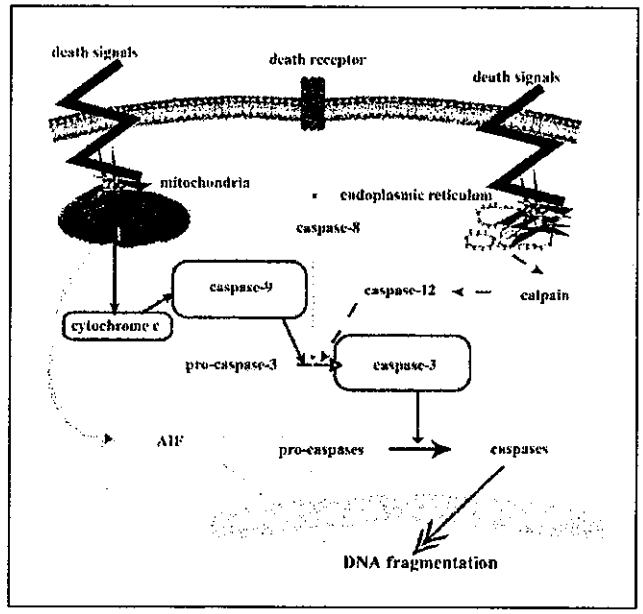
expression of cleaved caspase-8, indicating that the death receptor-mediated pathway is not a major pathway of apoptotic cell death in these cells after treatment with CDDP. However, expression of cleaved caspase-9 was observed in damaged marginal cells. In addition, immunohistochemistry for cytochrome c showed that there was release of cytochrome c from the mitochondria to the cytoplasm in damaged marginal cells. These findings indicate that the majority of damaged marginal cells die via apoptosis initiated by permeabilization of mitochondrial membranes. However, expression of activated caspase-9 was not observed in all of the affected marginal cells. Therefore, there is a possibility that other apoptotic pathways activate caspase-3 in damaged marginal cells. Recent studies have indicated that the mode of action of CDDP involves endoplasmic reticulum stress [24]. It is believed that endoplasmic reticulum stress results in release of calpain which in turn activates caspase-12 leading to activation of caspase-3 (fig. 5) [11]. In addition, carboplatin, a second-generation cisplatin analogue, reportedly causes activation of calpain in nerve fibers in the inner ear [25]. Overall, further studies are required in order to define the signaling pathways involved in CDDP-induced apoptosis of marginal cells.

Reactive Radical Species in Damage of Marginal Cells

Immunohistochemical analysis demonstrates that CDDP induced production of NT and HNE in the SV, indicating the occurrence of protein peroxidation by peroxynitrite and lipid peroxidation by hydroxy radicals in the SV. Notably, strong expression of these two molecules in the SV was observed on day 3 when apoptosis of marginal cells was most likely to be induced. In addition, expression of both molecules in the SV became weaker when the numbers of apoptotic cells in the SV decreased. These findings suggest that peroxynitrite and hydroxy radicals are involved in the induction of apoptotic cell death in the SV. Moreover, both peroxynitrite and HNE have been shown to induce mitochondrial membrane permeabilization [26]. Immunohistochemical analysis for caspases and apoptosis-related proteins indicated that damage to mitochondria might play a key role in the induction of apoptotic death of marginal cells. Therefore, both peroxynitrite and hydroxy radicals may be mediators for induction of apoptosis by CDDP in marginal cells. Peroxynitrite is formed by the reaction of NO with superoxide (fig. 6) [15]. NO is produced by a group of enzymes called NOS. Mammalian systems contain three well-characterized isoforms of NOS. Amongst the three isoforms of NOS, iNOS shows the highest activity. In fact,



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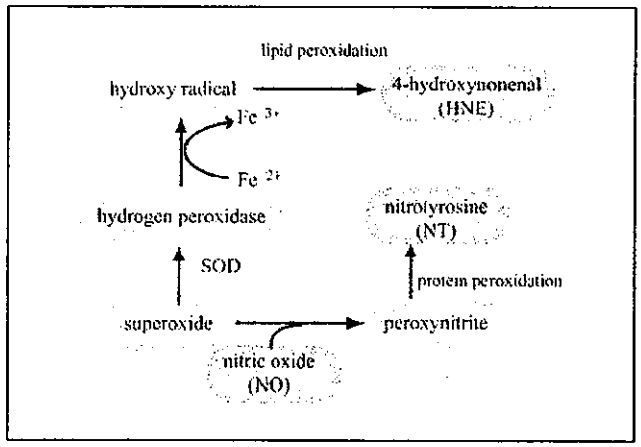

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Fig. 5. Schematic diagram showing the pathways of apoptosis.
Fig. 6. Generation of reactive radical species and their products. SOD = Superoxide dismutase.

overproduction of NO by iNOS is implicated in a number of pathogenic conditions [27] including CDDP-induced degeneration of the SV [28]. Thus, we have examined expression of iNOS in the SV following CDDP treatment. The pattern of expression of iNOS in the SV appears to follow the same time course as that of NT in this study. In fact, overproduction of NO by iNOS may be linked with generation of peroxynitrite in the SV. We suggest that inhibition of iNOS activity may therefore be a cue for protection of the SV from CDDP toxicity.

The present findings have shown that there is generation of HNE in the SV following CDDP treatment, indicating generation of hydroxy radicals. It has been shown that hydroxy radical peroxidation of polyunsaturated fatty acids in cellular membranes results in the production of HNE (fig. 6), which causes further lipid peroxidation and acts as a pro-apoptotic mediator [26]. Therefore, inhibition of hydroxy radical generation is crucial for stopping this chain reaction. Superoxide is metabolized to hydroxy radicals in the presence of iron ions [17]. In addition, superoxide can form peroxynitrite by reaction with NO (fig. 6). Therefore, inhibition of the production of hydroxy radicals from superoxide may result in an increase in peroxynitrite. Reduction of both hydroxy radicals and peroxynitrite by, for example, treatment with an iNOS inhibitor and a hydroxy radical scavenger may be effective for the protection of the SV from CDDP toxicity.

The present findings suggest that CDDP toxicity causes apoptosis initiated by mitochondrial damage in the SV. Apoptosis occurs mainly in marginal cells and is mediated by the formation of peroxynitrite and hydroxy radicals. We suggest that reduction of these reactive radical species may lead to stabilization of mitochondrial membrane permeability, leading to protection of the SV from CDDP toxicity.

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Radixin deficiency causes deafness associated with progressive degeneration of cochlear stereocilia

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Ezrin/radixin/moesin (ERM) proteins cross-link actin filaments to plasma membranes to integrate the function of cortical layers, especially microvilli. We found that in cochlear and vestibular sensory hair cells of adult wild-type mice, radixin was specifically enriched in stereocilia, specially developed giant microvilli, and that radixin-deficient ($Rdx^{-/-}$) adult mice exhibited deafness but no obvious vestibular dysfunction. Before the age of hearing onset (~2 wk), in the cochlea and vestibule of $Rdx^{-/-}$ mice, stereocilia developed normally in which ezrin was concentrated. As these $Rdx^{-/-}$ mice grew, ezrin-based cochlear stereocilia progressively degenerated, causing deafness, whereas ezrin-based vestibular stereocilia were maintained normally in adult $Rdx^{-/-}$ mice. Thus, we concluded that radixin is indispensable for the hearing ability in mice through the maintenance of cochlear stereocilia, once developed. In $Rdx^{-/-}$ mice, ezrin appeared to compensate for radixin deficiency in terms of the development of cochlear stereocilia and the development/maintenance of vestibular stereocilia. These findings indicated the existence of complicate functional redundancy in situ among ERM proteins.

Introduction

Deafness is one of the most prevalent forms of sensory impairment in humans, and it affects ~1 in 1,000 children (for reviews see Morton, 1991; Fortnum and Davis, 1997). Various gene mutations are known to cause hereditary hearing loss in humans (for reviews see Holme and Steel, 1999; Petit et al., 2001; Tekin et al., 2001; Zuo, 2002). Hearing is achieved through a number of steps: first, acoustic stimuli reaching the cochlea deflect the stereocilia on the apical surface of the inner and outer hair cells in the organ of Corti, which in turn opens the mechanotransduction channels located at the tips of stereocilia. Potassium then floods through these open channels, resulting in plasma membrane depolarization, and an electrical signal is transmitted to the central nervous system (for reviews see Roberts et al., 1988; Hudspeth, 1989; Pickles and Corey, 1992). Thus, the stereocilia of cochlear hair cells are key elements in the transduction of acoustic stimuli into electrical signals. Stereocilia also occur in the hair cells of the vestibule to detect acceleration (Eatock et al., 1998; Corey, 2003). Deaf mouse models have provided crucial clues for understanding the molecular and cellular biology of cochlear sensory hair cells, and several forms of hearing loss are now understood to be caused by mutations of the cytoskeletal proteins of cochlear stereocilia (Probst and Camper, 1999; Gillespie and Walker, 2001; Steel and Kros, 2001; Call and Morton, 2002; Zuo, 2002; Belyantseva et al., 2003). In these mice, vestibular stereocilia were also defective, concomitantly causing imbalance.

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Key words: ERM; radixin; stereocilia; cochlea; deafness

Abbreviations used in this paper: ABR, auditory-evoked brainstem response; dB, decibel; ERM, ezrin/radixin/moesin; SPL, sound pressure level; VOR, vestibulo-ocular reflex.

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Stereocilia are specifically developed giant microvilli (finger-like projections of plasma membrane that are underlain by actin filaments). Microvilli show significant variations in morphology, diameter, and length depending on cell types; some are formed by densely packed rigid-looking actin filaments, whereas others are formed by loosely packed fragile-looking actin filaments (Furukawa and Fechheimer, 1997; Bartles, 2000; DeRosier and Tilney, 2000). Of these microvilli, the stereocilia of cochlear sensory hair cells are the most specialized with a characteristic morphology (DeRosier et al., 1980; Gillespie and Walker, 2001; Zuo, 2002). The stereocilia of vestibular sensory hair cells show a more fragile appearance (Sobin and Flock, 1983; Denman-Johnson and Forge, 1999). Besides the general microvilli cytoskeletal proteins such as actin, fimbrin, and myosin Ic, which were immunolocalized to stereocilia (Tilney et al., 1989; Drenckhahn et al., 1991; Skowron et al., 1998; Dumont et al., 2002), the positional cloning of deafness genes in humans and mice has enabled the identification of the novel cytoskeleton-associated constituents of stereocilia such as espin, harmonin, SANS, whirlin, unconventional myosins (VI/VIIa/XV), and cadherins (cadherin 23 and protocadherin 15); these proteins are most likely implicated during stereocilia formation and/or maintenance (Avraham et al., 1995; Gibson et al., 1995; Probst et al., 1998; Liang et al., 1999; Littlewood Evans and Müller, 2000; Zheng et al., 2000; Alagramam et al., 2001; Di Palma et al., 2001; Wada et al., 2001; Boeda et al., 2002; Karolyi et al., 2003; Mburu et al., 2003; Weil et al., 2003). Despite these advances, we are still far from having a comprehensive view of how the stereocilia of hair cells are built during development and maintained throughout life.

