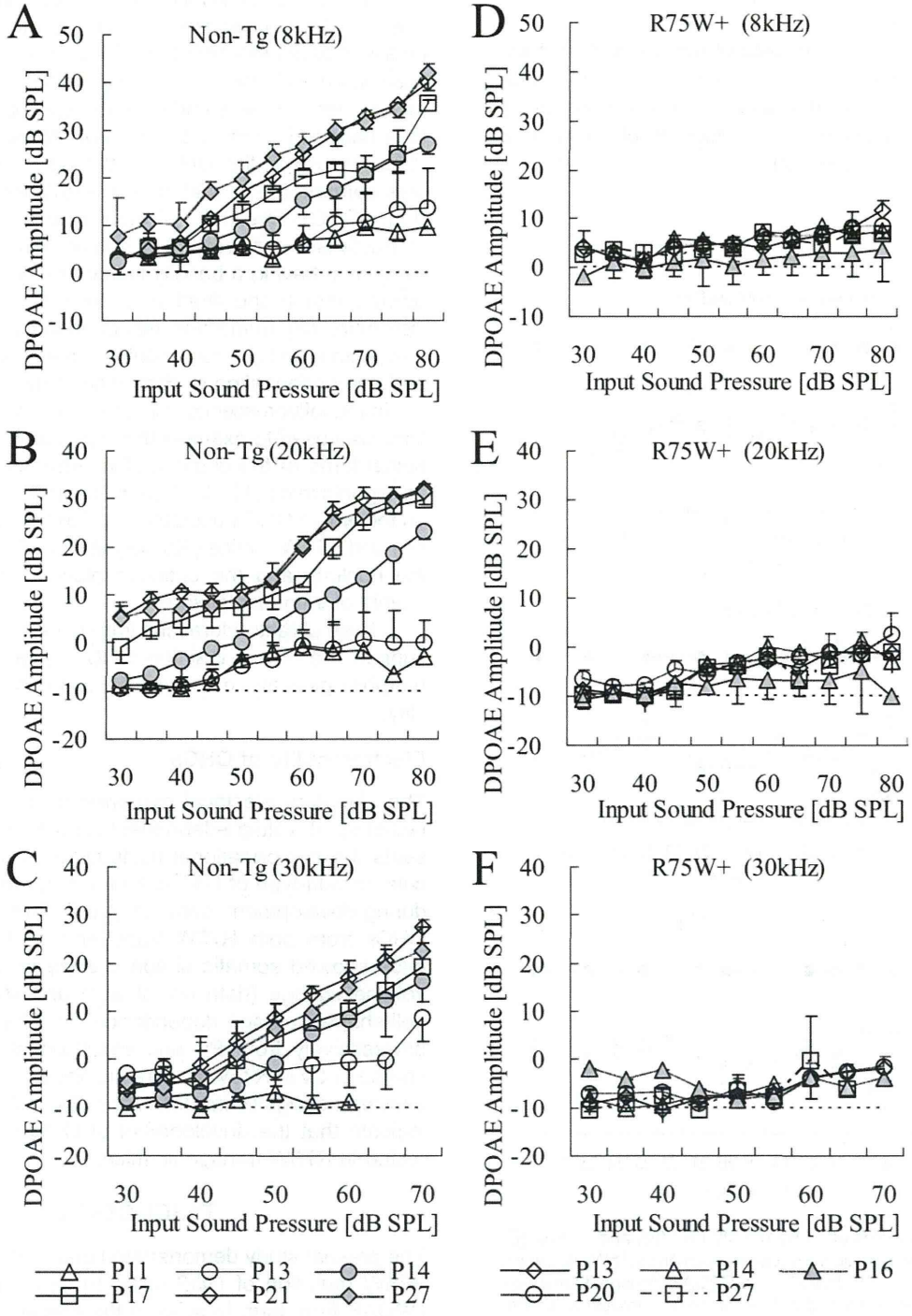

Fig. 1. Input/output function of the amplitudes of non-transgenic (A, B, C) and R75W transgenic (D, E, F) mice at 8 kHz, 20 kHz and 30 kHz frequencies (2f1–f2) from P11 to P27. DPOAE data were plotted as mean±SEM. The dotted line is the noise level. Non-Tg: non-transgenic mice, R75W+: R75W transgenic mice.

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ences of the DPOAE amplitudes of the non-transgenic mice in comparison to noise levels appeared at P12–14 for the different stimuli tested. In contrast, there were no statistically significant differences between noise level and DPOAE amplitudes at 8 kHz, 20 kHz, and 30 kHz throughout postnatal development in the R75W transgenic mice. Furthermore, no DPOAE was detected at any frequencies in R75W transgenic mice throughout postnatal development (Fig. 1D, E, F).

