Deafness in occludin-deficient mice with dislocation of tricellulin and progressive apoptosis of the hair cells

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ABSTRACT

Occludin is the first identified protein in the tight junction (TJ), but its function has remained for the most part obscure. TJs have been demonstrated to play important roles in the inner ear function, and occludin is expressed in all the epithelial TJs in the inner ear. Thus, we examined the inner ears of occludin-deficient (Occ−/−) mice. Although inner ears initially developed normally in Occ−/− mice, apoptosis occurs in hair cells in the organ of Corti around day 12 after birth, and deafness develops. Since hair cell degeneration was not observed in cochlear explant cultures of Occ−/− mice, environmental changes were considered to be the trigger of cell death. As for the vestibular system, both the morphologies and functions are normal in Occ−/− mice. These phenotypes of Occ−/− mice are very similar with those of claudin-14 or claudin-9 deficient mice, leading us to speculate on the existence of imbalance induced by TJ abnormalities, such as localized ionic components. Moreover, the occludin deficiency led to dislocalization of tricellulin, a gene responsible for human deafness DFNB49. The deafness in Occ−/− mice may be due to this dislocalization of tricellulin.

KEY WORDS: Occludin, Deafness, Tight junction, Cochlea, Hair cell, Tricellulin

INTRODUCTION

Multicellular organisms are composed of various compartments, and maintaining the environments of each compartment is essential for organs to fulfill their functions. In the inner ear, the different environments of endolymph and perilymph must be maintained (reviewed by Wangemann and Schacht, 1996). The endolymph and the perilymph are delineated by epithelial cells, and the leakage of solutes through a paracellular pathway is maintained (reviewed by Wangemann and Schacht, 1996). The TJ barrier has been demonstrated to play important roles in the inner ear function, and TJ transmembrane protein, was also identified as a responsible gene for human hereditary deafness DFNB49 (Chishti et al., 2008; Riazuddin et al., 2006) and knockin mice of tricellulin mutant gene, which mimics one of DFNB49-associated mutations, exhibited profound deafness (Nayak et al., 2013). Furthermore, a mutation in claudin-9 gene, another claudin member expressed in the organ of Corti (Kitajiri et al., 2004c), causes a severe deafness in mice (Nakano et al., 2009).

Occludin is a TJ membrane protein discovered earlier than claudin (Furuse et al., 1993), and is expressed in all the epithelial TJs, including the cochlea and the vestibule in the inner ear (Kitajiri et al., 2004c). However, its function remains unknown since no findings have been reported concerning its involvement in TJs barrier function (Saitou et al., 1998). Occludin knockout mice have been found to develop morphological abnormalities in various organs (Saitou et al., 2000). TJs may contribute to the morphogenesis of tissues through intercellular adhesion since intercellular interactions and adhesions, which organize the tissues, are essential for maintaining functions and morphogenesis of the tissues. In human, mutations of occludin cause brain calcification and renal dysfunction (O’Driscoll et al., 2010; LeBlanc et al., 2013).

The inner ear is considered useful for examining TJs and morphogenesis. In the organ of Corti of the cochlea, hair cells surrounded by supporting cells generate four clear rows (1 row by inner hair cells, 3 rows by outer hair cells), and stereocilia of each hair cell are arranged in an orderly fashion. This morphology provides a model to examine planner cell polarity in mammals (Montcouquiol et al., 2003), and the inner ear is a good target organ to observe morphogenesis. Differentiation and morphogenesis of the inner ear can be examined using expression markers (Hasson et al., 1995), and the period, during which functional development is completed after birth and environmental differences between endolymph and perilymph are generated, has been studied in detail (Lim and Anniko, 1985; Yamasaki et al., 2000). This information is valuable for examining TJ function, which is closely associated with the environments of endolymph and perilymph. Moreover, objective assessments of inner ear functions, including auditory perception and sense of equilibrium, can also be made on mice (Zheng et al., 1999; Iwashita et al., 2001).
Thus, in this study, inner ears of occludin knockout mice were examined in detail to further understand the TJ function in the inner ear.