Ezrin/radixin/moesin (ERM) proteins are three closely related proteins in the band 4.1 superfamily that are thought to function as cross-linkers between plasma membranes and actin filaments, thus integrating the actin cortical layer, especially the microvilli (Sato et al., 1992; Berryman et al., 1993; Mangeat et al., 1999; Tsukita and Yonemura, 1999; Bretscher et al., 2002). The COOH-terminal domains of these ERM proteins bind to the actin filaments (Algrain et al., 1993; Turunen et al., 1994; Martin et al., 1995), and the NH_2-terminal halves, the FERM domains (band 4.1/ezrin/radixin/moesin homology domains; Algrain et al., 1993; Henry et al., 1995; Bretscheret al., 2002), associate directly with the cytoplasmic domains of several integral membrane proteins such as CD43, CD44, and ICAM-1/2/3 (Tsukita et al., 1994; Helander et al., 1996; Serrador et al., 1997; Yonemura et al., 1998, 1999; Shaw, 2001). These NH_2-terminal halves are also indirectly associated with membrane transporters/channels such as the Na^+/H^+ exchanger 3, CFTR (cystic fibrosis transmembrane conductance regulator), β2-adrenergic receptor, and PDGF-receptor, via a cytoplasmic phosphoprotein, EBP50/NHE-RF (Reczek et al., 1997; Yun et al., 1997; Murthy et al., 1998; Short et al., 1998; Maudsley et al., 2000; Bretscher et al., 2002). The cross-linking activity of ERM proteins between actin filaments and plasma membranes is thought to be regulated by PIP_2 in the downstream of Rho (Hirao et al., 1996; Mackay et al., 1997; Heiska et al., 1998; Matsui et al., 1999; Yonemura et al., 2002).

The suppression of ERM protein expression in lymphocytes using antisense oligonucleotides results in the complete disappearance of microvilli, suggesting that they play an essential role in cortical microvilli formation (Takeuchi et al., 1994). To examine the physiological functions of ERM proteins at the entire body level (Ingraffea et al., 2002), moesin- and radixin-deficient mice have been generated (Doi et al., 1999; Kikuchi et al., 2002). Moesin-deficient mice showed no abnormalities when examined under laboratory conditions (Doi et al., 1999). By contrast, radixin deficiency caused congenital conjugated hyperbilirubinemia with losses of multidrug resistance protein 2, a bilirubin transporter, from the bile canalicular membranes of the hepatocytes with concomitant decrease of microvilli in number as well as in length (Kikuchi et al., 2002). In wild-type mice, radixin was the dominant ERM protein in the hepatocytes, and was not substituted by either ezrin or moesin in $Rdx^{-/-}$ mice.

Taking all of the above into consideration, it would seem important that the possible involvement of ERM proteins in the formation, maintenance, and functions of stereocilia on hair cells be examined. In this work, we found that radixin was the only detectable species of ERM protein in the stereocilia of the cochlea of adult wild-type mice, whereas vestibular stereocilia appeared to contain a detectable amount of ezrin in addition to radixin. Adult $Rdx^{-/-}$ mice exhibited profound deafness, but not imbalance; consistent with this, stereocilia were significantly defective in the outer and inner hair cells of the cochlea, but not in the hair cells of the vestibule. Furthermore, we found that in the cochlea of $Rdx^{-/-}$ mice, ezrin-based stereocilia appeared to develop normally from birth to postnatal day 14 (P14), the onset of hearing, after which they progressively degenerated. Thus, the radixin knockout provides a type of mouse deafness model caused by mutation in cytoskeletal components in stereocilia, which presents with progressive degeneration of cochlear (but not vestibular) stereocilia. The present paper provides a new insight into the ERM-based molecular mechanisms for the formation and maintenance of stereocilia, and the functional specificity/redundancy among ERM proteins in situ.

Results

Radixin is specifically enriched in stereocilia in the cochlear and vestibular sensory hair cells in adult wild-type mice

As a first step to examine the possible involvement of ERM proteins in the formation/maintenance of stereocilia on hair cells, the organ of Corti and the crista ampullaris of the vestibule were isolated from adult wild-type mice aged 5 wk, and then whole-mount stained with ezrin-, radixin-, and moesin-specific mAbs (Kondo et al., 1997; Doi et al., 1999). In the organ of Corti, only radixin was concentrated both in one row of the inner hair cell stereocilia and three rows of the outer hair cell stereocilia (Fig. 1, a–c): Individual stereocilia appeared resolved both by radixin staining in immunofluorescence microscopy (Fig. 1 d) and by scanning EM (Fig. 1 e). In addition to such intense signals, diffuse weak staining for ezrin and radixin was also detected in both hair and sup-

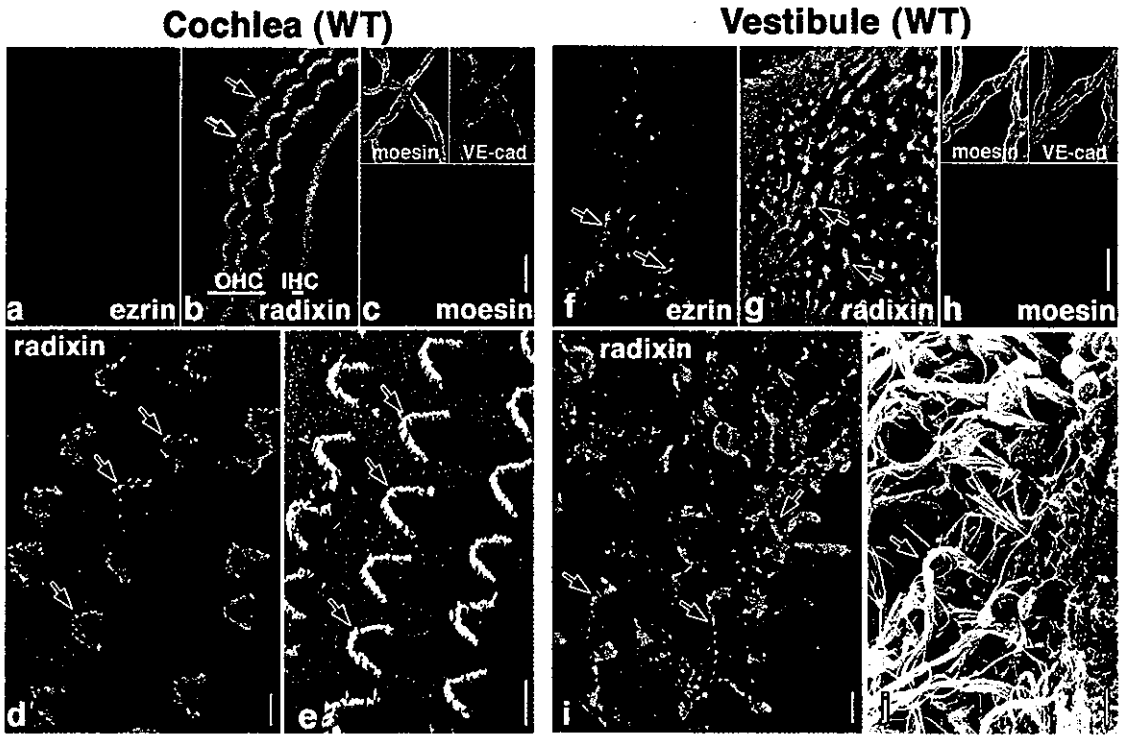

Figure 1. **ERM proteins in the cochlea and vestibule in adult wild-type mice at the age of 5 wk.** (a–c) Whole-mount immunofluorescence micrographs of the organ of Corti isolated from the cochlea with mAbs specific for ezrin, radixin, or moesin. The stereocilia of inner hair cells (IHC) and outer hair cells (OHC) are intensely stained with anti-radixin mAb (arrows). Ezrin and moesin are undetectable in the stereocilia, though moesin (moesin; green) is detected in large amounts in VE-cadherin–positive blood vessel endothelial cells (VE-cad; red) at a different focus plane (inset). Bar, 15 μm. (d and e) Comparison of the whole-mount radixin staining image with the scanning electron microscopic image of stereocilia on outer hair cells. Both radixin immunostaining (d) and scanning EM (e) reveal a highly organized array of stereocilia (arrows). Bars, 5 μm. (f–h) Whole-mount immunofluorescence micrographs of the crista ampullaris isolated from the vestibule with mAbs specific for ezrin, radixin, or moesin. Stereocilia are intensely stained with anti-radixin mAb, and weakly but reproducibly stained with anti-ezrin mAb (arrows). Moesin is detected only in the blood vessels at a different focus plane from radixin/ezrin-positive stereocilia (inset), as judged by the double-staining images for moesin (moesin; green) and VE-cadherin (VE-cad; red, inset). Bar, 15 μm. (i and j) Comparison of the whole-mount radixin staining image with the scanning electron microscopic image of stereocilia on vestibular hair cells. Radixin appears to concentrate along the entire length of long fragile stereocilia. Bars, 3 μm.

porting cells of the organ of Corti, whereas moesin was not detected in either; instead it was concentrated in the VE-cadherin–positive blood vessels (Berryman et al., 1993). In the crista ampullaris of the vestibule, radixin was concentrated in the stereocilia of the hair cells, but ezrin was also detectable in stereocilia, though faintly (Fig. 1, f–h). At higher magnification, individual long fragile vestibular stereocilia were also resolved both by radixin staining in immunofluorescence microscopy (Fig. 1 i) and by scanning EM (Fig. 1 j). Moesin was detected only in the VE-cadherin–positive blood vessels. These results indicated that radixin is the dominant ERM protein in the hair cell stereocilia of the inner ear, although the degree of contribution of ezrin appeared to be different between the cochlear and vestibular stereocilia.

Radixin deficiency causes deafness

Thus, it would be interesting to examine the physiological roles of radixin in hearing and balance in mice. First, we focused on the hearing ability of radixin-deficient ($Rdx^{-/-}$) mice. These mice showed a normal growth rate (Fig. 2 A) and were fertile, although they suffered from congenital conjugated hyperbilirubinemia (Kikuchi et al., 2002). Interestingly, when a sound stimulus was administrated in the form of a loud handclap, adult $Rdx^{-/-}$ mice (5–10-wk-old) showed no reflexive reaction, so-called Preyer's reflex, although $Rdx^{+/+}$ and $Rdx^{+/-}$ mice quickly moved their heads (Fig. 2 A). The auditory-evoked brainstem response (ABR) was then measured in two sets of heterogeneous $Rdx^{+/-}$ intercross adult littermates aged 5 and 10 wk (12 littermates in total) in response to a stimuli with a sound pressure level (SPL) of 70 decibels (dB) (20 kHz; Fig. 2 B). Of the 12 littermates, three showed no ABR; the others all showed a typical ABR waveform. Afterwards, these mice were genotyped, and these three littermates showing no ABR were all found to be $Rdx^{-/-}$ mice. This perfect correlation between the $Rdx^{-/-}$ genotype and a lack of ABR was reproducibly obtained in different series of measurements. In Fig. 2 C, the hearing thresholds of 10-wk-old mice were measured at various sound frequencies. Wild-type and $Rdx^{+/-}$ mice showed normal hearing thresholds (10–50 dB SPL), whereas $Rdx^{-/-}$ mice showed profound deafness (hearing threshold, >70–90 dB SPL).