The mean DPOAE thresholds of non-transgenic mice were abruptly reduced around P13–P14 to reach the adult level by P16. In contrast, the mean DOPAE thresholds of R75W transgenic mice stayed at high level throughout postnatal development (Fig. 2).

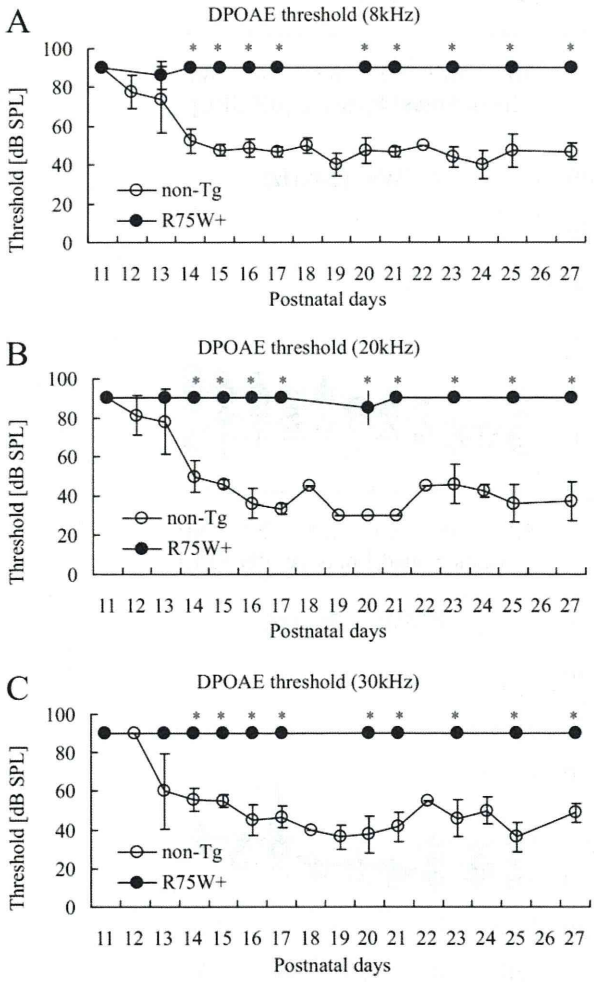

Fig. 2. DPOAE thresholds at 8 kHz (A), 20 kHz (B), and 30 kHz (C) frequencies of non-transgenic mice (open circle) and R75W transgenic mice (filled circle) from P11 to P27. The DPOAE threshold level was defined as the dB level at which the 2f1–f2 distortion product was more than 10 dB above the noise level. In the case of no DPOAE, the threshold level was defined as 90 dB. *: Significant difference between non-transgenic and transgenic mice ($P<0.05$). Non-Tg: non-transgenic mice, R75W+: R75W transgenic mice.

Histology and immunohistochemistry

The cytoarchitecture of the organ of Corti of the R75W transgenic mouse was remarkably different from that of the non-transgenic mouse (Fig. 3A, B). Transverse sections of the organ of Corti in R75W transgenic mouse revealed compression and squeezing of the OHC by the surrounding supporting cells, and Nuel's space around each OHC was occupied by Deiter's cells (Fig. 3B). Structural changes in the OHCs and adjacent cells are likely to restrict the electrically-induced motility of the OHC. The mesothelial cells associated with the basilar membrane in the transgenic mouse were cuboidal and more densely packed in contrast to a flattened layer in the control mouse. However, the ultrastructure of the OHCs in the non-transgenic mouse was comparable to that of the R75W transgenic mouse (Fig. 3C, D). The OHC of both mice showed consistent characteristic features; (i) a relatively high proportion of cytoplasm having a basally located nucleus, (ii) a smooth plasma membrane lined by a thick layer of subsurface cisternae, (iii) numerous mitochondria along the lateral membrane, and (iv) no vacuole formation in the cytoplasm and no condensation of chromatin in the nucleus.

Immunofluorescence microscopy of cross-cochlear sections was used to examine the distribution of prestin in the apical turns of the cochlea of non-transgenic and R75W transgenic mice at P12. Prestin labeling was clearly visible on the whole OHC basolateral wall in both the control (Fig. 4A) and R75W+ mice (Fig. 4B) at P12. On the other hand, the nucleus and the cuticular plate of both mice were devoid of immunostaining.

These ultrastructural and immunohistochemical results support the notion that the OHC are equipped with the morphological and molecular bases to produce electromotility.

