antibody overnight at room temperature, washed and incubated with a secondary biotinylated anti-rabbit IgG (Vector Labs, Burlingame, CA, USA). Immunostaining was visualized by incubation with the Vectastain ABC reagent (Vector Labs) followed by 3,3′-diaminobenzidine (DAB). Sections were not counterstained.

### Proteomic analysis

For proteomic analysis in the mouse, membranous cochlear and vestibular labyrinths were dissected separately from approximately 3-month-old (+/+) and *Coch* (−/−) mice. Protein lysates were prepared by incubation of tissues in lysis buffer [2% sodium dodecyl sulfate (SDS), 100 mM ammonium bicarbonate, 10 mM dithiothreitol (DTT), pH 8.5] at 90°C for 10 min, 37°C for 60 min, with subsequent sonication in 0.2% SDS, and incubation at 90°C for 10 min. Three rounds of sonication and boiling were performed, followed by alkylation with 30 mM iodoacetamide. The reaction was quenched with 10 mM DTT and samples were separated by SDS gel electrophoresis in 8–16% polyacrylamide gels. Gels were size-fractionated into four sections, destained with two washes of 50% methanol and 5% acetic acid, followed by three alternating washes of ammonium bicarbonate and acetonitrile. Gel slices were dried and subsequently suspended individually in trypsin (5.5 μg/ml in 50 mM ammonium bicarbonate) prior to incubation at 37°C for 18 h for digestion of proteins. Peptides were extracted with two rinses of 50 mM ammonium bicarbonate and two rinses of 50% acetonitrile and 0.1% formic acid. Samples were prepared for mass spectrometry by lyophilization and rehydration in 5% acetonitrile and 0.1% formic acid.

For proteomic analysis of the adult human inner ear, the only materials available were formalin-fixed, paraffin-embedded tissues. Sections of 8 μm in thickness were used from the same DFNA9-affected temporal bone as used for immunohistochemistry. For a human adult temporal bone control, formalin-fixed, paraffin-embedded, 8 μm thick sections were used from an 85-year-old female with otosclerosis, but showing no inner ear histopathology and with normal cochlear duct structures including the spiral ligament and spiral limbus.

Extraction of proteins from human paraffin-embedded sections was performed by adding heptane and incubating at room temperature for 60 min, followed by the addition of methanol to pellet-extracted proteins. Protein extracts were resolubilized and sonicated in 2% SDS, 100 mM ammonium bicarbonate, 10 mM DTT, pH 8.5. Reduction of proteins was achieved by boiling samples at 90°C for 20 min, followed by incubation at 37°C for 60 min and alkylation in 30 mM iodoacetamide for 60 min at room temperature. Trypsin digestion of proteins and preparation for mass spectrometry were performed as described for the mouse samples.

For mass spectrometry, samples were run on an LCQ DECA XP plus Proteome X workstation (Thermo Electron Corporation, San Jose, CA, USA). Peptide identifications were made using Sequest through the Bioworks Browser 3.1 (Thermo Electron Corporation). Database searches were made using the NCBI RefSeqHuman and RefSeqMurine databases using static carbamidomethyl-modified cysteines and differential oxidized methionines, followed by further searches using differential modifications.

### Reverse transcription–polymerase chain reaction

RT–PCR was performed with total RNA isolated from EBV-transformed cell lines from two P51S DFNA9 individuals, using the RNAeasy midi-kit (Qiagen, Leusden, the Netherlands). cDNA synthesis was performed as described (56) and PCR was done for 35 cycles under standard conditions using the following primers flanking the mutation: forward 5′-ACCAGAGGCTTGGACATCAG-3′ in exon 4 and reverse 5′-TTTGAGACTGGATGCCATTG-3′ in exon 5. Amplified products were gel-isolated and sequenced using an ABI PRISM Big Dye Terminator Cycle Sequencing V2 Ready reaction kit and the ABI 3730 DNA sequencing apparatus (Applied Biosystems, Foster City, CA, USA).

## SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online. The supplementary figure shows immunohistochemistry performed on human adult temporal bone sections with secondary antibody alone, as a negative control; no staining is detected.

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This manuscript is dedicated by Cynthia Morton to the memory of Craig Philip Morton, who passed away on July 9, 2005 from pancreatic cancer, and who upon his death donated his temporal bones to the NIDCD National Temporal Bone, Hearing and Balance Pathology Resource Registry (http://www.tbregistry.org/) having struggled with Meniere's disease during his too short life.

*Conflict of Interest statement*. None declared.

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# Spatiotemporal expression of Cochlin in the inner ear of rats during postnatal development

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ABSTRACT

Cochlin (encoded by *COCH*) constitutes 70% of non-collagenous protein in the inner ear, and the expression of cochlin is highly specific to the inner ear. Eleven missense mutation and one in-frame deletion have been reported in the *COCH* gene, causing hereditary progressive sensorineural hearing loss and vestibular dysfunction, DFNA9. These data imply that cochlin should bear an essential and crucial role in the inner ear function. However, the role of cochlin has not been fully clarified. We have investigated the spatiotemporal expression of cochlin in the inner ear of rats during postnatal development to better understand the functional role of cochlin. By immunohistochemistry, cochlin expression was faint in the cochlea and vestibule on the 6th day after birth (DAB6). At DAB70, strong expression of cochlin was detected in the spiral limbus and spiral ligament within the cochlea, and in the stromata of the maculae of otolithic organs and crista ampullaris within the vestibule. Immunoreactivity for cochlin increased during the postnatal development. Western blot analysis also showed an increase in the expression of cochlin isoforms. Furthermore, the dominant isoform of cochlin expressed changed from p63s to p40s between DAB24 and DAB70. These results suggest that the expression of cochlin may be related to the maturation of inner ear function, and the change in isoforms of cochlin expressed will provide important insight into the understanding of both cochlin function and formation of cochlin isoforms. This is the first to report about the spatiotemporal expression of cochlin in the developing rat inner ear.