RESULTS
Occludin deficiency causes deafness
We previously generated occludin-deficient (Occ−/−) mice. In these Occ−/− mice, the barrier function of intestinal epithelium was normal, but histological abnormalities were found in several tissues (Saitou et al., 2000). Interestingly, they showed no Preyer’s reflex, a motor reflex in response to auditory stimuli. When a sound stimulus was administrated in the form of a loud handclap, they showed no reflexive reaction (Fig. 1A). We then measured the auditory brainstem response (ABR) to stimuli of 70-decibel (dB) (20 kHz) sound pressure level (SPL) in two sets of Occ+/− intercross littermates (6 weeks old), which were later genotyped (Fig. 1B). Among 8 littermates in total, two showed no ABR, while the others showed a typical ABR waveform. Interestingly, only the two littermates showing no ABR were identified as Occ−/− mice. This perfect correlation between Occ−/− genotype and the lack of ABR was reproducibly obtained in different series of measurements. In Fig. 1C, the hearing thresholds of 6-week-old mice were measured at various sound frequencies. Occ+/+ and Occ−/− mice showed normal hearing thresholds (10–50 dB SPL), while Occ−/− mice showed profound deafness (hearing threshold, >70–90 dB SPL).

Occludin deficiency causes degeneration of the organ of Corti
Light microscopic observation with toluidine blue-stained Epon sections identified deformity of the organ of Corti in the 6-week-old Occ−/− mice (Fig. 1D). A collapse of the tunnel of Corti was observed, and outer hair cells were damaged or lost. There was no morphological change in Reissner’s membrane, tectorial membrane, spiral ligament or stria vascularis. Then, the apical surface of the Corti organ was observed by scanning electron microscopy (Fig. 2A). Up to 9 days after birth, the Occ−/− cochlea was indistinguishable from that of the control. However, subsequently rapid loss of OHC was observed.

At 12 days after birth, outer hair cells began to disappear rapidly, and at day 15, the outer hair cells had disappeared almost completely.
entirely, and also the inner hair cells showed changes, and began to disappear. These changes and the disappearance of hair cells were considered to be the cause of deafness in \textit{Occ}$^{-/-}$ mice.

**No structural changes in TJs of the inner ear were observed in \textit{Occ}$^{-/-}$ mice**

Whether structural changes in TJs occurred in \textit{Occ}$^{-/-}$ mice was examined using transmission electron microscopy, since occludin is a membrane protein localized at TJs (Furuse et al., 1993). However, kissing points, where tight junction strands between adjacent cells causing occlusion of plasma membrane, appeared normal, as in other organs of \textit{Occ}$^{-/-}$ mice (Saitou et al., 2000) (Fig. 2E), and TJs were apparently normal also in \textit{Occ}$^{-/-}$ mice.

Expression of claudin-14, which is expressed in TJs of the organ of Corti, and its mutations cause deafness (Wilcox et al., 2001; Ben-Yosef et al., 2003), was examined using whole mount immunostaining, but no changes were observed in \textit{Occ}$^{-/-}$ mice (Fig. 2B). In addition to claudin-14, claudin-1, -2, -3, -9, -10, -12 and -18 were expressed in the organ of Corti (Kitajiri et al., 2004c), but no change of their expression could be confirmed in \textit{Occ}$^{-/-}$ mice (claudin-9 and -12 in supplementary material Fig. S1).

To examine the barrier function of the \textit{Occ}$^{-/-}$ inner ear, we performed a tracer experiment as described previously (Kitajiri et al., 2004b). The perilymph compartment was perfused from the round to oval windows with an isotonic solution containing a primary amine-reactive biotinylation reagent (Mr=556.59), which is covalently cross-linked to the accessible cell surface. After 5 minutes incubation followed by perfusion with PBS, the cochlea was dissected out, fixed and frozen. Frozen sections of the cochlea were labeled with anti-ZO-1 (TJ scaffold protein) Ab in red and streptavidin in green to detect TJ and bound biotin, respectively (Fig. 3). As a result, there was no difference in the diffusion of tracer between the \textit{Occ}$^{-/-}$ organ of Corti and the \textit{Occ}$^{+/+}$ organ of Corti. In this study, the basilar membrane barrier that faces the perilymph, not an apical surface of the organ of Corti on which hair cells reside, was examined, and maintenance of the TJs barrier function of the organ of Corti in \textit{Occ}$^{-/-}$ mice was indicated. Although occludin was also expressed in marginal cells and basal cells of the stria vascularis, the barrier function of the stria vascularis was not affected either (data not shown). Historically, occludin deficiency does not cause evident loss of barrier function (Saitou et al., 1998), the data in this study have also suggested that occludin deficiency does not affect the TJ structure or barrier function.