Electromotility of OHCs

The signature electrical response of an adult OHC is a bell-shaped, voltage-dependent capacitance, which represents the conformational fluctuations of the motor molecule. In wild-type of C57BL/6J mice, *Cv* increased rapidly during development, saturating at P18 (Abe et al., 2007). OHCs from both R75W transgenic and non-transgenic mice showed somatic shape change in response to the voltage change (data not shown) and showed a typical bell-shaped voltage dependence (Fig. 5A). *Cv* increased progressively from P9 and saturated at P24. The time course of *Cv* in R75W transgenic and non-transgenic mice showed no significant difference (Fig. 5B). These results indicate that the development of OHC motility is not affected in R75W transgenic mice.

DISCUSSION

The present study demonstrated that a dominant-negative R75W mutation of *Gjb2* failed to generate a detectable DPOAE from birth in spite of the presence of OHCs and apparently normal electromotility. The DPOAE depends on two factors, an intact OHC system (Long and Tubis, 1988; Brown et al., 1989) and a positive endocochlear potential

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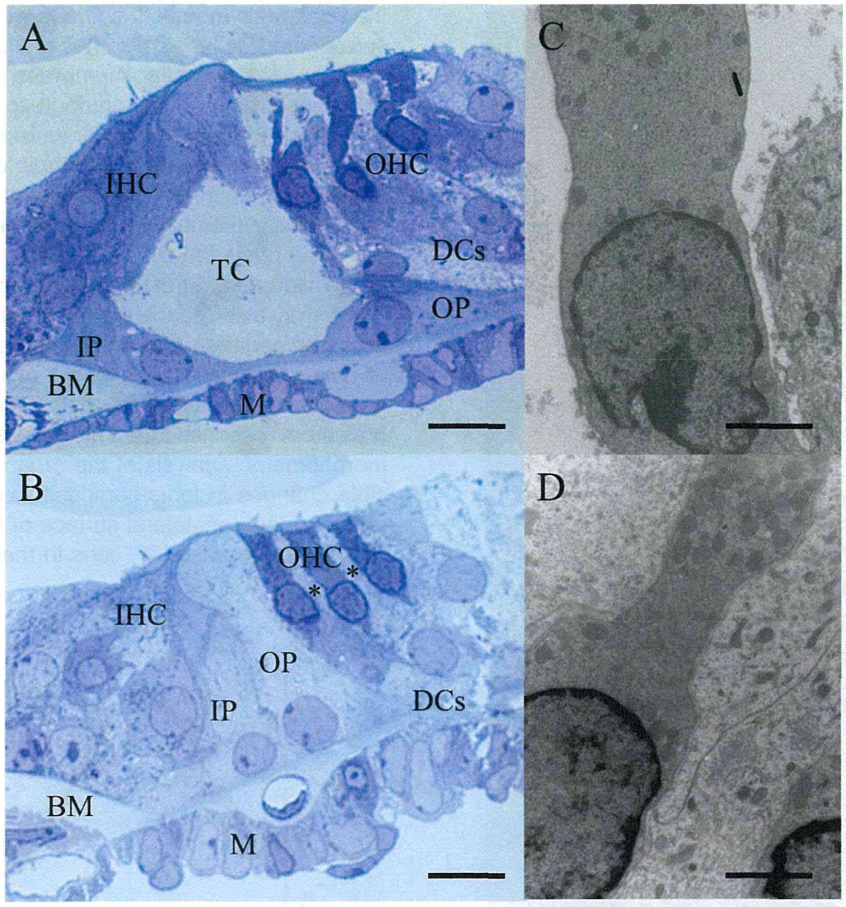

Fig. 3. Histology and transmission electron micrographs of non-transgenic (A, C) and R75W transgenic (B, D) mice. At P12, tunnel of Corti is detected in non-transgenic mice (A), but not (asterisk) in R75W transgenic mice (B). Nuel's space is formed in non-transgenic mice (A, C), but not in R75W transgenic mice (B, D). OHCs are detected in both non-transgenic (A) and R75W transgenic mice, but are squeezed by the surrounding Deiter's in R75W transgenic mice (B). The OHCs showed normal development, with preserved fine structure of the lateral wall, membrane-bound subsurface cisterna beneath the plasma membrane, and enriched mitochondria in both the non-transgenic (C) and R75W transgenic mice (D). Scale bars are 10 μm (A, B) and 2 μm (C, D). Abbreviations used: TC, tunnel of Corti; IP, inner pillar cell; OP, outer pillar cell; BM, basilar membrane; M, mesothelial cell.

(Brownell, 1990). The R75W transgenic mice have a normal endocochlear potential (Kudo et al., 2003). Furthermore, the OHC develops normally with apparently intact fine structure of the lateral wall, including normal membrane-bound subsurface cisterna beneath the plasma membrane. The characteristic phenotype observed in the R75W transgenic mice was the absence of the tunnel of Corti, Nuel's space, and spaces surrounding the OHC, related to abnormal development of the supporting cells.