The protein product of *COCH* gene, cochlin [8,9,13,14,16,19,18], is an extracellular matrix protein, and a major constituent of the inner ear, comprising 70% of the non-collagenous inner ear protein [7], and the expression of cochlin is highly specific to the inner ear [14]. Eleven missense mutation and one in-frame deletion have been reported in the *COCH* gene, causing hereditary progressive sensorineural hearing loss and vestibular dysfunction, DFNA9 [2–6,17,23,26]. In two-dimensional gel electrophoresis, cochlin in the inner ear tissue is composed of 16 different protein spots, with charge and size heterogeneity, and is classified into three groups (p63s, p44s, p40s) [7]. In addition, a short isoform of cochlin named cochlin-tomoprotein (CTP) was identified in the periylmph [9]. The deduced amino acid sequence of full-length cochlin (p63s) is a mosaic molecule consisting of a secretion signal peptide followed by two different types of domain, a Limulus factor C, cochlin and late gestation lung protein (LCCL) module and two von Willebrand factor A (vWF-A)-like domains, which are also found in combination with other motifs in proteins with diverse function [21]. p63s has an LCCL module and two vWF-A like domains, whereas p40s and p44s lack the LCCL module [7]. Interestingly, CTP, a 16-kDa short isoform that contains only an LCCL module without any vWF-A like domains, is found in the perilymph [9] (Fig. 1). Motif analysis of the *COCH* gene has suggested that cochlin may have a role in host defense through antibody-independent innate immunity (via its LCCL module) and may be critical for the highly structured architecture of the sensory organ (via its vWF-A like domains) [25]. These data imply that cochlin should play an essential and crucial role in the inner ear function. However, the functional role of cochlin has not been fully clarified. To better understand the function of cochlin, we have focused on the spatiotemporal expression

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of cochlin. Knowing when, where, and to what extent a protein is expressed in the developing inner ear will provide important clues to protein function. Here we demonstrate the spatial and temporal expression of cochlin in the rat inner ear during postnatal development using a cochlin-specific antibody. This is the first to report about the spatiotemporal expression of cochlin in the developing inner ear using immunohistochemistry and Western blot analysis.

Wistar rats were used at day after birth (DAB) 6 and 70 ($n = 5$ for each group) for immunohistochemistry and at DAB13, 17, 20, 24, and 70 ($n = 6$ for each group) for Western blot analysis. Each rat was anesthetized with sodium pentobarbital (50 mg/kg, i.p.) according to the ethical regulations for treatment of animals. In this study, we used the anti-vWF-A1 antibody that recognizes all three cochlin isoforms expressed in the inner ear tissue, and this antibody is referred to here as the anti-cochlin antibody (Fig. 1). The generation of this antibody has previously been described [9,18]. In brief, a 19-amino-acid (KADIAFLIDGSFNIGQRRF) peptide corresponding to residues 163–181 in the vWF-A1 domain was used to generate the antibody. The specificity of this antibody for the corresponding antigenic peptide was confirmed by dot blot analysis and a peptide absorption test (data not shown).

For immunohistochemistry, rats were sacrificed after intracardiac perfusion with cold saline, followed by 4% paraformaldehyde fixative. The temporal bones were excised and fixed for 2–3 h. Temporal bones were then decalcified in phosphate-buffered saline (PBS, pH 7.4) with 5% EDTA at −4 °C for 4–7 days. Temporal bones were embedded in paraffin, and serial sections of 3–4 μm thickness were cut using a microtome (Rotationsmikrotom, Leika, Nussloch, Germany). The immunoperoxidase method was used for the detection of cochlin using the Vectastain ABC kit (Vector laboratories, Burlingham, CA, USA). Paraffin sections were deparaffinized, and the sections were then treated with 0.3% $H_2O_2$ in methanol in order to block the endogenous peroxidase activity. After treatment with 10% normal goat serum, the sections were incubated with 1:1000 dilution of the anti-cochlin antibody overnight at 4 °C. The sections were then incubated for 60 min with a 1:500 dilution of the biotinylated goat anti-rabbit IgG antibody (E0432, Dako, Denmark) and then treated with the streptavidin-biotin-peroxidase complex (Vector laboratories, Burlingham, CA, USA). After rinsing in PBS, the reaction was developed with the substrate 3,3′-diaminobenzidine, rinsed twice in distilled water, and counterstained with Mayer's hematoxylin. Preimmune serum was used instead of the primary antibody as negative control.