Then, we compared light microscopic images of toluidine blue–stained sections prepared from Epon-embedded tissues of the inner ear between $Rdx^{+/+}$ and $Rdx^{-/-}$ P60 mice (Fig.

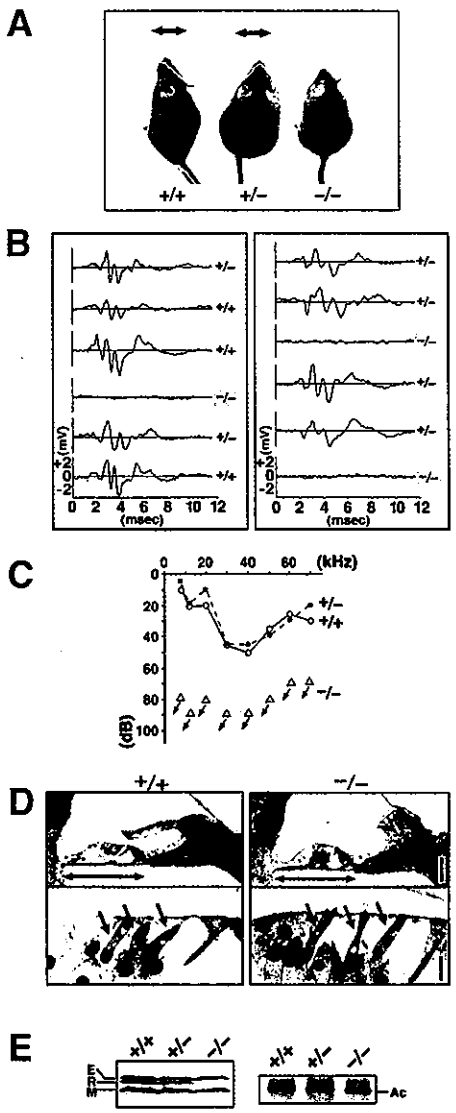

Figure 2. **Deafness of *Rdx*$^{-/-}$ mice.** (A) Loss of Preyer's reflex in *Rdx*$^{-/-}$ mice. Time-lapse photography captures Preyer's reflex in *Rdx*$^{+/+}$ and *Rdx*$^{+/-}$ mice, head movements (arrows), but not in *Rdx*$^{-/-}$ mice. Two successive frames after a loud handclap (1-s interval) were superimposed. These 10-wk-old mice show similar growth rates. (B) ABR to stimuli of 70-dB SPL (20 kHz) in two sets of *Rdx*$^{+/-}$ intercross littermates (left, 5-wk-old; right 10-wk-old). Among 12 littermates in total, three show no ABR, and were afterwards genotyped as *Rdx*$^{-/-}$ mice (blue squares). (C) Hearing thresholds of 10-wk-old *Rdx*$^{+/+}$, *Rdx*$^{+/-}$, and *Rdx*$^{-/-}$ mice at various sound frequencies. *Rdx*$^{+/+}$ and *Rdx*$^{+/-}$ mice show normal hearing thresholds (10–50 dB SPL), whereas *Rdx*$^{-/-}$ mice show profound deafness (hearing threshold, >70–90 dB SPL). (D) Toluidine blue–stained Epon semi-thin sections of the cochlea. No gross morphological difference is observed in the organ of Corti (double-headed arrows) including hair cells (single-headed arrows) between *Rdx*$^{+/+}$ and *Rdx*$^{-/-}$ mice. Bars, 50 μm (top panels); 10 μm (bottom panels). (E) Western blot analysis of isolated cochleae of the *Rdx*$^{+/+}$, *Rdx*$^{+/-}$, and *Rdx*$^{-/-}$ mice with anti-ERM pAb (TK89) that recognizes ezrin (E), radixin (R), and moesin (M) equally. In the *Rdx*$^{-/-}$ cochlea, radixin becomes undetectable without significant up-regulation of ezrin or moesin. Silver-stained bands of actin (Ac) in the same gels are present to show that an equal amount of cell lysate was applied in each lane.

2 D): No obvious gross morphological malformations were observed in *Rdx*$^{-/-}$ cochlea including hair cells, which was consistent with the scanning electron microscopic observation (see Fig. 3 A). However, as *Rdx*$^{-/-}$ mice became much older (>90 d), hair cells appeared to begin to be eliminated, leaving a fairly disorganized cell architecture of the organ of Corti (unpublished data). The expression of ERM proteins were examined in the *Rdx*$^{+/+}$, *Rdx*$^{+/-}$, and *Rdx*$^{-/-}$ cochleae isolated from P40 adult littermates by Western blotting with anti-ERM pAb (Fig. 2 E). Ezrin, radixin, and moesin were detected in both *Rdx*$^{+/+}$ and *Rdx*$^{+/-}$ cochleae, but *Rdx*$^{-/-}$ cochlea lacked radixin expression specifically and showed no significant up-regulation of ezrin or moesin.

Radixin deficiency causes progressive degeneration of stereocilia of cochlear hair cells after the onset of hearing

The question then naturally arose as to what is the consequence of radixin deficiency in the cochlear stereocilia in *Rdx*$^{-/-}$ mice. Isolated cochleae of P40 *Rdx*$^{+/+}$ and *Rdx*$^{-/-}$ littermates were examined by scanning EM (Fig. 3 A, a–f). When removing the lateral wall and tectorial membrane of the cochlea in P40 *Rdx*$^{-/-}$ mice to expose the organ of Corti, the cellular arrangement of the outer and inner hair cells as well as the supporting cells appeared normal, but very interestingly, the stereocilia in both outer and inner hair cells were significantly defective compared with those in *Rdx*$^{+/+}$ organ of Corti. Instead of being regularly arranged in a "W" shape, stereocilia of the outer hair cells of *Rdx*$^{-/-}$ mice were deformed to 1–3 residual knoblike protrusions on the apical surfaces (Fig. 3 A, d). Inner hair cells had several shorter fused protrusions instead of stereocilia (Fig. 3 A, f). When the organ of Corti isolated from these *Rdx*$^{-/-}$ cochleae was whole-mount stained with ezrin-, radixin-, and moesin-specific mAbs, ezrin, radixin, or moesin was not detected in the residual structures of outer and inner stereocilia, i.e., knoblike protrusions and shorter fused protrusions, respectively (Fig. 3 A, g–j). Diffuse ezrin staining in the organ of Corti and intense moesin staining in blood vessels did not appear to be affected. Considering that stereocilia play a central role in transducing acoustic stimuli into electrical signals in the cochlea, it is likely that these defects in cochlear stereocilia are responsible for the hearing impairments observed in adult *Rdx*$^{-/-}$ mice.

Cochlear hair cells of mice were reported to continue to develop after birth, and to become functionally mature at the onset of hearing around the age of 14 d (Lim and Anniko, 1985; Erven et al., 2002; Zuo, 2002). Therefore, we next attempted to distinguish whether in *Rdx*$^{-/-}$ mice cochlear stereocilia are defective in their development or degenerate after they have developed normally. As previously reported (Lim and Anniko, 1985; Zuo, 2002), in the cochleae of wild-type P1 mice, scanning EM showed that both the inner and outer hair cells already bore well-developed stereocilia in a characteristic arrangement (Fig. 3 B, a, c, and e). The central tubulin-based kinocilium of the W-shaped array of stereocilia on inner hair cells were especially prominent at this early stage. In addition to stereocilia, at this stage hair cells as well as supporting cells were covered with a large