The mammalian cochlea uses a unique mechanism for amplification of sound signals. Cochlear amplification is thought to originate from (1) somatic motility based on the cochlear motor prestin and (2) hair cell bundle motor related to mechanoelectrical channel (Robles and Ruggero, 2002). Distortion and cochlear amplification are believed to stem from a common mechanism. A recent study (Verpy et al., 2008) postulated that the main source of cochlear waveform distortions is a deflection-dependent hair bundle stiffness derived from stereocilin associated with the horizontal top connectors. However, the relationship between stereocilin and prestin is still unclear.

Somatic electromotility of the OHC is a voltage-dependent rapid alteration of OHC length and stiffness. The electromotility of the OHC is thought to amplify the motion of the basilar membrane at low sound pressure levels and compress it at high levels (Patuzzi et al., 1989; Ruggero and Rich, 1991; Kossl and Russell, 1992). Prestin, which resides in the basolateral membrane of the cochlear OHC (Yu et al., 2006), acts as a voltage-dependent motor protein responsible for OHC electromotility (Belyantseva et al., 2000; Zheng et al., 2000; Liberman et al., 2002). The present study demonstrated that the voltage-dependent, nonlinear capacitance representing the conformational fluctuations of the motor molecule progressively increased from P10 to P18 in *Gjb2* R75W transgenic mice. The developmental changes in the OHC electromotility observed in the *Gjb2* R75W transgenic mice resemble those of both the C57BL/6J mouse in a previous study (Abe et al., 2007) and the littermate non-transgenic mice in the present study.

At least three factors that could explain the discrepancy between the DPOAE and the OHC electromotility

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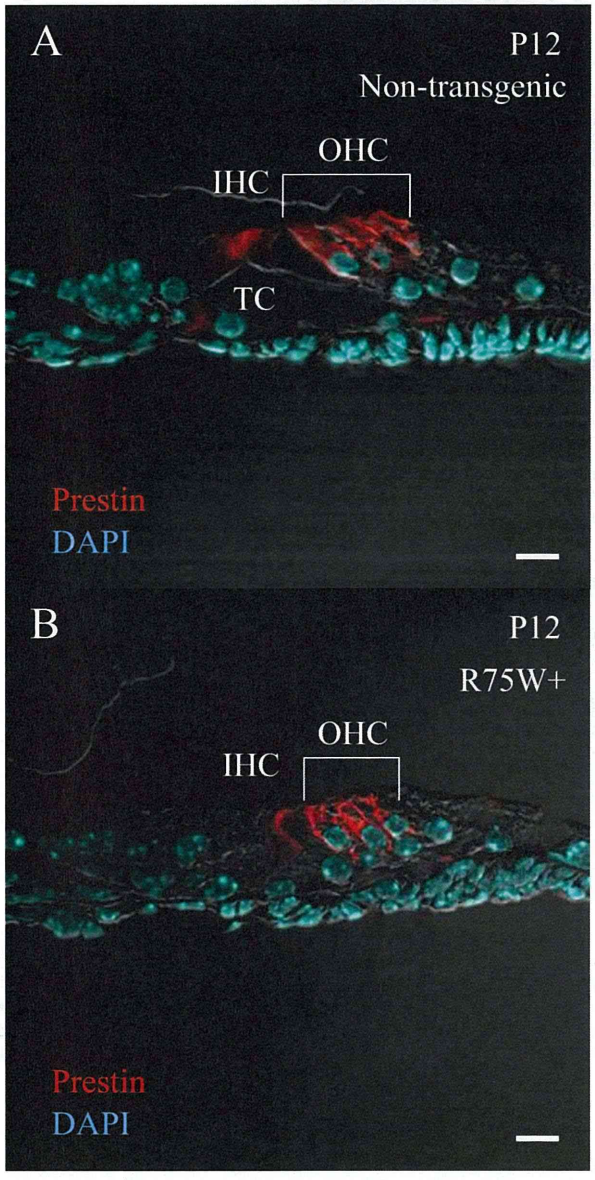

Fig. 4. A cross-sectional immunofluorescent analysis of prestin distributed in the apical turns of the cochlea of non-transgenic (A) and R75W transgenic mice (B) at P12. Prestin labeling (red) is clearly visible on the whole OHC basolateral wall in both the non-transgenic (A) and R75W transgenic mice (B) at P12. The extracellular space around the OHC in R75W transgenic mice is narrower than that in non-transgenic mice. On the other hand, the nucleus stained with DAPI (blue) and the cuticular plate of both mice are devoid of immunostaining. Abbreviations used: OHC, outer hair cell; IHC, inner hair cell. Scale bars are 10 μm (A, B).