For Western blot analysis, membranous cochlear and vestibular labyrinths were dissected from the rat inner ear. Protein lysates were prepared by a solubilization mixture containing 0.5% SDS and protease inhibitors (Complete mini EDTA (−), Boehringer-Mannheim, Mannheim, Germany) in 10 ml of PBS. We homogenized inner ear tissues using a mortar and pestle. The homogenate was centrifuged at 1000 × g for 15 min and the supernatant was stored at −70 °C until use. Protein concentrations were determined with a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). 0.1 μg of total inner ear protein was loaded onto a 10% SDS-polyacrylamide gel. Prior to loading, samples were diluted with 0.188 M Tris buffer to a total volume of 10 μl and then mixed with 5 μl of sample buffer (0.188 M Tris buffer, 2.39 mM SDS, 30% glycerol, and 15% of 2-mercaptoethanol). The samples were heated to 98 °C for 5 min and then loaded into each lane of the gel. Electrophoresis was performed on gels with 10% polyacrylamide (PAG mini Daiichi, Daiichi Pure Chemicals, Japan) in running buffer (25 mM Tris, 192 mM glycine, 1 g/l SDS, pH 8.3) at 20 mA for 2 h. The separated proteins were electrophoretically transferred onto a PVDF membrane (Immobilon-PSQ, Millipore, USA) using an Atto HorizBlot semi-dry transfer unit with a discontinuous buffer system, as recommended by the manufacturer (ATTO, Japan). Non-specific binding was blocked by incubating the membranes overnight at 4 °C in 5% skimmed milk and 0.2% polyoxyethylenesorbitan (Tween-20) dissolved in PBS. Membranes were then incubated in PBS containing 1% skimmed milk and 0.1% Tween-20 for 2 h at room temperature with the primary anti-cochlin antibody diluted at 1:2000. After being washed in 0.05% Tween-20 in PBS, membranes were incubated for 1 h at room temperature with Horse Radish Peroxidase-labeled goat anti-rabbit IgG (P0448, Dako, Denmark) diluted at 1:5000 in the same buffer used for the primary antibody reaction. The membranes were washed again and the reaction was developed with a chemiluminescence reaction kit (ECL advance, GE Healthcare, England). The level of protein expression of each isoform was measured by a densitometer LAS-3000 (Fuji Film, Japan), and the relative amount of each was calculated relative to the expression of DAB70 taken as 100. For normalization, samples were also analyzed using an anti-GAPDH (glyceraldehyde-3-phosphate dehydrogenase) antibody (IMGENEX, USA) as the primary antibody.

At DAB6, cochlin expression was not detected in the cochlea except for weak immunoreactivity in the spiral limbus (Fig. 2a). At DAB70, cochlin was highly expressed in the spiral ligament, the spiral limbus, the cells lining Rosenthal's canal and the channels of the osseous spiral lamina (Fig. 2b). In contrast, the organ of Corti, stria vascularis, Reissner's membrane and spiral ganglion cells were all negative for cochlin staining.

At DAB6, immunoreactivity for cochlin was not detected in the crista ampullaris (Fig. 2d). At DAB70, strong immunoreactivity for cochlin was detected in the stroma of the crista and in the area

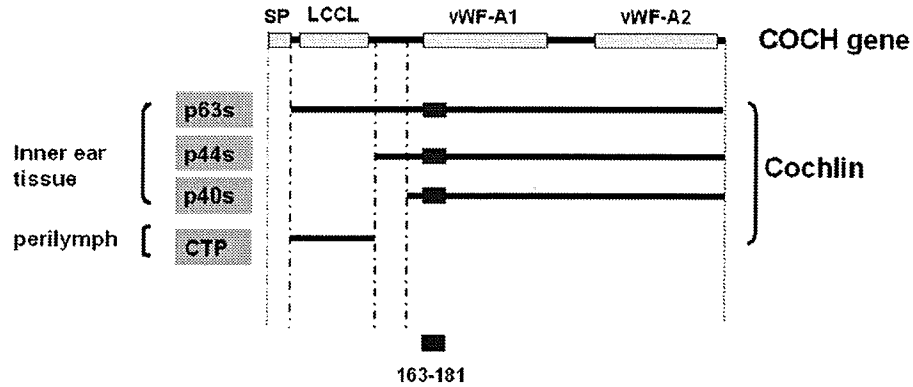


**Fig. 1.** Schematic representation of the deduced amino acid sequence of human *COCH* gene, which encodes the protein cochlin, shows a predicted signal peptide (SP), followed by a region in limulus factor C, cochlin and late gestation lung protein Lgl1 (LCCL) domain, and two vWFA-like domains (vWFA1 and vWFA2). The cochlin antibody used in this study was made against a small peptide in the N-terminus of the vWFA1 domain (black bold bar; amino acid residues 163–181), shown in the figure. This antibody recognizes all three isoforms of cochlin in the inner ear tissue (p63s, p44s, and p40s), and does not recognize cochlin-tomoprotein (CTP) in the perilymph.

beneath the planum semilunatum, dark cells and neurosensory epithelium. In contrast, apical surface of the sensory epithelia, including the cupula, subcupular space, sensory cells, transitional cells, dark cells, and supporting cells were negative for cochlin staining (Fig. 2e).

At DAB6, cochlin expression was not detected in the otolithic organs (Fig. 2g). At DAB70, strong expression of cochlin was detected in the stromata of all maculae, the lining of the membranous labyrinth and the channels in the bony labyrinth. In contrast, the apical surface of sensory epithelia, including the otoconia, gelatin layer, submembranous space, sensory cells, and supporting cells lacked cochlin staining (Fig. 2h).