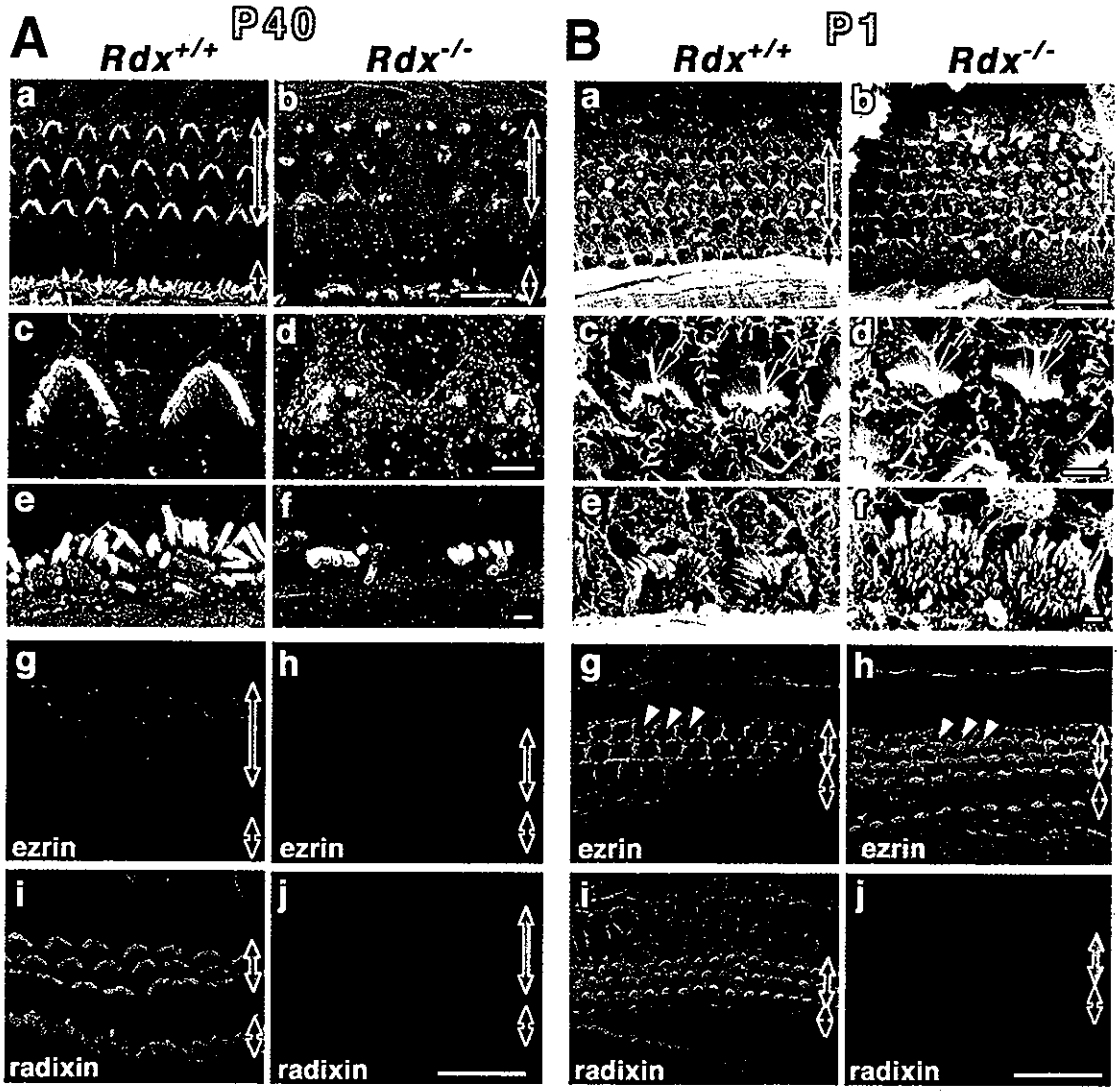

Figure 3. **Stereocilia on $Rdx^{+/+}$ and $Rdx^{-/-}$ cochlear hair cells of adult and newborn mice.** (A) Scanning electron micrographs (a–f) and whole-mount immunofluorescence micrographs for radixin and ezrin (g–j) of the $Rdx^{+/+}$ and $Rdx^{-/-}$ organ of Corti at 40 d of age (P40). The levels of outer hair cells (blue arrows) and inner hair cells (black arrows) are shown. At low magnification in the scanning electron micrograph of the $Rdx^{+/+}$ organ of Corti (a), the luminal surface is characterized by three rows of outer hair cells (blue arrow) and one row of inner hair cells (black arrow). Outer hair cells bear stereocilia arranged regularly in the form of a letter W (c), and inner hair cells have more disorganized arrays of stereocilia (e). In the $Rdx^{-/-}$ organ of Corti (b), no significant abnormalities are detected in the cellular arrangements on the luminal surface, but the morphology of the stereocilia on both the outer (blue arrow) and inner (black arrow) hair cells is severely affected. In $Rdx^{-/-}$ outer hair cells, instead of regularly arranged stereocilia, 1–3 residual knoblike protrusions are observed on their apical surface (d). $Rdx^{-/-}$ inner hair cells bear several shorter fused irregularly shaped protrusions instead of long stereocilia (f). By immunostaining, in the $Rdx^{-/-}$ organ of Corti (h and j), no staining for radixin is detected without any increase in the staining intensities for ezrin. Bars, 10 μm (a and b); 2 μm (c and d); 1 μm (e and f); 20 μm (g–j). (B) Scanning electron micrographs (a–f) and whole-mount immunofluorescence micrographs for radixin and ezrin (g–j) of the $Rdx^{+/+}$ and $Rdx^{-/-}$ organ of Corti at 1 d of age (P1). Scanning electron microscopic images of the cochlea of P1 $Rdx^{-/-}$ mice (b, d, and f) are indistinguishable from those in the $Rdx^{+/+}$ mice (a, c, and e). In addition to stereocilia arrays carrying central tubulin-based kinocilium (arrows in c and d), hair cells as well as supporting cells are covered with large numbers of short conventional microvilli that have mostly disappeared in adult mice. Whole-mount immunostaining reveals that in the P1 $Rdx^{+/+}$ organ of Corti, radixin is highly enriched in stereocilia of inner and outer hair cells as well as the apical surface of hair and supporting cells (i), but in contrast to adult mice ezrin is also detected in stereocilia, weakly but clearly (g, arrowheads). In the $Rdx^{-/-}$ organ of Corti, instead of radixin, ezrin is highly concentrated at stereocilia on both inner and outer hair cells (h, arrowheads). Bars, 10 μm (a and b); 2 μm (c and d); 1 μm (e and f); 20 μm (g–j).

number of short conventional microvilli that disappeared in adult mice (Fig. 3 A). To our surprise, the scanning electron microscopic images of the cochlea of P1 $Rdx^{-/-}$ mice were indistinguishable from those in the $Rdx^{+/+}$ mice (Fig. 3 B, b, d, and f). The question is how stereocilia were developed normally in the absence of radixin in P1 $Rdx^{-/-}$ mice. Whole-mount immunostaining was then performed (Fig. 3 B, g–j). In P1 $Rdx^{+/+}$ mice, radixin was highly enriched in stereocilia of inner and outer hair cells, but in contrast to adult mice, ezrin was also detected in stereocilia, weakly but clearly (Fig. 3 B, g and i). Furthermore, the P1 $Rdx^{+/+}$ cochlea was characterized by punctate radixin and ezrin signals

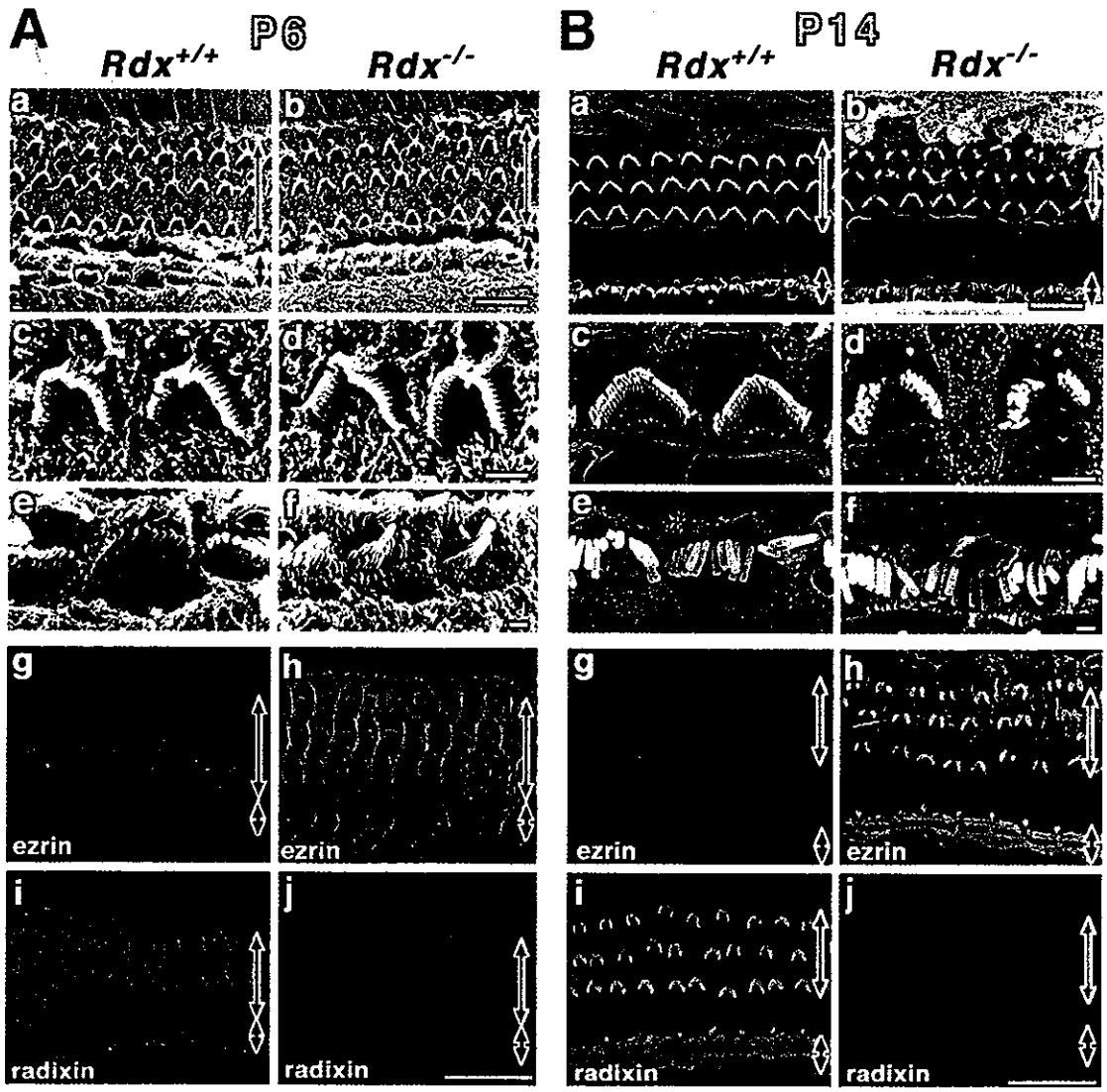

Figure 4. **Postnatal degeneration of stereocilia on $Rdx^{-/-}$ cochlear hair cells.** (A) Scanning electron micrographs (a–f) and whole-mount immunofluorescence micrographs for radixin and ezrin (g–j) of the organ of Corti isolated from $Rdx^{+/+}$ and $Rdx^{-/-}$ mice at 6 d of age (P6). The levels of outer hair cells (blue arrows) and inner hair cells (black arrows) are shown. Scanning EM does not detect any significant differences between $Rdx^{+/+}$ (a, c, and e) and $Rdx^{-/-}$ cochlea (b, d, and f). In contrast to P1 $Rdx^{+/+}$ cochlea, in P6 $Rdx^{+/+}$ cochlea, whole-mount immunostaining detects a weak signal of ezrin at radixin-enriched stereocilia on inner hair cells (black arrow), and a trace of ezrin in outer hair cells (blue arrow) (g and i). In $Rdx^{-/-}$ cochlea (h and j), ezrin is still highly concentrated at stereocilia on both inner hair cells (black arrow) and outer hair cells (blue arrow). Bars, 10 μm (a and b); 2 μm (c and d); 1 μm (e and f); 20 μm (g–j). (B) Scanning electron micrographs (a–f) and whole-mount immunofluorescence micrographs for radixin and ezrin (g–j) of the organ of Corti isolated from $Rdx^{+/+}$ and $Rdx^{-/-}$ mice at 14 d of age (P14). Scanning EM detects the initial sign for the degeneration of stereocilia on outer hair cells (blue arrow) (a–d). The central part of the W-shaped row of stereocilia is lost, leaving discontinuous and disorganized arrays of stereocilia in $Rdx^{-/-}$ cochlea. At this stage, no significant defects can be observed in stereocilia on the inner hair cells (black arrow) (b and f). In P14 $Rdx^{+/+}$ cochlea, ezrin is only weakly detected at radixin-enriched stereocilia on inner hair cells (black arrow), and is not detected in outer hair cells (blue arrow) (g and i). In $Rdx^{-/-}$ cochlea, ezrin was still concentrated in the degenerating stereocilia in large amounts in outer and inner hair cells (h). Bars, 10 μm (a and b); 2 μm (c and d); 1 μm (e and f); 20 μm (g–j).

on the apical surface of hair cells and supporting cells, which may correspond to numerous conventional microvilli. Of course, in the organ of Corti in P1 $Rdx^{-/-}$ littermates, radixin was undetectable (Fig. 3 B, j), but instead of radixin, ezrin was markedly increased in stereocilia of both inner and outer hair cells as well as in conventional microvilli on hair and supporting cells (Fig. 3 B, h). Therefore, ezrin appeared to compensate for radixin deficiency in the development of hair cell stereocilia, at least up to 1 d after birth. Subsequently, these ezrin-based stereocilia degenerated postnatally.