**Fig. 2.** Scanning electron micrographs of the organ of Corti and expression of claudin-14 in the organ of Corti. (A) Scanning electron micrographs of the organ of Corti. Up to postnatal day 9 (P9), both inner and outer hair cells appeared to develop normally in \textit{Occ}$^{-/-}$ mice. However, at postnatal day 12 (P12), outer hair cells began to disappear from the surface of the \textit{Occ}$^{-/-}$ organ of Corti, and be replaced by supporting cells. At postnatal day 15 (P15), the outer hair cells had disappeared almost entirely, and also the inner hair cells showed changes, and began to disappear from the surface. (B) Expression of claudin-14 in the organ of Corti. The whole mount double immunofluorescence micrographs for claudin-14 (a–f, green) and occludin (g,h, red) of the \textit{Occ}$^{+/+}$ and \textit{Occ}$^{-/-}$ organ of Corti. In every stage of degeneration (P9–P15; postnatal days 9–15), claudin-14 was expressed normally at the junctional complex along the apical borders of the organ of Corti even in \textit{Occ}$^{-/-}$ mice. Scale bars: 10 μm.

**Fig. 3.** Tracer permeability assay of the organ of Corti of 6-week-old mice. An isotonic solution containing freshly made biotinylation reagent was injected into the perilymph space from the round window of the cochlea, and after 5 minutes incubation followed by being washed with PBS, the cochlea was dissected out, fixed and frozen. Frozen sections were triple stained with anti-ZO-1 Ab in red, streptavidin in green and DAPI in blue to detect TJ and bound biotin, respectively. In this study, the basilar membrane barrier that faces the perilymph, not an apical surface of the organ of Corti on which hair cells reside, was examined, and there was no difference between the \textit{Occ}$^{+/+}$ and \textit{Occ}$^{-/-}$ organ of Corti. n=3. Scale bar: 50 μm.
Environmental factors induce apoptosis in hair cells of the Occ<sup>−/−</sup> organ of Corti

For detailed study of morphological changes in the organ of Corti, the organs of Corti undergoing morphogenesis were whole mount stained with markers. Radixin was used as a marker for stereocilia, and myosin VIIa for hair cells (Kitajiri et al., 2004a; Hasson et al., 1995). As a result, the number of hair cells was decreased in the Occ<sup>−/−</sup> organ of Corti (Fig. 4A).

To monitor the process of cell death within the organ of Corti, we performed TUNEL experiments in P12, which is on the process of degeneration. As a result, in the Occ<sup>−/−</sup> mice, degenerating OHCs were labeled, which was never been seen in the Occ<sup>+/+</sup> mice (Fig. 4B).

To clarify whether degeneration of hair cells of Occ<sup>−/−</sup> mice is caused by a signal intrinsic to the cell or by extracellular conditions, we maintained explants derived from the organ of Corti of Occ<sup>−/−</sup> and Occ<sup>+/+</sup> mice for up to 12 days (from P3 to P15) in culture medium. At P15, immunostaining with anti-radixin and anti-myosin VIIa antibodies revealed the survival of explants of Occ<sup>−/−</sup> hair cells, as observed with the explants of Occ<sup>+/+</sup> mice (Fig. 5). This is in sharp contrast to the organ of Corti of Occ<sup>+/−</sup> mice in vivo, in which most of the outer hair cells and some inner hair cells had disappeared by P15 (Fig. 2A, Fig. 4A). These findings suggest that the apoptosis in hair cells of Occ<sup>−/−</sup> mice was induced by some environmental factors.

Occ<sup>−/−</sup> mice have normal vestibular function

The expression of occludin is also observed in TJs in the vestibule (Kitajiri et al., 2004c), leading us to speculate on a possible imbalance in Occ<sup>−/−</sup> mice. Thus, we measured the vestibulo-ocular reflex. A mouse was mounted on a turntable and rotated sinusoidally, and the eye position was recorded by a CCD camera. However, as shown in Fig. 6A, the sinusoidal curve of eye velocity shows no difference among Occ<sup>+/+</sup>, Occ<sup>−/−</sup>, or Occ<sup>+/−</sup> mice. Indeed, the vestibulo-ocular reflex gain of the Occ<sup>−/−</sup> mouse was normal at all frequencies of head rotation stimulus (Fig. 6B). Scanning electron microscopy consistently revealed that the appearance of sensory epithelia in the crista ampularis was indistinguishable between Occ<sup>+/+</sup> and Occ<sup>−/−</sup> mice (Fig. 6C).