arising from the failure of development of the supporting cells can be proposed. First, mature OHCs are supported by underlying Deiter's cells, flanked on the lateral edge by a several rows of Hensen's cells, and anchored by the reticular lamina at their apical surface. The three-dimensional structure of the OHCs enable the longitudinal changes driven by transmembrane potential changes. In the transgenic mouse, the OHCs were compressed by the surrounding Deiter's cells, thus restricting motility. Second, vibration of the basilar membrane may be related to its thickness, which would contribute to the sensitivity and the production of the otoacoustic emissions (Kossl and Vater, 1985) and further to the tonotopic changes of the developing gerbil cochlea (Schweitzer et al., 1996). The thickened basilar membrane observed in the transgenic mice might suppress the DPOAE by reducing the basilar membrane vibration. Structural changes in the basilar membrane may also reduce the sound-induced vibration of the cochlear partition, thus inhibiting deflection of stereocilia on inner hair cells. This could explain why *Gjb2* R75W transgenic mice show remarkable elevation of the auditory brainstem response threshold (Inoshita et al., 2008). Third, morphometric analysis of the organ of Corti suggest possible changes in ionic composition of the cortilymph surrounding the basolateral surface of the OHCs (Inoshita et al., 2008). Increased K^+ ions in the cortilymph would de-

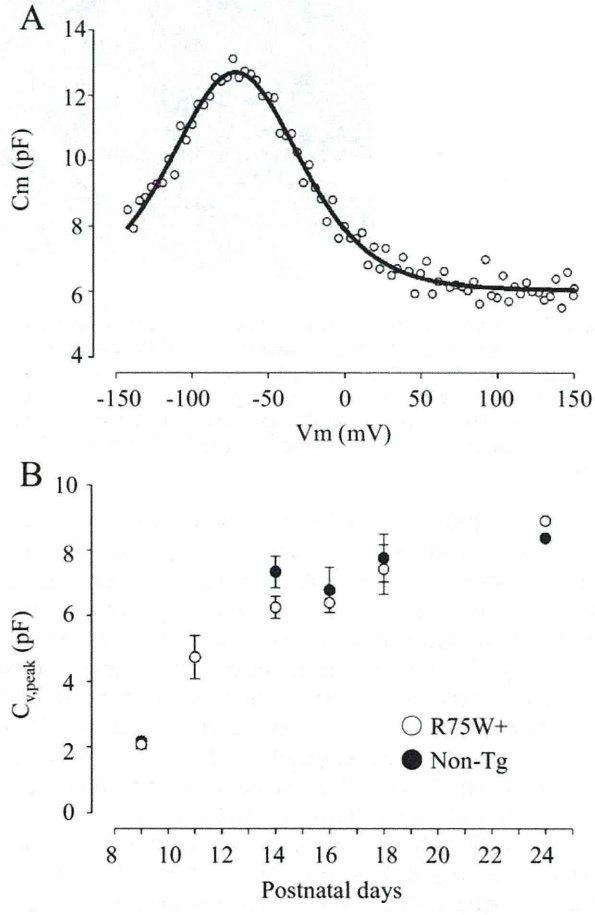

Fig. 5. Electrical responses of isolated OHC. C_m is expressed as a function of V_m at P14 in the R75W transgenic mouse (A). Fitted parameters are Q_{max}=0.704 pC, z=0.89. C_v, peak is expressed as a function of postnatal day (B). The number of cells in non-transgenic (closed circle) and R75W transgenic mice (open circle) was (from P9 to P24) 1–2, 0–3, 2–3, 5–2, 3–3, and 1–1, respectively. Standard error is plotted. Non-Tg: non-transgenic mice, R75W+: R75W transgenic mice.

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polarize the OHCs, and decreased driving force across the mechanosensitive channels could affect OHC electromotility. The progressive degeneration of OHCs observed in the adult R75W transgenic mice (Kudo et al., 2003) may be brought about by disturbed homeostasis of the cortilymph.

The secondary hair cell loss in adult R75W transgenic mice (Kudo et al., 2003; Inoshita et al., 2008) implies that the restoration of hearing requires the regeneration of hair cells in addition to introduction of the *Gjb2* gene. The present study clearly showed both morphological and functional maturation of OHC until late in development, suggesting that a dominant-negative R75W mutation of *Gjb2* does not affect the genes that determine or control the differentiation of the OHC. Therefore, gene transfer of *Gjb2* into the supporting cells before hair cell degeneration could be used to treat deafness. Transgene expression has been accomplished in the supporting cells of the neonatal mouse cochlea using adeno-associated viral vectors without causing additional damage to the cochlea (Iizuka et al., 2008). Therefore, the present study provides a new strategy to restore hearing in *Gjb2*-based mutation.