We then pursued the possible postnatal degeneration process of stereocilia in $Rdx^{-/-}$ mice (Fig. 4). At P6, scanning EM did not detect any significant differences between $Rdx^{+/+}$ and $Rdx^{-/-}$ cochlea, indicating that there was still no sign for stereocilia degeneration (Fig. 4 A, a–f). However, in $Rdx^{+/+}$ cochlea, ezrin became weaker during P1 to P14, almost undetectable from stereocilia at ~P14, and radixin was

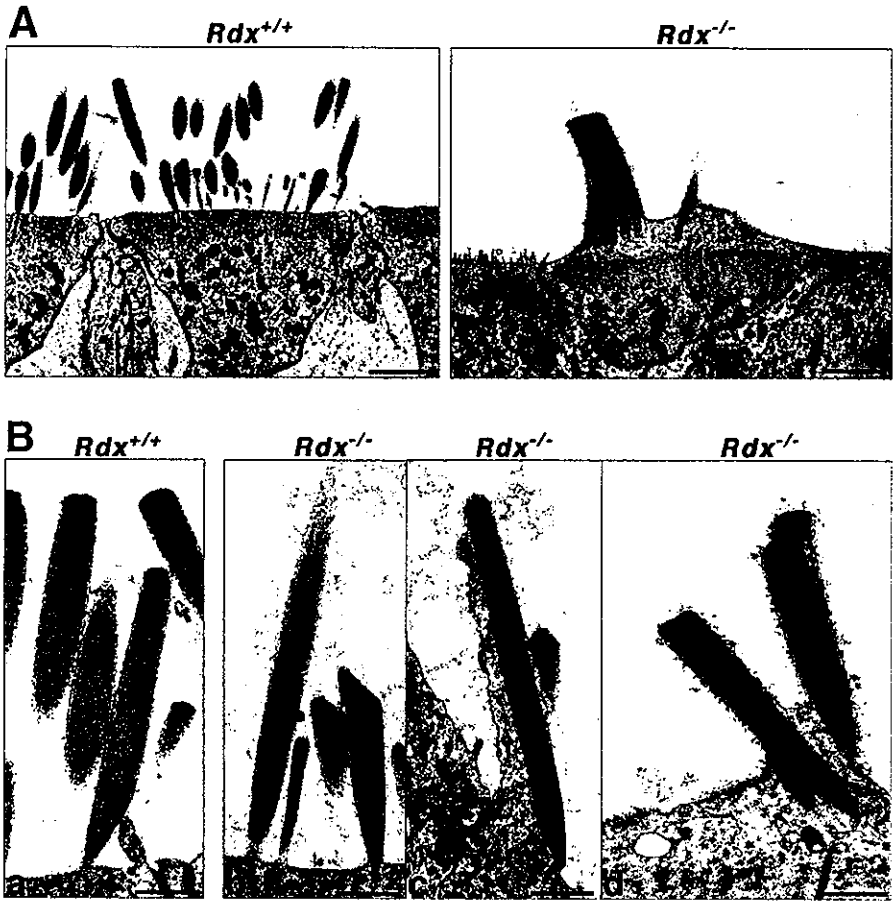

Figure 5. **Ultrathin-section electron micrographs of stereocilia of cochlear outer hair cells of *Rdx*$^{+/+}$ and *Rdx*$^{-/-}$ mice aged 3 wk.** (A) Low power electron micrographs. All of the *Rdx*$^{+/+}$ hair cells bear numerous stereocilia with normal appearance, whereas *Rdx*$^{-/-}$ hair cells were frequently characterized by abnormal 1–3 residual knoblike cellular protrusions on their apical surfaces. These images may correspond to the scanning electron micrographs of Fig. 3 A, c and d, respectively. Bars, 2 μm. (B) *Rdx*$^{+/+}$ radixin-based stereocilia (a) and *Rdx*$^{-/-}$ ezrin-based stereocilia at various degeneration stages (b–d). *Rdx*$^{-/-}$ ezrin-based stereocilia contained highly-organized bundles of actin filaments as cores (b) that were indistinguishable in appearance from those in *Rdx*$^{+/+}$ radixin-based stereocilia (a), and during the process of degeneration, these stereocilia as well as core bundles appeared to be fused to form abnormal thick and short protrusions (c and d). Bars, 0.5 μm.

dominantly detected among ERM proteins in stereocilia (Fig. 4 A, g and i). Supporting cells were intensely stained for ezrin and radixin. In P6 *Rdx*$^{-/-}$ cochlea, ezrin was highly concentrated in stereocilia on both inner and outer hair cells (Fig. 4 A, h) with no radixin staining (Fig. 4 A, j). Around age P14 (onset of hearing in mice), in *Rdx*$^{-/-}$ cochlea, the sign for stereocilia degeneration began to be detected on outer hair cells by scanning EM (Fig. 4 B, a–f): The central part of the W-shaped row of stereocilia was lost, leaving discontinuous and disorganized arrays of stereocilia. Ezrin was still concentrated in these degenerating stereocilia in large amounts, though in *Rdx*$^{+/+}$ hair cells, radixin, but not ezrin, was enriched at stereocilia (Fig. 4 B, g–j). We concluded that in *Rdx*$^{-/-}$ cochleae, ezrin can counterbalance radixin deficiency to normally develop stereocilia up to the onset of hearing, but not to maintain these once-developed stereocilia to transmit acoustic stimuli into electrical signals.

To clarify the interior structure of ezrin-based stereocilia in *Rdx*$^{-/-}$ cochleae, the cochleae of *Rdx*$^{-/-}$ mice aged 3 wk were examined by ultrathin-section EM (Fig. 5). In these mice, stereocilia at various stages of degeneration were observed. Ezrin-based stereocilia contained highly organized bundles of actin filaments as cores that were indistinguishable in appearance from those in wild-type radixin-based stereocilia, and during the process of degeneration these stereocilia as well as core bundles appeared to be fused to form abnormal thick and short protrusions.

Finally, a natural question is what are the membrane-binding partners for radixin/ezrin in cochlear stereocilia. To elucidate an answer, we first performed immunoprecipitation experiments with anti-radixin or anti-ezrin mAb using isolated *Rdx*$^{+/+}$ or *Rdx*$^{-/-}$ cochlea; however, the results were inconclusive, probably due to the limitation of the material and the low insolubility of radixin and ezrin. We also performed a gel overlay blot with labeled radixin/ezrin, but again failed to detect any bands specifically associated with radixin/ezrin.

Ezrin compensates radixin deficiency in the development/maintenance of stereocilia in the vestibular hair cells

As shown in Fig. 1, in the vestibule of wild-type mice aged 5–10 wk, radixin was predominantly concentrated in stereocilia among ERM proteins, but distinct from cochlear stereocilia, these stereocilia contained detectable amounts of ezrin. The question then arose as to the structure and function of vestibular stereocilia in adult *Rdx*$^{-/-}$ mice. Structurally, scanning EM identified no abnormality in the number/density and morphology of stereocilia at the crista ampullaris of the vestibule of adult *Rdx*$^{-/-}$ mice (Fig. 6 A). Also, scanning EM identified no abnormality in the otolith organs (the utricle and saccule) of adult *Rdx*$^{-/-}$ mice (unpublished data). Functionally, *Rdx*$^{-/-}$ vestibular stereocilia appeared to be normal because adult *Rdx*$^{-/-}$ mice showed no signs of imbalance in their behavior as far as we examined up to 100 d after birth. Then, their vestibulo-ocular reflex (VOR) was measured, in which the function of horizontal semicircular canals, but not otolith organs (the utricle and saccule), can be evaluated. Heads of *Rdx*$^{+/+}$, *Rdx*$^{+/-}$, or *Rdx*$^{-/-}$ mice aged

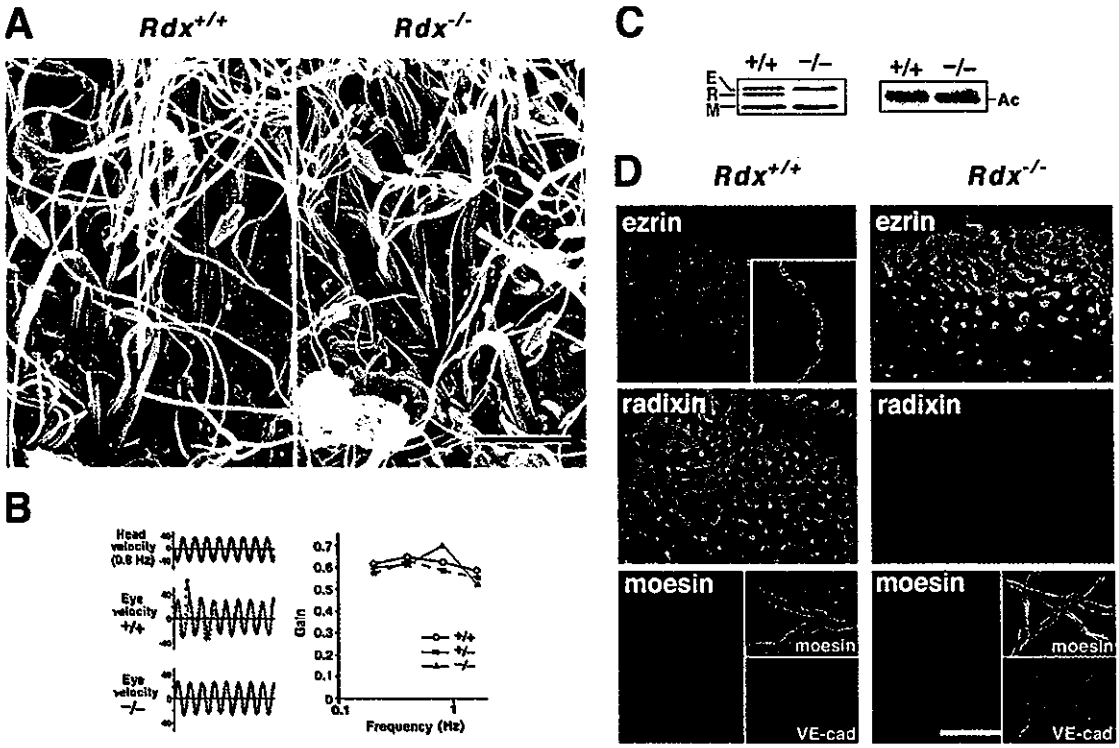

Figure 6. **The vestibule and the balance function of 5-wk-old *Rdx*$^{-/-}$ mice.** (A) Scanning EM of the *Rdx*$^{+/+}$ and *Rdx*$^{-/-}$ crista ampullaris of the vestibule. The appearance of stereocilia (arrows) was indistinguishable between *Rdx*$^{+/+}$ and *Rdx*$^{-/-}$ mice. Bar, 5 μm. (B) VOR. A mouse head was rotated sinusoidally and the eye position recorded by a CCD camera (left). The eye velocity was calculated from the change of eye position, and was fitted with sinusoidal curves (red lines). The gain was obtained by dividing the peak eye velocity by the peak head velocity. No difference is detected in the VOR between *Rdx*$^{+/+}$ and *Rdx*$^{-/-}$ mice. The VOR gains of *Rdx*$^{-/-}$ mice are also normal at any frequency of the head rotation stimulus in *Rdx*$^{+/+}$ and *Rdx*$^{-/-}$ mice (right). (C) Western blot analysis of isolated *Rdx*$^{+/+}$ and *Rdx*$^{-/-}$ vestibule (mainly crista ampullaris) with anti-ERM pAb (TK89) that recognizes ezrin, radixin, and moesin with almost the same affinity. In *Rdx*$^{-/-}$ vestibule, radixin (R) became undetectable without significant up-regulation of ezrin (E) or moesin (M). Silver-stained bands of actin (Ac) are presented on the right to show that equal amounts of cell lysate were applied in each lane. (D) Whole-mount immunostaining of the crista ampullaris isolated from the *Rdx*$^{+/+}$ and *Rdx*$^{-/-}$ vestibules with ezrin-, radixin- and moesin-specific antibodies. In the *Rdx*$^{+/+}$ crista ampullaris, radixin is highly concentrated in the stereocilia where ezrin is detected weakly but reproducibly (inset, a frozen section stained with anti-ezrin antibody). Moesin is detected only in the blood vessels at a different focus plane from stereocilia (inset), as judged by the double staining images for moesin (moesin; green) and VE-cadherin (VE-cad; red) (inset). By contrast, in the *Rdx*$^{-/-}$ crista ampullaris, the concentration of ezrin in the stereocilia is significantly increased. All samples were treated under completely identical conditions, paying special attention to ensure that the signals were not saturated. Bar, 40 μm.