Occludin deficiency leads to dislocalization of tricellulin in cochlea

Tricellulin is a recently identified constituent of TJ, and is the first marker of the tricellular tight junction (tTJ) where three epithelial cells meet in polarized epithelia (Ikenouchi et al., 2005). It has COOH-terminal sequence similar to occludin, and is necessary to maintain the epithelial barrier (Ikenouchi et al., 2005). The mutations of tricellulin were reported to cause human deafness DFNB49 (Riazuddin et al., 2006), and occludin was reported to support tricellular localization of tricellulin (Ikenouchi et al., 2008). The importance of tricellular localization of tricellulin is also suggested by human deafness DFNB42 (Borck et al., 2011). The responsible gene of DFNB42 encodes ILDR-1, which is required for the localization of tricellulin at tricellular contacts (Higashi et al., 2013).

Thus, we examined if occludin deficiency cause dislocalization of tricellulin in cochlea. Two parts of cochlea, stria vascularis and organ of Corti, were dissected out from Occ<sup>+/−</sup> and Occ<sup>−/−</sup> mice, and whole mount double immunofluorescence staining were performed (Fig. 7). In Occ<sup>+/−</sup> cochlea, tricellulin was localized at
tTJ, as reported previously (Riazuddin et al., 2006). But in Occ\(^{-/-}\) cochlea, tricellulin was mislocalized and its signal at bicellular TJs was increased. The dislocalization of tricellulin was observed in other tissues of Occ\(^{-/-}\) mice (supplementary material Fig. S2).

**DISCUSSION**

Occludin is a molecule that was first identified as a membrane protein of TJs, but its functions remain unclear (Furuse et al., 1993; Saitou et al., 1998; Saitou et al., 2000). TJs were demonstrated to play roles in compartmentalization in multicellular organisms (Schneeberger and Lynch, 1992; Anderson and Van Itallie, 1995; Balda and Matter, 1998; Tsukita et al., 2001), and detailed study of its physiological functions requires functional analysis in living tissues. In the inner ear, there are compartments of endolymph and perilymph, and maintenance of the environmental differences between them is essential for inner ear functions (reviewed by Wangemann and Schacht, 1996). Functional assessments were made in the inner ear, for which TJs play important roles and detailed assessments of functions and morphology can be made (Kitajiri et al., 2004b).

Although inner ears initially developed normally in Occ\(^{-/-}\) mice, apoptosis occurs in outer hair cells in the organ of Corti followed by in inner hair cells, and deafness developed. Cell death occurred at around day 12 after birth in cochlear hair cells, the same period when the ionic environment in the endolymph and high resting endocochlear potential (EP) appear (Yamasaki et al., 2000). Since hair cell degeneration was not observed in cochlear explant cultures of Occ\(^{-/-}\) mice, environmental changes were considered to be the trigger of cell death. As no abnormalities were observed in the vestibula, in which high resting potential comparable with EP dose not exist, there is a possibility that cochlear hair cells of Occ\(^{-/-}\) mice cannot tolerate the high EP.

Occludin is not essential for TJ formation (Saitou et al., 1998), and the mice in this study underwent no structural changes in the TJs. Since morphological abnormalities of many organs were observed in the occludin knockout mice, occludin is considered to be essential for maintaining the morphology of other organs (Saitou et al., 2000). There has been some reports that occludin is involved in the regulation of TJ permeability in response to cytokines (Marchiando et al., 2010; Van Itallie et al., 2010), suggesting that occludin play a role in the modulation of barrier function. Recently, it has been reported that mutations of occludin cause brain calcification and renal dysfunction (O’Driscoll et al., 2010; LeBlanc et al., 2013), which further suggests the occludin involvement in TJ barrier function.