We examined the expression of the cochlin isoforms by Western blot analysis of the inner ear during postnatal development of the rat (Figs. 3 and 4). We performed three independent experiments. Representative data of three different experiments are shown in Fig. 3, and the average relative expression of each isoform is as shown in Fig. 4. At DAB13, no immunoreactivity for cochlin was detected. The expression of cochlin gradually increased from DAB17 to DAB24. We also observed changes in the expression pattern of each isoform. The level of expression of p63s was the highest among all three isoforms between DAB17 and DAB24. At DAB70, by contrast, the expression of p63s had decreased and p40s was the most dominant isoform. The expression of p44s was lower than that of the other isoforms throughout the developmental process.

Mutations in the *COCH* gene have been shown to correlate with DFNA9, an autosomal dominant nonsydromic hearing disorder that causes sensorineural deafness and vertigo [11,15,22,20]. Sequence analysis of individuals with DFNA9 has demonstrated the existence of eleven missense mutations and one in-frame deletion in *COCH* [2–6,17,23,26]. The onset of symptoms is relatively late and eventually leads to profound deafness and vestibular failure. Vestibular disorder may occur and progressive hearing loss can show fluctuations and asymmetry.

We have conducted a series of studies elucidating the different cochlin isoforms and the pathogenesis of DFNA9. In the previous studies describing the cochlin isoform structure in the inner ear, we used 4 different antibodies [9]. In the present work we used the anti-cochlin antibody (previously named anti-vWF-A1 antibody), which showed the best performance in the immunohistochemical analysis of cochlin expression in the rat inner ear during postnatal development. Studying gene expression during the postnatal development of the rat inner ear provides certain advantages, because functional maturation of the inner ear occurs during the postnatal period. In the present studies, we used the DAB70 rat as an adult as in other studies published elsewhere [1,24]. As a result, we found a marked change in cochlin expression in the connective tissues of the inner ear during postnatal development (Fig. 2). These results suggest cochlin might play an important role in maturation of the inner ear, and areas of the inner ear that expressed cochlin also contain various proteins that are thought to play an important role of the inner ear function [12,16,21]. Among these proteins, we interest in the correlation between cochlin and type II collagen. Our recent immunohistochemical study of cochlin and type II collagen in the rat semicircular canal using electron microscopy has shown that the co-localization of these proteins in the same fibrillar substance [16]. These results indicate that cochlin may play a role in the structural homeostasis of the inner ear acting in concert with the fibrillar type II collagen bundles.

We used Western blot analysis for a temporal study of cochlin expression during development. Cochlin expression was not detected at DAB13, but gradually increased from DAB17 to DAB24, when the total amount of cochlin reached adult levels (Figs. 3 and 4). These results indicate that temporal expression of

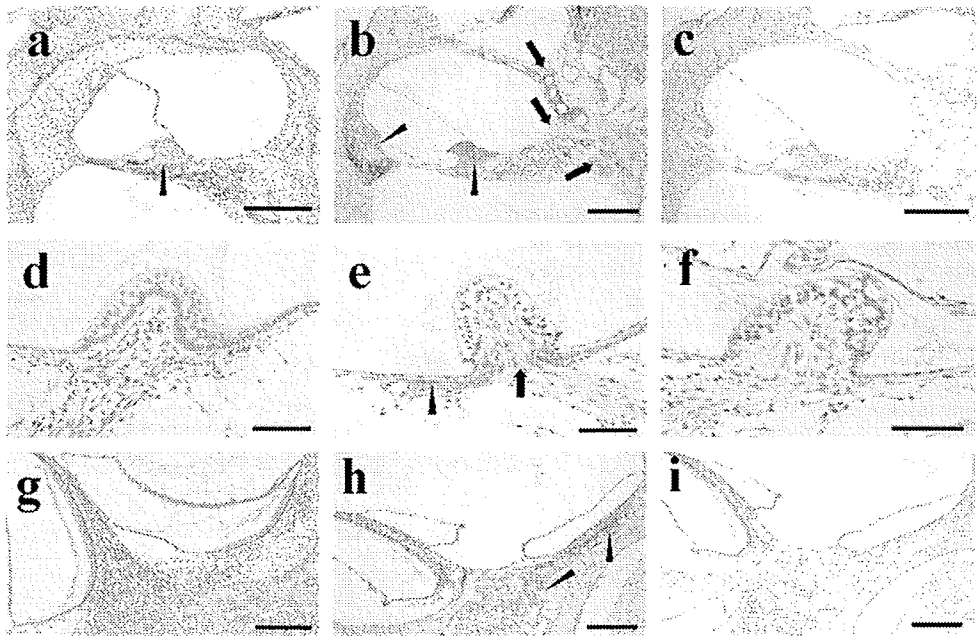


**Fig. 2.** (a–c) Representative immunohistochemistry for cochlin expression in the developing rat cochlea. (a) At 6th day after birth (DAB6), immunoreactivity for cochlin was faint in the spiral limbus (arrow head). (b) At DAB70, immunoreactivity for cochlin was strong in the area of the spiral limbus (arrow head), spiral ligament (arrow head), cells lining Rosenthal's canal and perivascular rings around blood vessels in the modiolus (arrow). (c) Negative control of DAB70 did not show immunoreactivity for cochlin in any of these structures. Scale bars in (a)–(c) = 200 μm. (d–f) Representative immunohistochemistry for cochlin expression in the developing rat crista ampullaris of the horizontal semicircular canal. (d) At DAB6, no immunoreactivity for cochlin was detected. (e) At DAB70, immunoreactivity for cochlin was strong in the stroma of the crista (arrow), the area beneath the planum semilunatum and the dark cells (arrow head). (f) Negative control of DAB70 did not show immunoreactivity for cochlin in any of these structures. Scale bars in (d)–(f) = 50 μm. (g–i) Representative immunohistochemistry for cochlin expression in the developing rat maculae. (g) At DAB6, no immunoreactivity for cochlin was detected. (h) At DAB70, immunoreactivity for cochlin was strong in the stromata of all maculae and the channels in the bony labyrinth (arrow head). (i) Negative control of DAB70 did not show immunoreactivity for cochlin in any of these structures. Scale bars in (g)–(i) = 200 μm.