~5–10 wk were rotated sinusoidally and eye positions were recorded using a CCD camera at various head rotation frequencies. As shown in Fig. 6 B, the VOR gains of *Rdx*$^{-/-}$ mice were normal at all head rotation frequencies.

Western blotting of the isolated vestibule with anti-ERM pAb revealed no significant up-regulation of ezrin or moesin in adult *Rdx*$^{-/-}$ mice (Fig. 6 C). We then whole-mount stained the crista ampullaris isolated from adult *Rdx*$^{+/+}$ and *Rdx*$^{-/-}$ vestibule with ezrin-, radixin-, and moesin-specific mAbs (Fig. 6 D). In the vestibule of *Rdx*$^{+/+}$ mice, radixin was specifically and highly enriched in the stereocilia of the crista ampullaris with a small amount of ezrin, whereas in the *Rdx*$^{-/-}$ crista ampullaris, instead of radixin, ezrin was highly concentrated in stereocilia, although this up-regulation of ezrin in stereocilia could not be detected by immunoblotting of the whole vestibule (Fig. 6 C). Therefore, in the vestibule, ezrin appears to be able to compensate radixin deficiency not only in developing stereocilia, but also in maintaining them to transmit acceleration stimuli into electrical signals.

Discussion

The physiological functions of ERM proteins, ezrin, radixin, and moesin, have attracted increasing interest, but the relevance of the existence of these three closely related proteins in mammalian cells remains elusive (Berryman et al., 1993; Takeuchi et al., 1994; Doi et al., 1999; Tsukita and Yonemura, 1999; Ingraffea et al., 2002; Kikuchi et al., 2002). In most cultured mammalian cells, all ERM proteins are coexpressed and codistributed (Sato et al., 1992; Berryman et al., 1993). Using antisense oligonucleotides, ERM proteins were shown to be functionally redundant, without a quantitatively compensatory increase in their expression levels, in cell–cell/cell–substrate adhesion and the formation of microvilli in cultured cells (Takeuchi et al., 1994). At the whole-body level, the expression levels and combinations of ERM proteins varied significantly depending on cell type (Sato et al., 1992; Berryman et al., 1993; Franck et al., 1993; Doi et al., 1999; Ingraffea et al., 2002), and to date no clear example has been found to indicate the functional

redundancy among ERM proteins. Radixin deficiency causes hyperbilirubinemia due to the loss of multidrug resistance protein 2 from the bile canalicular membranes, where microvilli were decreased in number as well as in length (Kikuchi et al., 2002). Radixin was the dominant ERM protein in the liver of wild-type mice, but no compensatory up-regulation of expression level of ezrin or moesin was detected in the $Rdx^{-/-}$ liver.

To discuss the functions of ERM proteins and their possible redundancy in situ, the inner ear provides an advantageous system, as in it there are two distinct types of stereocilia, cochlear and vestibular stereocilia, which play central roles in transmitting acoustic and acceleration stimuli, respectively, into electrical signals. (Roberts et al., 1988; Hudspeth, 1989; Pickles and Corey, 1992; Eatock et al., 1998; Corey, 2003). In wild-type cochlea, before ~P14, ezrin was detectable in addition to large amounts of radixin in stereocilia, and then progressively disappeared, leaving purely radixin-based stereocilia. In the $Rdx^{-/-}$ cochlea, ezrin was significantly up-regulated, and purely ezrin-based stereocilia developed normally up to ~P14 and then progressively degenerated. By contrast, in the $Rdx^{+/+}$ vestibule, stereocilia contained a small amount of ezrin in addition to large amounts of radixin throughout life, whereas in the $Rdx^{-/-}$ vestibule, stereocilia containing up-regulated ezrin developed and was maintained normally throughout the life. These findings would provide the first clear example that these members of the ERM family, ezrin and radixin, function redundantly in situ at the whole-body level: ezrin compensates for radixin deficiency functionally in stereocilia. In $Rdx^{+/+}$ mice, the cochlear hair cells up to ~P14 and the vestibular hair cells throughout the life expressed both radixin and ezrin in large and small amounts, respectively, and probably due to the difference in their expression levels or affinity to plasma membranes, radixin was the main contributor to the formation of stereocilia. In these hair cells, in the absence of radixin ($Rdx^{-/-}$ mice), a larger amount of ezrin was recruited, which appeared to compensate for radixin deficiency in the formation of stereocilia.

The molecular mechanism behind the progressive degeneration of the cochlear stereocilia after the onset of hearing (~P14) remained elusive. At present, two explanations appear possible. First, if the expression of ezrin is genetically programmed to be suppressed in cochlear hair cells after the onset of hearing in the wild-type mice, and if this program is not changed by radixin deficiency, in $Rdx^{-/-}$ mice, cochlear hair cells lack both ezrin and radixin after the onset of hearing, resulting in progressive degeneration of stereocilia. As the total lack of ERM proteins was reported to cause cell death (Kondo et al., 1997), this mechanism can explain why cochlear hair cells were eliminated in older $Rdx^{-/-}$ mice. The second possibility is that sound stimulation induces the progressive degeneration of ezrin-based stereocilia in the $Rdx^{-/-}$ cochlea. It would be possible that these ezrin-based stereocilia are more labile against the noise stimulation (i.e., frequent deflection) than the wild-type radixin-based stereocilia due to the qualitative or quantitative difference between radixin in wild-type stereocilia and ezrin in $Rdx^{-/-}$ stereocilia. Consistently, the noise-induced loss of stereocilia was reported in the wild-type mice as well as humans (Ou et al., 2000). As deflection frequency for vestibular stereocilia is expected to be much lower than for cochlear stereocilia, $Rdx^{-/-}$ vestibule ezrin-based stereocilia could be maintained without degeneration. According to Western blotting of the whole cochlea and vestibule, there were no signs for up-regulation of the expression of ezrin (or moesin), but as it was technically difficult to examine the expression of ERM proteins in individual sensory hair cells, the two above explanations cannot be further evaluated for now. Furthermore, to understand the molecular mechanism behind the progressive degeneration of the cochlear stereocilia, identification of the specific molecular partners of radixin and ezrin, including integral membrane proteins, within stereocilia would be important, although it is again technically difficult due to the small number of stereocilia in the cochlea. Indeed, as described in the results, we performed immunoprecipitation and gel overlay experiments, but were unable to identify the radixin- or ezrin-binding partners in stereocilia. To be successful, any future work will need to overcome several technical difficulties.

Mutations in the actin-bundling protein espin have been reported in *jerker* mice and DFNB36, a form of nonsyndromic deafness in humans (Grüneberg et al., 1941; Zheng et al., 2000; Belyantseva et al., 2003). Adult *jerker* mice exhibit impaired hearing and balance dysfunctions, as well as short cochlear and vestibular stereocilia. Analyses of various aged *jerker* mice revealed apparently normal cochlear stereocilia development until the onset of hearing, after which the stereocilia progressively degenerate (Sjöström and Anniko, 1992). This aspect of *jerker* mice is very similar to $Rdx^{-/-}$ mice, but *jerker* mice are completely different from $Rdx^{-/-}$ mice in terms of the balance dysfunction. Because espin is likely to play a role in bundling actin filaments in stereocilia (Loomis et al., 2003), it was suggested that the organization of core actin filaments is critical for the maintenance of stereocilia after the onset of hearing. Furthermore, genetic studies of deafness in mice and humans have identified several genes for other cytoskeleton-related proteins, harmonin, myosin VI/VIIa, whirlin, and SANS, and for cadherin-related proteins, cadherin 23 and protocadherin 15, as causal genes (Avraham et al., 1995; Gibson et al., 1995; Probst et al., 1998; Self et al., 1998; Littlewood Evans and Müller, 2000; Zheng et al., 2000; Alagramam et al., 2001; Di Palma et al., 2001: Petit et al., 2001; Boeda et al., 2002; Mustapha et al., 2002; Karolyi et al., 2003; Kikkawa et al., 2003; Mburu et al., 2003; Weil et al., 2003). In these mice, the formation of not only cochlear, but also vestibular stereocilia was affected, resulting in the deafness associated with imbalance. All these cytoskeleton-related proteins, including ERM proteins and epsin, may act in cooperative ways to form and maintain dynamic stereocilia in cochlear and vestibular sensory hair cells.

To date, three mouse models in which there is profound hearing loss and no vestibular dysfunction have been reported: the deafness (dn) gene mutant mice (Bock and Steel, 1983), Beethoven mice (Vreugde et al., 2002), and the claudin 14-knockout mice (Ben-Yosef et al., 2003). $Rdx^{-/-}$ mice can now be added as a new member of this list. $Rdx^{-/-}$ mice should provide a valuable resource for further analysis of the functional redundancy of ERM proteins at the whole-body level, and of the molecular mechanisms behind the formation and maintenance of highly specialized microvilli, stereocilia;

the elucidation of which will shed light on the pathogenesis of human deafness and imbalance.

Materials and methods

Antibodies

Rabbit anti-ERM pAb, TK89, recognized COOH-terminal domains of all ERM proteins almost equally (Kondo et al., 1997; Doi et al., 1999). We previously raised and characterized rat anti-ezrin (M11), anti-radixin (R21), and anti-moesin mAb (M22) (Kondo et al., 1997; Doi et al., 1999). Goat anti-VE-cadherin (C-19) was obtained from Santa Cruz Biotechnology, Inc.

Generation of $Rdx^{-/-}$ mice

Rdx$^{-/-}$ mice were generated as previously reported (Kikuchi et al., 2002). Two independent mouse J1 ES clones (129/Sv) in which the radixin gene was correctly disrupted were injected into C57BL/6 blastocysts, and the resulting chimeras were mated with C57BL/6 mice (Doi et al., 1999).

ABR measurements

ABR measurements were performed in a soundproof room (Zheng et al., 1999). In general, the ABR waveforms were recorded for 12.8 ms at a sampling rate of 40,000 Hz using 50–5,000-Hz filter settings; waveforms recorded from 1,024 stimuli at a frequency of 9 Hz were averaged. ABR waveforms were recorded in decreasing 5-dB SPL intervals from a maximum amplitude until no waveforms could be visualized.

VOR measurements

VOR was measured as described by Iwashita et al. (2001). Head movements were transduced to DC signals using a small angular velocity sensor (Gyrostar; Murata Corporation) that was fixed on the turntable. Eye movements were detected by LED and a CCD camera (C53500; Tokyo Electronic Industry), and eye velocities were calculated online by downloading them onto a computer through a video capture board. Both the head and eye velocity curves were fitted with sinusoidal curves using the least squares criterion, and the gain of eye velocity relative to the head velocity was obtained.