CONCLUSION

OHC from the dominant-negative R75W mutation of *Gjb2* showed normal development and maturation, and isolated OHC clearly showed voltage-dependent, nonlinear capacitance with characteristic subcellular features. However, the DPOAE, which serves as an index for *in vivo* cochlear amplification, was remarkably suppressed in the mutant mice. This may result from disturbed development of the supporting cells surrounding the OHCs. The present study confirmed that the normal development of the supporting cells is indispensable for the cellular function of the OHC.

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←前号に続く

3．Cell therapy targeting cochlear fibrocytes

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Cell therapy targeting cochlear fibrocytes

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Recently, a number of clinical studies for cell therapy have been reported and clinically used for several intractable diseases. Inner ear cell therapy for sensorineural hearing loss also has been studied using some laboratory animals, although the successful reports for the hearing recovery were still few.

Cochlear fibrocytes play important roles in normal hearing as well as in several types of sensorineural hearing loss due to inner ear homeostasis disorders. Recently, we developed a novel rat model of acute sensorineural hearing loss due to fibrocyte dysfunction induced by a mitochondrial toxin[1), 2)]. In this model, we demonstrate active regeneration of the cochlear fibrocytes after severe focal apoptosis without any changes in the organ of Corti. To rescue the residual hearing loss, we transplanted mesenchymal stem cells into the lateral semicircular canal; a number of these stem cells were then detected in the injured area in the lateral wall. Rats with transplanted mesenchymal stem cells in the lateral wall demonstrated a significantly higher hearing recovery ratio than controls. The mesenchymal stem cells in the lateral wall also showed connexin 26 and connexin 30 immunostaining reminiscent of gap junctions between neighboring cells[3)]. These results indicate that reorganization of the cochlear fibrocytes leads to hearing recovery after acute sensorineural hearing loss in this model and suggest that mesenchymal stem cell transplantation into the inner ear may be a promising therapy for patients with sensorineural hearing loss due to degeneration of cochlear fibrocytes.

Key words：cochlear fibrocyte, inner ear cell therapy, mesenchymal stem cell
和文キーワード：蝸牛線維細胞，内耳細胞療法，間葉系幹細胞

Mammalian cochlear fibrocytes of the mesenchymal nonsensory regions play important roles in the cochlear physiology of hearing, including the transport of potassium ions to generate an endocochlear potential in the endolymph that is essential for the transduction of sound by hair cells[4), 5), 6)]. It has been postulated that a potassium recycling pathway toward the stria vascularis via fibrocytes in the cochlear lateral wall is critical for proper hearing, although the exact mechanism has not been definitively proven[5)]. One candidate model for this ion transport system consists of an extracellular flow of potassium ions through the scala tympani and scala vestibuli and a transcellular flow through the organ of Corti, supporting cells, and cells of the lateral wall[7), 8)]. The fibrocytes within the cochlear lateral wall are divided into type I to V based on their structural features, immunostaining patterns, and general location[8)]. Type II, type IV, and type V fibrocytes resorb potassium ions from the surrounding perilymph and from outer sulcus cells via the Na, K-ATPase. The potassium ions are then transported to type I fibrocytes, strial basal cells and intermediate cells through gap junctions, and are secreted into the intrastrial space through potassium channels. The

secreted potassium ions are incorporated into marginal cells by the Na, K-ATPase and the Na-K-Cl cotransporter, and are finally secreted into the endolymph through potassium channels.

Degeneration and alteration of the cochlear fibrocytes have been reported to cause hearing loss without any other changes in the cochlea in the Pit-Oct-Unc (POU)-domain transcription factor Brain-4 (Brn-4) deficient mouse[9] and the otospiralin deficient mouse[6]. Brn-4 is the gene responsible for human DFN3 (Deafness 3), an X chromosome-linked nonsyndromic hearing loss. Mice deficient in Brn-4 exhibit reduced endocochlear potential and hearing loss and show severe ultrastructural alterations, including cellular atrophy and a reduction in the number of mitochondria, exclusively in spiral ligament fibrocytes[9, 10]. In the otospiralin deficient mouse, degeneration of type II and IV fibrocytes is the main pathological change and hair cells and the stria vascularis appear normal[6]. Furthermore, in mouse and gerbil models of age-related hearing loss[11, 12, 13], degeneration of the cochlear fibrocytes preceded the degeneration of other types of cells within the cochlea, with notable pathological changes seen especially in type II, IV, and V fibrocytes. In humans, mutations in the connexin 26 (Cx26) and connexin 30 (Cx30) genes, which encode gap junction proteins and are expressed in cochlear fibrocytes and non-sensory epithelial cells, are well known to be responsible for hereditary sensorineural deafness[14, 15]. These instances of deafness related to genetic, structural and functional alterations in the cochlear fibrocytes highlight the functional importance of these fibrocytes in maintaining normal hearing.