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**Fig. 6.** The balance function of 6-week-old Occ\(^{-/-}\) mice. (A) The vestibulo-ocular reflex to 0.8 Hz sinusoidal head rotation. The eye position during the head rotation stimulus was recorded by a CCD camera and the eye velocity was calculated. The sinusoidal curves of eye velocities were indistinguishable between Occ\(^{+/+}\), Occ\(^{+/-}\) and Occ\(^{-/-}\) mice. (B) The gain was obtained by dividing the peak eye velocity by the peak head velocity. The vestibulo-ocular reflex gains of Occ\(^{-/-}\) mice are also normal at any frequency of the head rotation stimulus in Occ\(^{+/+}\) and Occ\(^{+/-}\) mice. (C) Scanning electron micrographs of the 6-week-old crista ampullaris of the vestibule. The appearance of stereocilia and hair cells are totally normal in Occ\(^{-/-}\) mice. Scale bar: 5 μm.
Generation of mice lacking claudin-14, a membrane protein expressed in the organ of Corti, was reported (Ben-Yosef et al., 2003). In these mice, hair cells in the organ of Corti initially developed normally, but degenerated within 3 weeks after birth and developed deafness. However, the EP was not reduced, and collapse of the barrier function has not been demonstrated. Mice harboring a mutation in claudin-9 gene, which is another component of TJ in the organ of Corti, also exhibit severe deafness with progressive degeneration of hair cells (Nakano et al., 2009). The phenotypes of these mice are very similar with those of occ−/− mice in the following points: morphological changes of the organs of Corti, preceding degeneration in outer hair cells, degeneration timing, and no degeneration observed in explant cultures. In addition, since the expression of claudin-14 and claudin-9 is maintained in the cochlea of occ−/− mice, occludin deficiency does not cause changes in the expression or localization of claudin-14 or claudin-9, resulting in the same abnormalities. Considering that three mutant mice lacking TJ components exhibit similar phenotype, some defect in TJ function would be the cause of hair cell degeneration, leading to hearing loss.

It has been reported that tricellulin fail to localize at tricellular tight junctions (tTJs) in the occludin-deficient cells (Ikenouchi et al., 2005). Antibodies against tricellulin did not localize at tTJs, whereas in occludin-deficient tissue tricellulin is found in bTJs in addition to tTJs. Scale bars: 10 μm.

Fig. 7. The dislocalization of tricellulin in occ−/− cochlea. The organ of Corti (upper panels) and stria vascularis Corti (lower panels) of P15 occ+/+ and occ−/− mice were labeled with anti claudin-14 (green) and anti tricellulin (red) antibodies. In occ−/+ tissue tricellulin is mainly detected in tTJs, whereas in occludin-deficient tissue tricellulin is found in bTJs in addition to tTJs. Scale bars: 10 μm.

MATERIALS AND METHODS

Antibodies

We previously raised and characterized rat anti-mouse occludin mAb (MOC37), rabbit anti-mouse claudin-14 pAb, rat anti-mouse radixin mAb (R21), rat anti-mouse ZO-1 mAb and rat anti-mouse tricellulin mAb (Saitou et al., 1997; Kitajiri et al., 2004c; Kitajiri et al., 2004b; Hirao et al., 1996; Ikenouchi et al., 2005). Rabbit anti-ZO-1 pAb was purchased from Zymed Lab (San Francisco, California, USA). Rabbit anti-human myosin VIIa pAb was provided by Dr Tama Hasson (University of California, Los Angeles, CA) (Hasson et al., 1995).

Generation of occ−/− mice

occ−/− mice were generated as previously reported (Saitou et al., 2000). Two independent mouse J1 ES clones (129/Sv), in which the occludin gene was correctly disrupted, were injected into C57BL/6 blastocysts, and the resulting chimeras were mated with C57BL/6 mice (Doi et al., 1999).

Auditory Brainstem Response (ABR) measurements

ABR measurements were performed in a soundproof room according to the method described previously (Zheng et al., 1999; Kitajiri et al., 2004b; Kitajiri et al., 2004a). In general, 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 the maximum amplitude until no waveforms could be visualized.