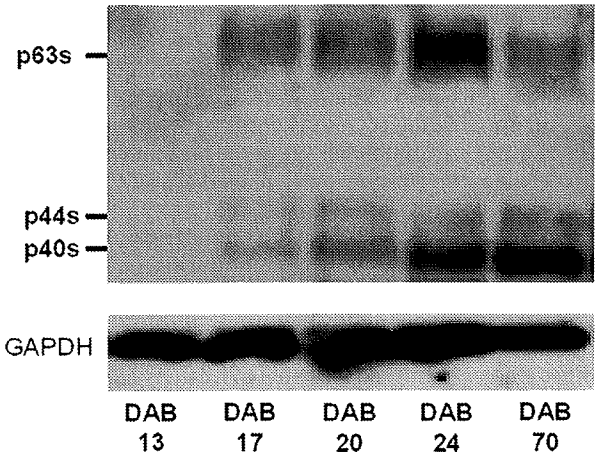


**Fig. 3.** Representative image of Western blot analysis of the rat inner ear homogenates from DAB13 to DAB70 ($n=6$ for each group; 0.1 μg per lane). p63s was not expressed at DAB13, but its expression gradually increased up to DAB24. At DAB70, however, expression of p63s was lower than at DAB24. p44s was weakly expressed from DAB17 to DAB70. p40s was not expressed at DAB13, but its expression gradually increased up to DAB70.

cochlin in the inner ear is similar to that of other proteins expressed in the fibrocytes of inner ear, such as connexin 26 [24], Na-K ATPase [27], and GLAST [10]. In the rat, temporal expression of these three proteins shows a sigmoidal time course with a rapid increase during DAB10 to DAB20 when the auditory function, as measured by electrophysiological methods, reaches that of the adult. These results indicate that cochlin might interact with other functional proteins in the inner ear, and might play an important role in maturation of the inner ear. Furthermore, Western blot analysis suggests that expression of the cochlin isoforms has unique characteristics during postnatal development. The expression of p63s was the highest, and the total amount of cochlin gradually increased until DAB24. From DAB24 to DAB70, there were no marked changes in the total amount of cochlin, but the dominant isoform of cochlin shifted from p63s to p40s (Figs. 3 and 4). Because the morphology and function of the inner ear reach adult levels by DAB24 in the rat, the isoform shift between DAB24 and DAB70 may provide important information for our understanding of cochlin function. One possibility is that the full-length cochlin (p63s) may be metabolized to form shorter isoforms (p40s, p44s and CTP); thus, the relative amount of the p63s isoform would become smaller. In addition, the LCCL module-containing isoform in the inner ear, full-length cochlin (p63s), may play a critical role in the early stage of postnatal development, whereas the shorter isoforms, p40s and p44s, may serve as extracellular matrix protein in the mature inner ear (Fig. 1). We are now performing further studies to clarify the transcriptional and post-translational modification of cochlin expression during development of the inner ear.

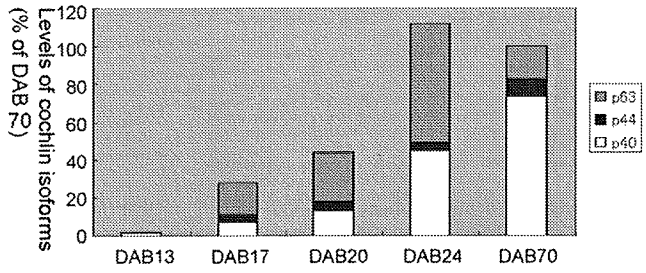


**Fig. 4.** Levels of expression of the cochlin isoforms in the rat inner ear during postnatal development. We performed 3 independent experiments. The level of expression of each isoform was measured by a densitometer relative to the expression at DAB70 taken as 100%. The data are an average of 3 independent experiments except for DAB20, where the average data of 2 experiments are given.

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# Ultrastructural co-localization of cochlin and type II collagen in the rat semicircular canal

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**Abstract**

Cochlin and type II collagen are major constituents of the inner ear extracellular matrix. To investigate the morphological relation of cochlin and type II collagen in the rat semicircular canal, immuno-electronmicroscopic analysis was performed using the post-embedding immunogold method. Immunolabeling for cochlin was detected in the fibrillar substance underlying the supporting epithelium of the sensory cells and beneath the epithelial cells facing the endolymph in the semicircular canals. Immunolabeling for type II collagen was observed in the same fibrillar substance in the subepithelial area. The co-localization of cochlin and type II collagen in the fibrillar substance in the subepithelial area indicate that cochlin may play a role in the structural homeostasis of the vestibule acting in concert with the fibrillar type II collagen bundles.