Generation of the animal model to study cochlear fibrocyte

To study the role of cochlear fibrocytes in hearing loss and hearing recovery, we developed an animal model of acute sensorineural hearing loss due to acute cochlear energy failure by administering the mitochondrial toxin 3-nitropropionic acid (3NP) into the rat round window niche[1, 2]. 3NP is an irreversible inhibitor of succinate dehydrogenase, a complex II enzyme of the mitochondrial electron transport chain[16, 17]. Systemic administration of 3NP has been used to produce selective striatal degeneration in the brain of several mammals[18, 19]. Our model with 3NP administration into the rat cochlea showed acute sensorineural hearing loss and revealed an initial pathological change in the fibrocytes of the lateral wall and spi-

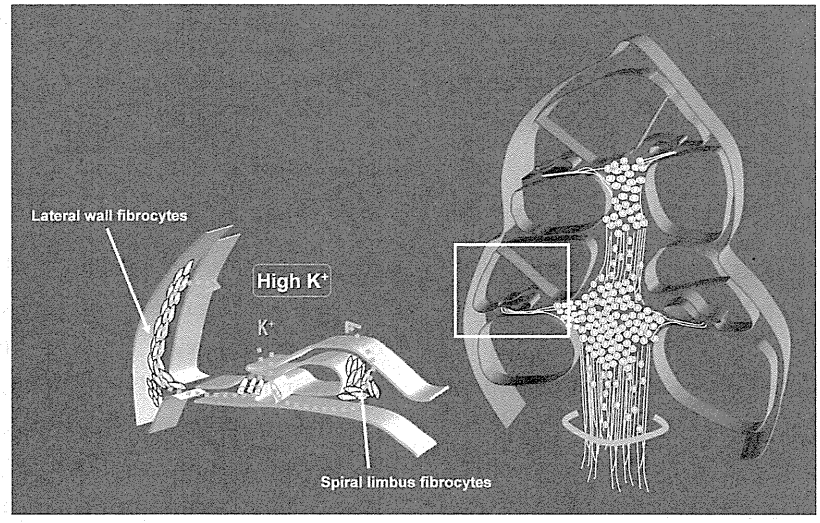

Figure. 1

The localization and the function of cochlear fibrocytes. In mammalian cochlea, ATP-dependent potassium recycling pathways have been well known as the essential mechanism for normal sound input. Cochlear fibrocytes in lateral wall and spiral limbus play a critical role in this potassium recycling system. They transport K+ into the endolymph and keep high K+ concentration mainly by Na+/K+-ATPase and gap junction.

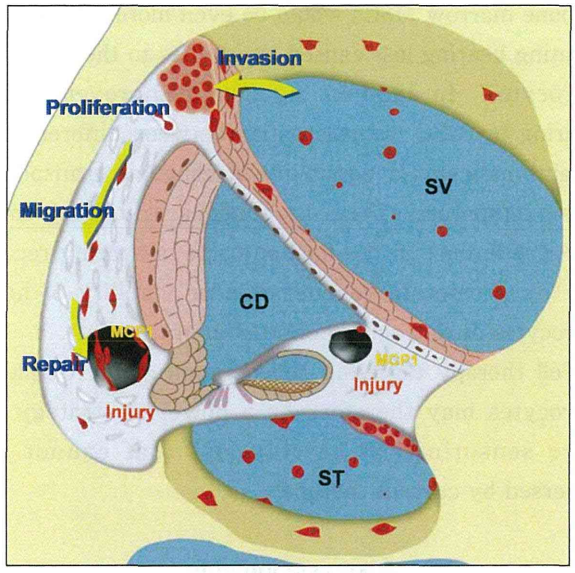

Figure. 2

A summary of the histological observations and our hypothesis for the migration of the transplanted MSCs. Arrows indicate the hypothetical route of MSC migration to the injured area. Some MSCs formed a cell mass around the scala tympani. A number of MSCs successfully invaded the lateral wall. The invading MSCs migrated and proliferated in the lateral wall. Cell migration may be induced by some chemokines such as MCP1 which was detedctd in our DNA microarray analysis. The MSCs which reached the injured area continued to proliferate and repaired the disconnected gap junction network. SV, scala vestibuli; CD, cochlear duct; ST, scala tympani. The schematic illustration was cited and modified from Am J Pathol, 171: 214-226, 2007 Kamiya, et al.

ral limbus without any significant damage to the organ of Corti or spiral ganglion. Furthermore, depending on the dose of 3NP used, these hearing loss model rats exhibited either a permanent threshold shift (PTS) or a temporary threshold shift (TTS). In the following study, we used doses of 3NP that induce TTS to explore the mechanism of hearing recovery after injury to the cochlear fibrocytes, and examined a novel therapeutic approach to repair the injured area using mesenchymal stem cell (MSC) transplantation.