Vestibulo-Ocular Reflex (VOR) measurements

VOR was measured as described previously (Iwashita et al., 2001; Kitajiri et al., 2004a). Head movements were transduced to DC signals using a small angular velocity sensor (Gyrostar, Murata Corporation, Japan), which was fixed on the turntable. Eye movements were detected by LED and a CCD camera, 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 occ−/− or occ−/+ mice, and the round and oval windows were opened, together with the small holes in the cochlear apical turn and superior semicircular canal. The perilymphatic space was gently perfused with 10% trichloroacetic acid (TCA) from the round to oval windows (Hayashi et al., 1999; Kitajiri et al., 2004c). Then the samples were immersed in 10% TCA for 1 hour, washed 3 times with phosphate buffered saline (PBS), and decalcified with 5% EDTA in PBS for 3 days. They were microdissected and mounted on slide glasses for whole mount staining. Some specimens were immersed in 30% sucrose in PBS for 1 day, and frozen in liquid nitrogen. Frozen sections −10 m thick were cut from these samples and air-dried on slide glasses. The whole mount samples and frozen sections were treated with 0.2% Triton X-100 in PBS for 15 min, and soaked in 1% bovine serum albumin (BSA) in PBS. The whole mounts and sections were then incubated with primary Abs for 30 min at room temperature. They were then washed three times with PBS, followed by a 30-min incubation with Cy3- or Alexa Fluor® 488-conjugated secondary antibody. After a wash with PBS, they were embedded in 95% glycerol-PBS containing 0.1% paraformaldehyde and 1% n-propylgallate. 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.

TUNEL method

Apoptotic cells were detected by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) method. After microdissection
for whole mount staining, the specimens were permeabilized in 0.5% Triton X-100 in PBS for 30 min. TUNEL staining was performed using an ApopTag Fluorescein Direct In Situ Apoptosis Detection Kit (Intergen Company, Purchase, NY) according to the supplier’s instruction. The specimens were observed using a Delta Vision system.

Scanning electron microscopy
Temporal bones obtained from Oec+/+ or Oec−/− mice were fixed using perilymphatic perfusion as described above with 1% gluteraldehyde in 0.1 M phosphate buffer (pH 7.2). They were then washed with phosphate buffer and post-fixed in 1% OsO4 for 2 hours, 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 electron microscopy
Samples were fixed as described above, dehydrated with ethanol and embedded in Polybed 812 (Polysciences). Ultrathin sections were cut, doubly stained with uranyl acetate and lead citrate and viewed with a JEM 1010 transmission electron microscope (JEOL).

Explant culture
Cochleae from the temporal bones of 3-day-old Oec−/− or Oec−/− mice under deep anesthesia with ether were dissected in PBS without calcium. After microdissection, cochleae were placed onto the sterile filter membrane (Millicell, 12 mm, Millipore, MA, USA) in standard medium composed of Minimum Essential Medium (Invitrogen Corp., CA, USA) added with 3 g/l glucose and 0.3 g/l penicillin G potassium salt (Nacalai Tesque Inc., Kyoto, Japan) into a 24 well culture plate (Asahi Techno Glass Corp., Tokyo, Japan). Cochleae were then incubated at 37°C in a humidified atmosphere of 95% air and 5% CO2.

Tracer permeability assay
Temporal bones were removed from 6-week-old mice, and the round and oval windows were opened in PBS containing 1 mM CaCl2. As mentioned above, the perilymph space was carefully perfused with 100 µl of 10 mg/ml EZ-Link™ Sulfo-NHS-LC-Biotin (Pierce Chemical Co., Rockford, IL) in PBS containing 1 mM CaCl2 for 5 minutes (Chen et al., 1997), followed by perfusion for 5 times with PBS containing 1 mM CaCl2. The temporal bones were then fixed by perilymphatic perfusion with 10% TCA for 1 hour, and processed for immunofluorescence microscopy. The distribution of injected biotin was visualized by incubating frozen sections with streptavidin-immunofluorescence microscopy. The terminal of occludin is required for tight junction barrier function in early Xenopus embryos. J. Cell Biol. 138, 891-899.


Fig. S1. Expression of claudin-9 and -12 in the organ of Corti. The whole mount immunofluorescence micrographs for claudin-9 and claudin-12 of the Occ+/+ and Occ-/- organ of Corti in P15 mice. Claudin-9 and claudin-12 were expressed normally at the junctional complex along the apical borders of the organ of Corti even in Occ-/- mice. Scale bars: 10 μm.

Fig. S2. The dislocalization of tricellulin in other tissues (kidney, colon and liver) of Occ-/- mice. Kidney, colon and liver of 6-week-old Occ+/+ and Occ-/- mice were labeled with anti tricellulin (red) and anti zo-1 antibodies. In Occ+/+ tissues tricellulin is predominantly concentrated in tTJs, whereas in all occludin-deficient tissues tricellulin is found in bTJs in addition to tTJs. Scale bars: 5 μm.