*Keywords:* Hereditary hearing impairment; DFNA9; *COCH*; Vestibular organ; Immunogold method

A number of genetic loci for familial syndromic or non-syndromic sensorineural hearing losses have been mapped [22]. Mutations of the *COCH* gene, which encodes cochlin, are associated with an autosomal dominant progressive sensorineural hearing loss and vestibular disorders including Meniere's disease like symptoms at the DFNA9 locus [3,6,7,15,16,19,23,24]. Cochlin has von Willebrand factor type A (vWFA) like domains which are present in a variety of secreted proteins, both soluble and insoluble, are involved in the production of extracellular matrix and have been shown to bind fibrillar collagens, glycoproteins and proteoglycans [4,5]. Although, the function of cochlin is not yet known, our recent studies demonstrated immunoreactivity for cochlin in the type II collagen rich areas of the vestibular organ [10]. Type II collagen is also an extracellular matrix material in the acellular structures and subepithelial connective tissue of the inner ear [21]. The interaction between cochlin and type II collagen has been suggested in histopathological findings of DFNA9 [14]. To precisely examine the morphological relation of cochlin and type II collagen, in the present study we performed immuno-electronmicroscopic evaluation of cochlin and type II collagen using the immunogold method.

Rabbit polyclonal antibody used in this study was raised against vWFA like domain 1 of cochlin and has been previously described [11]. Briefly, a 19-mer (KADIAFLIDGSFNIGQRRF) peptide corresponding to residues 163–181 in the vWF-A1 domain was used to generate antibody. This sequence is present in all three cochlin isoforms and is completely homologous with the sequences in both human and mouse proteins, as deduced from the sequence of the corresponding genes and the sequence of bovine cochlin. This antibody recognizes all the three cochlin isoforms in the inner ear. The specificity of this antibody for the corresponding antigenic peptide was confirmed by dot blot analysis and a peptide absorption test (data not shown). Antibody against type II collagen (Millipore, Billerica, MA) was purchased commercially.

Wistar rats (100–200 g body weight) were anesthetized with pentobarbital (50 mg/kg body weight, i.p.) according to the ethical regulations for treatment of animals. A fixative of 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate

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buffer (PB, pH 7.4) was perfused from the left ventricle, and then the temporal bones were isolated. The temporal bones were immediately immersed in the same fixative. Thereafter, the vestibular organs were dissected under a stereomicroscope and further fixed for 2 h at 4 °C. The specimens were immersed into 50 mM $NH_4Cl$ in 0.1 M PB for 2 h and were washed in 0.1 M PB overnight at 4 °C. Embedding in Lowicryl K4M (Electron Microscopy Sciences, Fort Washington, PA) was done in accordance with the manufacturer's instruction with slight modification. Briefly, the specimens were dehydrated in a graded series of ethanol as follows: 30% ethanol for 30 min at 4 °C; 50% ethanol for 1 h at −20 °C; 70% ethanol for 1 h at −20 °C; 100% ethanol for 1 h at −20 °C. The specimens were then infiltrated with Lowicryl K4M at −20 °C; with 100% ethanol:Lowicryl K4M (1:1) for 1 h; with 100% ethanol:Lowicryl K4M (1:2) for 1 h; with 100% Lowicryl K4M for 1 h and with 100% Lowicryl K4M overnight. The tissue subsequently was embedded in Lowicryl K4M and polymerized by ultraviolet light irradiation for 24 h at −35 °C followed by 48 h at room temperature. Ultrathin sections were cut using an ultra-microtome and mounted onto nickel grids (400 mesh).

The grid with sections was immersed in a droplet (25 μl) of 1% bovine serum albumin (BSA; Sigma, St. Louis) in phosphate-buffered saline (PBS, 0.9% NaCl in 6.7 mM phosphate buffer, pH 7.2) for 1 h at room temperature, then incubated in a droplet of the optimal concentrations of rabbit antibodies against cochlin (3.3 μg/ml in BSA/PBS) or type II collagen (4 μg/ml in BSA/PBS), overnight at 4 °C. After rinsing in PBS, the sections were incubated in colloidal gold conjugated goat anti-rabbit IgG secondary antibody (15 nm in diameter, BB international, Cardiff, UK) at 1:50 dilution in BSA/PBS for 1 h at room temperature. Subsequently, the sections were washed with PBS and distilled water, and counter stained with uranyl acetate for 3 min, and lead citrate for 30 s. These sections were then observed under a JEOL JEM-1220 electron microscope. Pre-immune rabbit IgG (4 μg/ml in BSA/PBS) was used instead of the primary as negative control.

Immunoreactivity for cochlin was seen in the fibrillar substance in the region beneath the sensory cells (Fig. 1) and subepithelial area in the crista ampullaris (Fig. 2a). Immunoreactivity was detected in the banded bundles which have a parallel array in the area of fibrocytes (Fig. 2b). No immunoreactivity was detected in any epithelial cells or fibrocytes of the crista ampullaris.

Immunoreactivity for Type II collagen was detected on the fibrillar substance in the subepithelial area of the crista ampullaris (Fig. 3). Immunoreactivity for cochlin and type II collagen was seen in the same fibrillar substance (Fig. 2 with Fig. 3, respectively).

Negative control using pre-immune IgG did not exhibit any immunoreactivity on the fibrillar substance of the crista ampullaris (Fig. 4).