Immunofluorescence microscopy

Temporal bones were removed from 1-, 6-, 14-, or 40-d-old mice, and together with the small holes in the cochlear apical turn and superior semicircular canal, the round and oval windows were opened. The lymphatic space was gently perfused with 10% TCA through a round window (Hayashi et al., 1999; Kitajiri et al., 2004). Samples were then immersed in 10% TCA for 1 h at 4°C, washed three times with PBS, and decalcified with 5% EDTA in PBS for 3 d at 4°C. The cochlea and vestibule were then carefully microdissected, treated with 0.2% Triton X-100 in PBS for 15 min, and soaked in 1% BSA in PBS at RT. They were then incubated with rat anti-ezrin (M11), anti-radixin (R11), or anti-moesin (M22) mAb for 30 min at RT. Samples were washed three times with PBS, followed by a 30-min incubation with Cy3- (Jackson ImmunoResearch Laboratories, Inc.) or Alexa Fluor® 488–conjugated secondary antibody (Molecular Probes, Inc.). After a wash with PBS, they were embedded in 95% glycerol-PBS containing 0.1% paraphenylendiamine and 1% *n*-propylgalate. Fluorescence images were obtained with a confocal microscope (model LSM 510 META; Carl Zeiss MicroImaging, Inc.) or with a DeltaVision optical sectioning microscope (version 2.10; Applied Precision, Inc.), equipped with an Axioplan2 (Plan Apochromat 63×/1.40 NA oil immersion objective; Carl Zeiss MicroImaging, Inc.) or IX70 (PlanApo 60×/1.40 NA oil immersion objective; Olympus) microscope, respectively.

Immunoblotting

The membranous labyrinths of 5-wk-old mice were dissected under a microscope. Whole organ of Corti and vestibules isolated from each mouse were sonicated in 100 μl SDS sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 20% glycerol, 2% 2-mercaptoethanol, and 0.01% bromophenol blue), applied to SDS-PAGE, and immunoblotted by a blotting detection kit with biotinylated Ig and streptavidin-conjugated alkaline phosphatase (Amersham Biosciences).

Scanning EM

Temporal bones obtained from 1-, 6-, 14-, or 40-d-old mice were fixed using perilymphatic perfusion as described above with 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2). They were then washed with phosphate buffer and post-fixed in 1% OsO_4 for 2 h, after which they were once again treated with perilymphatic perfusion. The organ of Corti or crista ampullaris was microdissected, dehydrated, critical-point dried, sputter coated, and observed by scanning EM (model S-800 microscope; Hitachi Co.).

Ultrathin-section EM

Samples were processed as previously described, using 2% formaldehyde, 2.5% glutaraldehyde, and 0.1 M sodium cacodylate buffer (pH 7.4) as a fixative (Yonemura et al., 2002).

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Note added in proof. While this paper was being reviewed, Pataky et al. presented evidence that radixin is a constituent of stereocilia in hair cells (Pataky, F., R. Pironkova, and A.J. Hudspeth. 2004. *Proc. Natl. Acad. Sci. USA.* 101:2601–2606).

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Mini Review

内耳への神経幹細胞移植

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Transplantation of neural stem cells into the inner ear

Hearing and balance disorders are included in most common disabilities, and the majority is incurable because of the low capability for regeneration of the inner ear. New therapeutic approaches including cell transplantation are therefore subjected to overcome degenerative diseases of inner ears. Firstly, transplantation of neural stem cells (NSCs) into inner ears has been attempted as well as the retina. Grafted NSCs can survive and migrate into inner ear tissues including sensory epithelia after transplantation into inner ears of newborn rats. Histological analysis following transplantation of NSCs into injured, matured inner ears of mice demonstrates the potential of NSCs for replacement of inner ear hair cells or primary neurons. *In vitro* analysis also support the hypothesis that NSCs can differentiate into inner ear hair cells. In addition, NSC-derived cells have the potential for producing several neurotrophins in the inner ear, suggesting the potential of NSC transplantation for protection of inner ear tissues from degeneration.

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Key words transplantation, inner ear, neural stem cell, regeneration, protection

はじめに

内耳は聴覚および平衡覚の末梢受容器であり，内耳障害は感音難聴，めまいの主な原因の一つとされている．高度の内耳障害は聾や高度の平衡障害を来し，日常生活を著しく障害する．これら内耳障害の頻度は決して低いものではなく，例えば感音難聴は60歳以上の人口の約60%に存在し，身体障害者に該当する高度内耳障害者は，本邦で40万人以上存在する．このように多くの高度内耳障害者が存在する要因として，内耳の脆弱性があげられる．哺乳類の内耳は，一旦傷害されると機能的に再生することは，極めて困難とされている．このような背景から，現在内耳障害に対する有効な治療法はほとんどなく，ごく限られた場合にのみ機能回復が期待できる．人工内耳は，蝸牛に電極を挿入し，ラセン神経節を有毛細胞にかわり，刺激することにより聴覚を再獲得させる医療機器として広く用いられている．しかし，適応は高度感音難聴者に限定され，また得られる聴力も自然な聴力とは異なる．

内耳における聴覚や平衡覚の受容には有毛細胞が不可欠である．有毛細胞は，音響や振動といった物理的な信号を電気信号に変換する役割を果たしている．また，有毛細胞が受容した信号を脳に伝える一次ニューロンも内耳に存在し，内耳感覚受容に不可欠な細胞である．しかし，有毛細胞とこれと連なる一次ニューロンは，哺乳類での再生は極めて困難であり，ごく限られた再生能力が前庭感覚上皮に存在することが報告されているのみである[1]．しかしながら，近年の分子生物学および幹細胞医学の発展とその内耳への応用により，哺乳類内耳再生が不可能なものでなくなりつつある．このような新しい研究の流れの一つとして，内耳への遺伝子導入がある．有毛細胞の分化運命決定機構に関係する遺伝子を感覚上皮の支持細胞に導入することにより，有毛細胞が再生することが報告されている[2,3]．しかし，機能的再生を証明する報告はなされていない．一方，内耳で再生能力が低い要因として，再生細胞の元となる幹細胞や前駆細胞といえる細胞が内

耳に存在しても[4,5]，その数が不十分であることが想定されている．そこで内耳有毛細胞や神経細胞の元になる細胞を内耳へ移植してやり，再生を誘導する方法が注目されている．

内耳は再生しない，あるいは再生する能力が極めて低いという前提に基づけば，内耳をいかにして保護するかということも重要となる．一部の神経栄養因子が高い内耳保護効果を有することが報告されており[6,7]，いかにして内耳に長期的に神経栄養因子を投与することができるかが一つの課題となっている．中枢神経系では，これら神経栄養因子を分泌する能力がある細胞を移植することにより，長期的に神経栄養因子を中枢神経系に投与する方法が開発されつつある[8]．内耳への細胞移植は，この目的にも応用できることが期待できる．そこで，どのような細胞をどのような方法で内耳に移植すれば，期待される効果が得られるのかが問題となる．内耳は複雑な構造を持つ脆弱な組織からなるため，特に，いかに移植するのかが大きな問題となる．

神経幹細胞は内耳に生着するか

神経幹細胞は，胎生期だけではなく，成熟後の脳からも分離できることが知られている．さらに，神経幹細胞は脳，脊髄のみならず，網膜にも生着することが報告されている[9]．そこで，神経幹細胞の内耳への生着能力を調べる目的で新生ラットの蝸牛に神経幹細胞を直接注入し，組織学的解析を行った．すると，移植された神経幹細胞が蝸牛内に生着し，一部の移植細胞は感覚上皮内にも生着していることが確認された[10]．この結果は，神経幹細胞が内耳環境下で生着しうることを示唆し，神経幹細胞が内耳移植に用いることができる細胞であり，神経幹細胞の内耳生着後の分化能力が注目されるところとなった．

内耳への細胞移植経路

成熟した内耳は骨に囲まれた複雑な形態を持つ器官であり，手術操作に対しても脆弱な組織である．このような特徴を有する内耳に細胞を移植するためには，その移植方法を十分に検討する必要がある．そこで内耳に細胞を移植する2つの経路を考案し，それぞれの侵襲について聴覚障害を指標とした研究を行った[11]．移植経路として，蝸牛に直接細胞を注入する方法と末梢前庭器の一つである半規管から細胞を注入する方法について検討した．胎生11.5日のGFPマウス脳室からNeuro sphere法を用いて，分離・培養した神経幹細胞を移植細胞とした．成熟したマウス内耳に細胞を蝸牛側壁もしくは半規管から注入し，移植前後の聴力を聴性脳幹反応を用いて評価し，移植細胞の蝸牛での生着について検討を加えた．いずれの移植方法でも内耳に神経幹細胞由来の細胞を確認することができたが，蝸牛から細胞を移植した場合，約50dB SPLの聴覚閾値上昇が認められた（図1）．一方，半規管から細胞移植を行った場合，聴力閾値の上昇は10dB SPL以内にとどまり，低侵襲であることが判明した．つまり，内耳への侵襲を考慮した場合，半規管からの移植が優れていることといえる．一方，移植細胞の生着部位を詳細に検討すると，蝸牛から移植した場合，感覚上皮に接する中央階に細胞が高頻度に認められるのに対して，半規管から移植した場合は，中央階に存在する細胞の割合が顕著に低下することが分かった．したがって，内耳に低侵襲に細胞を移植する方法としては，半規管からの移植が優れるが，感覚上皮に近い中央階に細胞を導入するという観点からは，蝸牛に直接細胞を移植する方法を用いる方が合理的と考えられた．そこで，実験的に内耳有毛細胞の再生への潜在能力を検証する場合には，蝸牛からの注入を用い，神経栄養因子投与目的の移植には半規管から移植する方法を選択することとした．

神経幹細胞の分化能力

内耳有毛細胞の再生を目的とした場合，移植する神経幹細胞が内耳有毛細胞に分化する能力を持たなければならない．そこで，*in vitro*で神経幹細胞の分化誘導を促し，有毛細胞としての性質を持つ細胞に分化するのか否かを免疫組織学的に検討した．神経幹細胞を分化誘導の条件下で培養すると，多くの細胞は神経系の細胞に分化する傾向を示した．しかし，少数であるが，有毛細胞のマーカーであるmyosin VIIaおよびBrn 3c双方に陽性を示す細胞が認められた（図2）[12]．つまり，詳細な誘導のメカニズムは不詳であるが，神経幹細胞は内耳有毛細胞の方向にも分化しうることが判明した．したがって，神経幹細胞を内耳に移植した場合，条件が整えば，内耳で有毛細胞に分化し，感覚上皮の再生に寄与する可能性があることが期待された．

神経幹細胞移植による内耳再生

他の組織でも移植細胞の生着は，傷害を受けた組織に移植した場合の方が良好であることが知られている[9]．全く傷害を与えていない網膜上皮には移植した神経幹細胞が上皮内に侵入しえないが，上皮に傷害を与えることにより数多くの移植細胞が網膜上皮内に生着することが示されている．そこで，耳毒性薬物であらかじめ内耳感覚上皮に傷害を与えた後に，内耳に神経幹細胞を移植し，その分化傾向を免疫組織学的に解析した．感覚上皮への移植