Mesenchymal Stem Cell (MSC) Transplantation

MSCs are multipotent cells that can be isolated from adult bone marrow and can be induced to differentiate into a variety of tissues *in vitro* and *in vivo*[20]. Human MSCs transplanted into fetal sheep intraperitoneally undergo site-specific differentiation into chondrocytes, adipocytes, myocytes, cardiomyocytes, bone marrow stromal cells, and thymic stroma[21]. Furthermore, when MSCs were transplanted into postnatal animals, they could engraft and differentiate into several tissue-specific cell types in response to environmental cues provided by different organs[22]. These transplantability features of MSCs suggested the possibility that they could restore hearing loss in 3NP-treated rats to the normal range. Recently, experimental bone marrow transplantation into irradiated mice suggested that a part of spiral ligament which consists of cochlear fibrocytes was derived from bone marrow cells or hematopoietic stem cells[23]. This indicates that bone marrow derived stem cells such as MSC may have a capacity to repair the injury of cochlear fibrocytes.

MSC transplantation accelerated hearing recover

The 3NP-treated rats showed complete hearing recovery at low frequencies; however, there remained a residual hearing loss at higher frequencies. Considering that the cochlear fibrocytes that were injured in this model are mesenchymal in origin, we transplanted rat MSCs into the cochlea to attempt to rescue the residual hearing loss. We used MSC which we previously established and demonstrated their potential as MSC, and we further confirmed the surface antigen expression of the cells used for transplantation in flow cytometry which showed similar expression pattern to human and murine MSCs. This suggests that the cells maintained the capacity as rat MSC at the moment of transplantation. Because there is no barrier in the inner ear perilymph between the cochlear and vestibular compartments, cells delivered from the lateral semicircular canal by perilymphatic perfusion are considered to have reached the cochlea. Within the perilymph of the cochlea, these cells presumably spread through the scala vestibuli toward the apical turn of the cochlea, and then, after passing through the helicotrema where the scala vestibuli communicates with the scala tympani, kept moving through the scala tympani toward the basal turn. There is no other way in which MSCs can spread within the cochlear perilymph.

Invasion of MSC to lateral wall tissue

Our study clearly demonstrates that rat MSCs were

successfully transplanted into the inner ear of 3NP-treated rats by perilymphatic perfusion from the lateral semicircular canal. A number of MSCs were detected on the surface of the ampullary crest facing the perilymph and some of them were detected within the tissue of the ampullary crest, indicating that MSCs survived at least for 11 d after the perfusion and had maintained their ability to invade and migrate into the inner ear tissue. In the cochlea, a number of MSCs formed cell masses on the surface of the scala timpani, where the majority of the surrounding tissue is bone tissue, suggesting that these MSCs did not invade the cochlear tissue. In the scala vestibuli, a small number of MSCs were also found attached to the surface of the bone and the Reissner membrane. However, in the apical part of the lateral wall, a number of MSCs were observed within the tissue, suggesting that MSCs had successfully invaded the lateral wall from the perilymph. This area may be an optimum site for MSC invasion. Furthermore, we performed DNA microarray analysis of the cochlear lateral wall RNAs in 3NP-treated rats and found a significant increase in the expression of the small inducible cytokine A2 gene encoding monocyte chemoattractant protein 1 (MCP1), which has been reported as a chemokine that induces migration of neural stem cells[24]. This may suggests that the MSC migration to the injured area of the lateral wall in this study may also be induced by chemokines because most MSCs were obsereved in the lateral wall in basal turn which had a prominent damage, but not in the apical turn.

Conclusion

Bone marrow MSCs have greater advantages for clinical use in human subjects than other multipotential stem cells, such as embryonic stem cells, because MSCs can be collected from the patient's own bone marrow for an autologous transplantation with little physical risk, no rejection risk, and few ethical problems. In the present transplantation, many MSCs were confirmed to have invaded the lateral wall and to have contributed to recovery of hearing loss despite transplantation between different rat strains. Therefore, we expect that autologous transplantation of bone marrow MSCs would be even more effective in treating hearing loss caused by injuries to the cochlear fibrocytes. In addition, significant improvement of hearing by MSC transplantation between different rat strains indicates a possibility of allogenic transplant. Even temporary effects by allogenic transplant may cause difference in the final outcome of hearing recovery by promoting regeneration or viability of host fibrocytes during acute period of injury.

Cell therapy targeting regeneration of the cochlear fibrocytes may therefore be a powerful strategy to cure sensorineural hearing loss that cannot be reversed by current therapies.

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