The present study is the first to report the localization of cochlin, ultrastructurally. Here, we found that cochlin was co-localized with type II collagen in the fibrillar substance in the extracellular area of crista ampullaris. Temporal bone studies on DFNA9-affected subjects have revealed a unique histopathology showing loss of cellularity and aggregation of abundant homogeneous acellular eosinophilic deposits in the cochlear and vestibular labyrinths [13,15]. Recently, Robertson et al. conducted immunohistochemichal study of the DFNA9-affected inner ear, and found eosinophilic deposits in the collapsed ampulla that stained with the anti-cochlin antibody (the same antibody as used in the present study). Proteomic analysis of the same subject detected cochlin as one of the primary proteins [17]. This study showed that the eosinophilic substance was mutated cochlin itself and was not another component of the inner ear, a cochlin-interacting protein, or some other downstream effect of the *COCH* mutations. This work strongly supports the hypothesis of DFNA9 pathogenesis that mutated cochlin accumulates in the inner ear, present as acellular eosinophilic deposits, and this accumulation leads to neuroepithelial and neural degeneration in the inner ear.

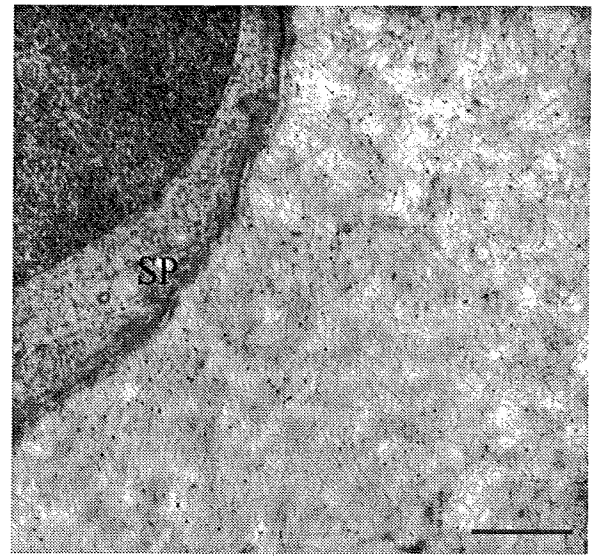


Fig. 1. Immuno-electronmicroscopy for cochlin expression in the inner ear. Immunolabeling for cochlin was seen on the fibrillar substance beneath the supporting cells in the crista ampullaris. SP = supporting cell of the sensory cells; bar = 1 μm.

A latticework of type II collagen fibers compose the extracellular matrix of cartilage with retaining of proteoglycan aggregate [9]. Co-localization of cochlin and type II collagen suggests that cochlin may also bind to the type II collagen to form an intact structure in the inner ear. An investigation of DFNA9 affected inner ear using electron microscopy has shown the absence of major fibrillar type II collagen [14] in the spiral ligament where cochlin is expressed abundantly in normal subjects [20]. The disturbance of the correct interaction between cochlin and type II collagen is one of the downstream effects of cochlin mutation, and results in loss of type II collagen fibers.

In DFNA9 affected inner ear, a thickening of the ampullary wall with abnormal eosinophilic substance is one of the striking findings in the vestibular organ. The thin layer under the epithelial cells of ampullary wall contains both type II collagen [12] and cochlin [10], indicating that the abnormal interactions between mutated cochlin and type II collagen may have contributed to the marked thickening of the wall. Interestingly, an