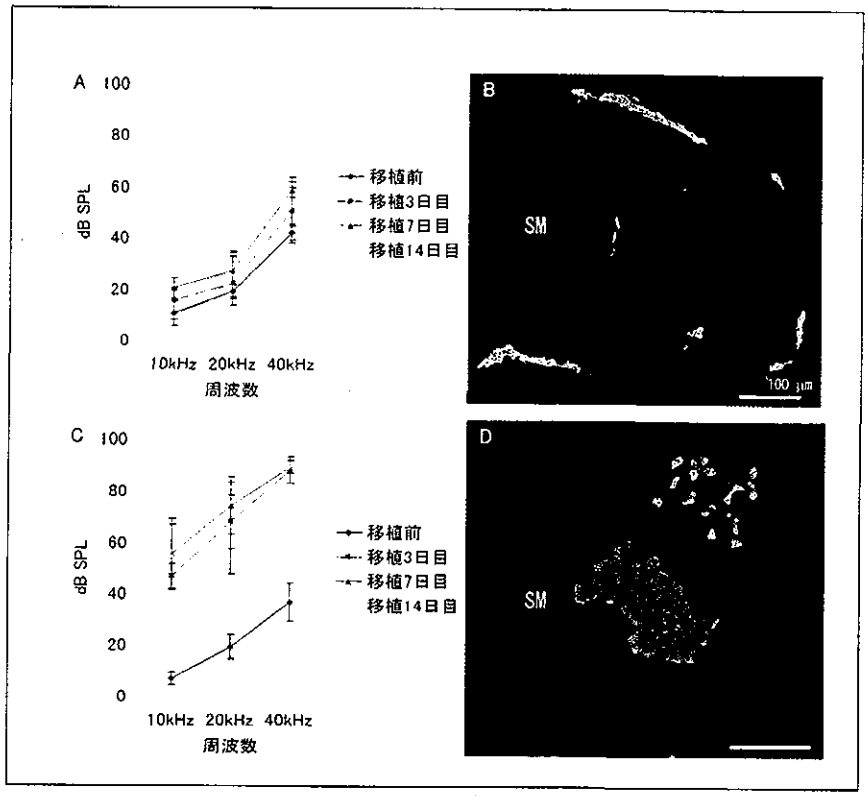

図1 マウス内耳への神経幹細胞移植後の聴力変化と蝸牛内神経幹細胞の局在

A: 半規管から細胞移植後の聴覚閾値（中央値±標準偏差）の変化．閾値上昇は約10dBにとどまる．B: 蝸牛第2回転での移植細胞（緑色蛍光）の局在．蝸牛内に移植細胞を認めるが，中央階（SM）には認められない．C: 蝸牛から直接細胞を移植した後の聴覚閾値の変化．移植直後より約50dBの閾値上昇を認め，回復傾向を認めない．D: 蝸牛から直接移植した場合，移植細胞（緑色蛍光）は中央階（SM）にも認められる．（文献11)より一部改変）

図2 神経幹細胞から誘導された有毛細胞phenotype

myosin VIIa（赤色蛍光）の発現を細胞質，Brn3c（緑色蛍光）発現を核に認める細胞を認める．（文献12)より一部改変）

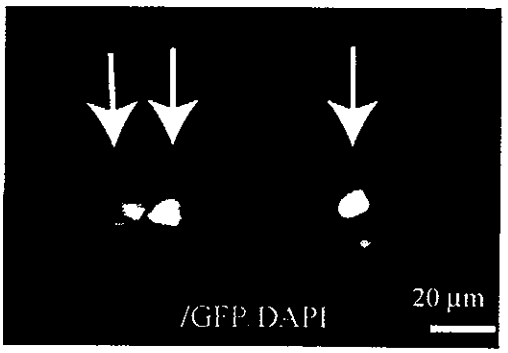

図3 前庭感覚上皮内に生着した神経幹細胞由来細胞

前庭感覚上皮内にmyosin VIIa（赤色蛍光）陽性，GFP（緑色蛍光）陽性の細胞を認める（矢印）．青色蛍光はDAPIによる核染色を示す．（文献13)より一部改変）

細胞の移行を期待する観点から，蝸牛側壁から直接細胞を注入する方法を用いた．結果，移植細胞の生着が蝸牛，前庭感覚上皮内およびラセン神経節に認められた[11]．この結果を無処置の内耳に移植した場合と比較すると，内耳組織内への神経幹細胞の移行が良好であり，内耳においても，組織傷害が移植細胞の生着に影響を及ぼすことが推察された．有毛細胞のマーカーであるmyosin VIIa陽性細胞は，前庭感覚上皮に生着した細胞にのみ認められた（図3）．したがって，内耳に移植された神経幹細胞は，内耳感覚上皮内に生着し，有毛細胞の特徴を有する細胞に分化しうることが明らかとなった．この結果は，神経幹細胞移植が内耳有毛細胞再生に応用できる可能性を示す

ものといえる．しかし，有毛細胞のマーカー陽性の細胞は前庭感覚上皮内に生着した移植細胞の5%にしか認められなかった．したがって，移植方法もしくは移植細胞に何らかの工夫を加えることが，機能的再生の実現には必要と考えられる．

神経幹細胞は神経細胞にも分化することから，内耳への神経幹細胞移植は，蝸牛軸に存在する聴覚の一次ニューロンであるラセン神経節再生にも応用できる可能性がある．ラセン神経節は有毛細胞が受容した音刺激を脳に伝える役割を担う細胞であり，高度感音難聴者における聴覚再獲得の唯一の手段である人工内耳の臨床有用性の獲得には不可欠という側面も持つ．人工内耳は蝸牛内に電極を挿入し，ラセン神経節を直接刺激することによって聴覚を再獲得する電子器機であり，現在では本邦を含め世界中で聾からの回復の手段として広く用いられている．耳毒性薬物により，ラセン神経節に変性を誘導するマウスモデルを用い[14]，ラセン神経節が本来存在する蝸牛軸に神経幹細胞を移植し，その分化傾向を免疫組織学的に検討した[15]．移植された神経幹細胞は蝸牛軸に広く生着し，神経およびグリア細胞に分化していることが確認された．神経とグリアの比率は，損傷脊髄に移植された場合[16]と同様にグリア細胞が多い傾向を示した．したがって，神経幹細胞移植はラセン神経節再生にも応用可能であると考えられる．しかし，機能的再生には効率よく神経細胞に，特に蝸牛求心性神経としての性質を持つ細胞に誘導する工夫が必要である．

神経幹細胞移植による神経栄養因子投与

先述したように内耳への細胞移植は，内耳保護を目的に，内耳へ長期的に神経栄養因子を投与する方法としても応用することが期待できる．内耳への侵襲が低い半規管からの移植経路を用い，移植細胞の神経栄養因子分泌能力について組織学的に検討した[17]．すると，マウス内耳に生着した神経幹細胞の約90%がグリア細胞株由来神経栄養因子（GDNF）陽性であり，約半数の細胞が脳由来神経栄養因子（BDNF）陽性であることが判明した[17]．GDNFおよびBDNFの内耳保護作用はすでに報告されており[6,7]，神経幹細胞移植は，内耳への神経栄養因子投与方法として応用可能であると考えられる．

おわりに

内耳障害の治療に細胞移植を応用しようとする試みは，未だ緒についたところである．我々が内耳に細胞を移植するという新しいコンセプトを提示してから3年が経過し，本邦のみならず，細胞移植による内耳再生に関する研究が広く行われつつある．神経幹細胞のみならず，胚性幹細胞[18]，骨髄由来間葉系細胞[19]，内耳幹細胞[5,20,21]を内耳再生に応用する研究が行われている．しかし，現在のところ，哺乳類内耳に細胞移植を行い，有毛細胞再生を誘導したという結果は我々の報告のみにとどまっている．内耳幹細胞は内耳組織の再生を考慮すると理想的な細胞と思われるが，いかにして細胞のソースを得るかという問題がある．一方，胚性幹細胞は万能細胞として，内耳再生についても期待がもたれるが，自己由来の細胞を用いることはできない．神経幹細胞は自己由来細胞を得られる可能性がある細胞であるが，現実的には問題がある．また，有毛細胞再生に関しては，有毛細胞の誘導効率に問題があり，内耳再生に用いる移植細胞として，どの細胞がベストなのかについては今後の研究成果を待つ必要がある．また，内耳での移植細胞に対する免疫反応についての研究も今後検討されるべき課題といえる．しかし，内耳細胞移植に関する一連の研究結果は，内耳細胞移植の内耳治療への応用への期待を抱かせるものであり，今後の研究成果に期待がもたれる．

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Original Contribution

Elevation of superoxide dismutase increases acoustic trauma from noise exposure

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Abstract

The generation of superoxide has been implicated as a cause of cochlear damage from excessive noise. Cu/Zn superoxide dismutase (SOD1) generally will protect against superoxide-mediated tissue injury but protection by this enzyme against noise trauma is controversial. This study assessed auditory function in C57BL/6 mice overexpressing SOD1 or treated with lecithinized SOD1 (PC-SOD1). Noise exposure caused significantly higher threshold shifts in PC-SOD1-treated animals than physiological saline-treated animals. Cochlear tissues of PC-SOD1-treated animals exhibited significant elevation of the levels in the SOD activity, not in the catalase activity, in comparison with those of saline-treated animals. Likewise, transgenic mice overexpressing SOD1 tended to suffer higher threshold shifts than nontransgenic littermates from noise exposure. The findings indicate that increasing SOD1 enhances auditory dysfunction following noise exposure.

Keywords: Reactive oxygen species; Hearing loss; Acoustic trauma; Redox regulation; SOD; Free radicals

Introduction

Hearing impairment is one of the most frequent disabilities in industrialized countries and exposure to intense sound is one of the major causes for hearing deficits. Depending on the intensity of the exposure, acoustic overstimulation induces a temporary threshold shift with complete recovery or a permanent threshold shift (PTS).[1] PTS involves the loss of sensory hair cells and spiral ganglion neurons [1]. In contrast to the avian auditory epithelium, the mammalian cochlea is unable to regenerate new hair cells, resulting in irreversible hearing loss. Protection of hair cells and spiral ganglion neurons from cell death is therefore crucial for the prevention of PTS due to acoustic trauma.

Reactive oxygen species (ROS) play a key role in mechanisms for induction of cochlear damage under various pathological conditions [2–6]. Superoxide is readily generated in the inner ear following acoustic overstimulation [2]. Superoxide can react in an iron-catalyzed Fenton reaction to form destructive hydroxyl radicals or combine with nitric oxide to form the highly toxic peroxynitrite. Therefore, the regulation of superoxide levels is important cochlear degeneration caused by ROS. Superoxide dismutase (SOD) is an enzyme that converts superoxide to hydrogen peroxide and has three subtypes including copper/zinc-superoxide dismutase (SOD1), manganese-superoxide dismutase (SOD2), and extracellular superoxide dismutase (SOD3).

Abbreviations: ABR, auditory brainstem response; EDTA, ethylenediaminetetraacetic acid; GPx, glutathione peroxidase; PC-SOD1, lecithinized copper/zinc superoxide dismutase; PTS, permanent threshold shift; ROS, reactive oxygen species; SOD1, Cu/Zn superoxide dismutase; SPL, sound pressure level.

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