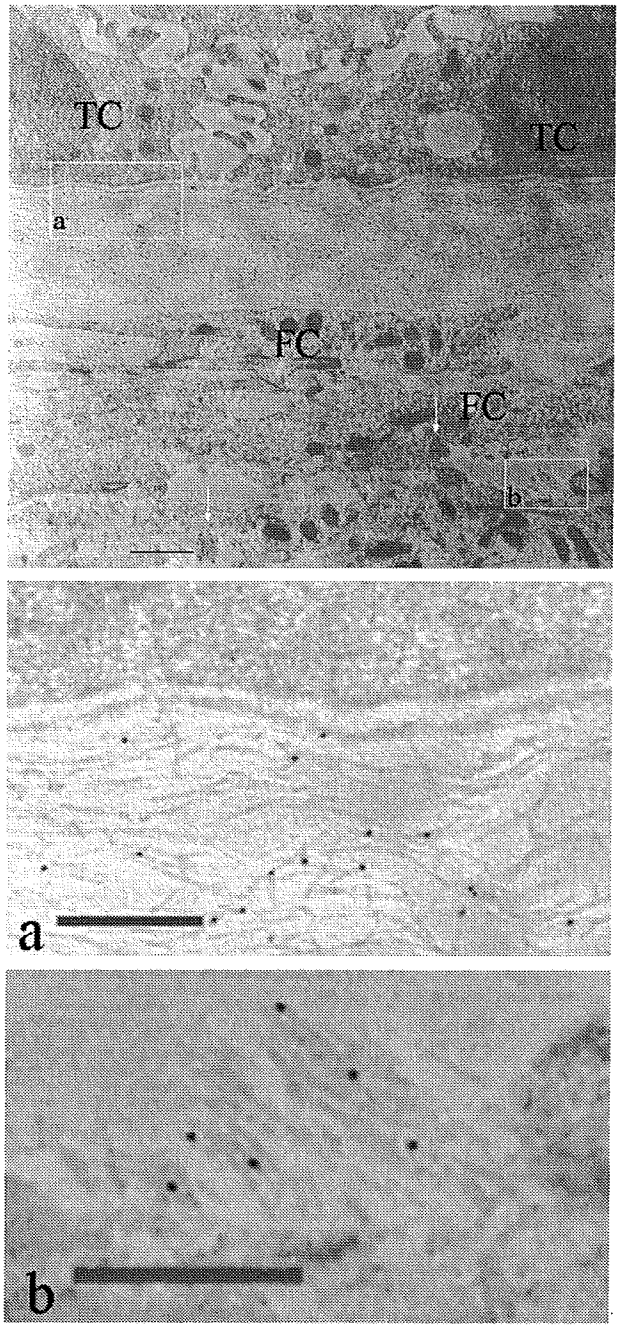


Fig. 2. Immuno-electronmicroscopy for cochlin expression in the inner ear. Immunolabeling for cochlin was seen on the fibrillar substance in the region beneath the transitional cells and on the banded bundles in fibrocytes area (arrows). Gold particles cannot be detected in any epithelial cells or fibrocytes of the crista ampullaris. TC = transitional cell; FC = fibrocyte; bar = 1 μm. Higher magnification of the open square (a) area in this figure. Gold particles labeled the fibrillar substance. Bar = 0.5 μm. Higher magnification of the open square (b) area in this figure. Gold particles were also seen in the banded bundles which have a parallel array in the area of fibrocytes. Bar = 0.5 μm.

increase in the levels of cochlin with a parallel decrease in type II collagen has been reported in the trabecular meshwork in human glaucoma as well as in an animal model of glaucoma (DBA/2J mice) [1,2]. The precise pathogenesis or genetic abnormalities in DBA/2J mice is not yet known, but DBA/2J mice also exhibit very early progressive sensorineural hearing loss [25], suggesting a relationship between auditory function and cochlin/type II collagen interactions.

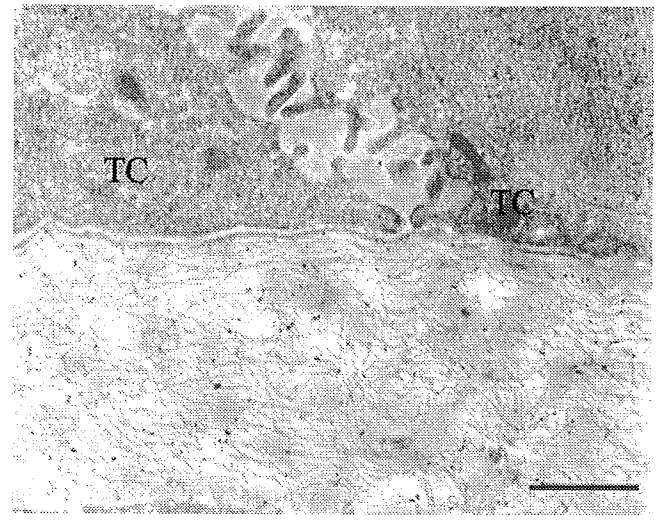


Fig. 3. Immuno-electronmicroscopy for type II collagen expression in the inner ear. Immunolabeling for type II collagen was seen on the fibrillar substance in the area of subepithelial cells in the semicircular canal. TC = transitional cell; bar = 1 μm.

In our previous study at the light microscopic level, cochlin staining was seen in the crista ampullaris of the semicircular canals and the stromata of the maculae of otolithic organs [10]. These areas also exhibited high expression of *COCH* mRNA, in particular in the fibrocytes underlying the sensory epithelium [20]. The current study indicates an absence of cochlin from fibrocytes and a presence of cochlin on the extracellular matrix, showing that cochlin is a secretory protein, which is in concert with the previous assumption. From a motif analysis, cochlin has two tandem von Willebrand factor A-like domains which are present in majority of extracellular matrix proteins [4,5]. Transient transfection of *COCH* gene into 293T or Hela cells reveled that cochlin is secreted out into the culture medium [8,18].

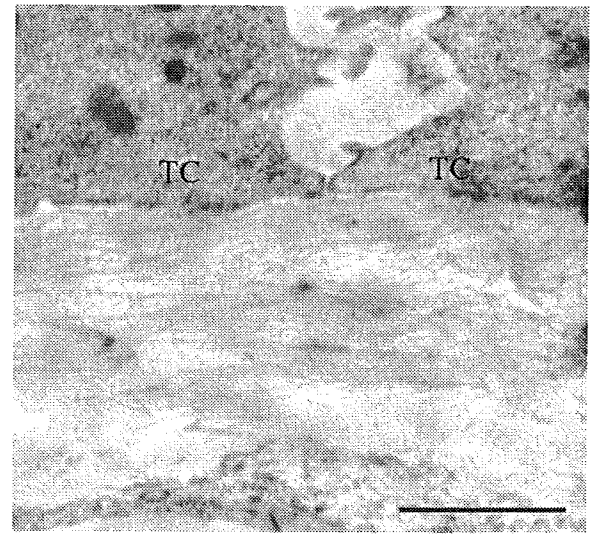


Fig. 4. Control. No gold particle was seen on the fibrillar substance in the region beneath the transitional cells. TC = transitional cell; bar = 1 μm.

In conclusion, we suggest that cochlin could cross-link type II collagen fibers in the semicircular canal and is responsible for the structural integrity of the vestibule in order to withstand the stress associated with fluid movement. Further studies are needed to determine the nature of interaction between cochlin and type II collagen.

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