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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

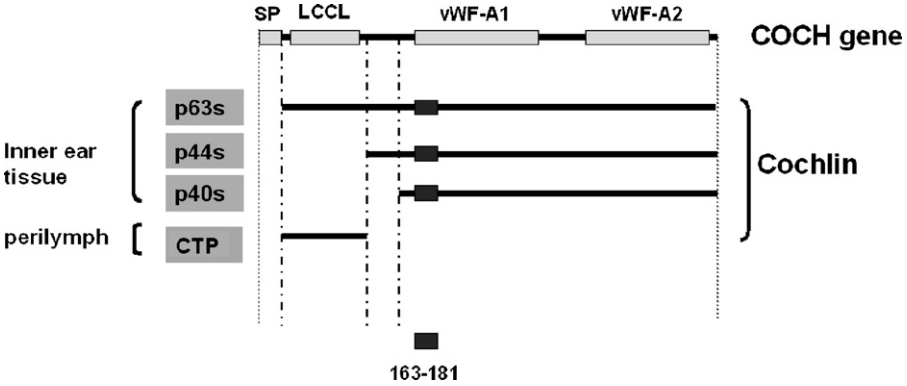

Fig. 1. Schematic representation of the deduced amino acid sequence of human *COCH* gene, which encodes the protein cochlin, shows a predicted signal peptide (SP), followed by a region in limulus factor C, cochlin and late gestation lung protein Lgl1 (LCCL) domain, and two vWFA-like domains (vWFA1 and 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

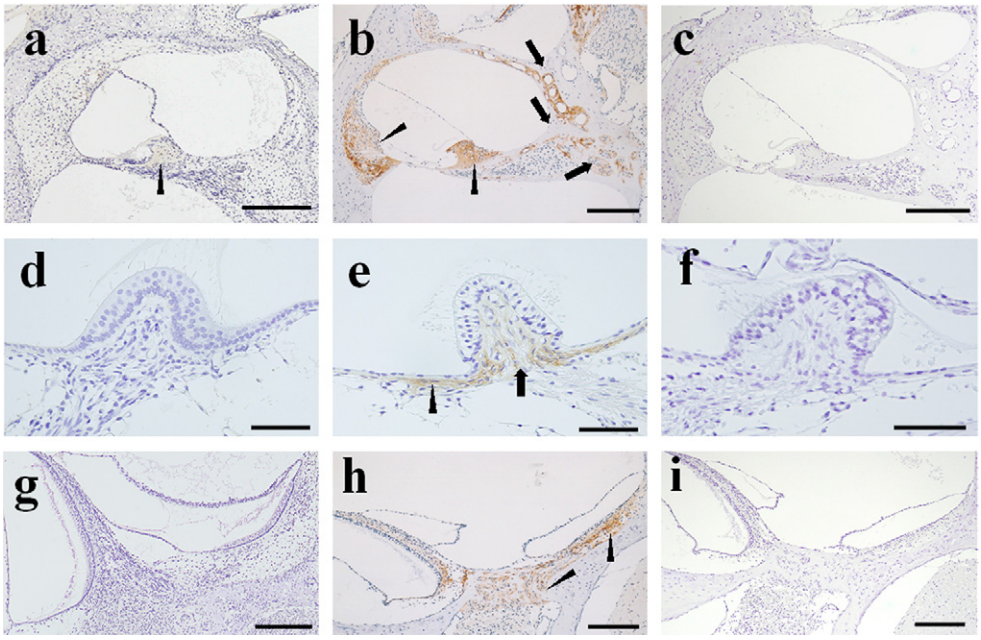

Fig. 2. (a–c) Representative immunohistochemistry for cochlin expression in the developing rat cochlea. (a) At 6th day after birth (DAB6), immunoreactivity for cochlin was faint in the spiral limbus (arrow head). (b) At DAB70, immunoreactivity for cochlin was strong in the area of the spiral limbus (arrow 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.

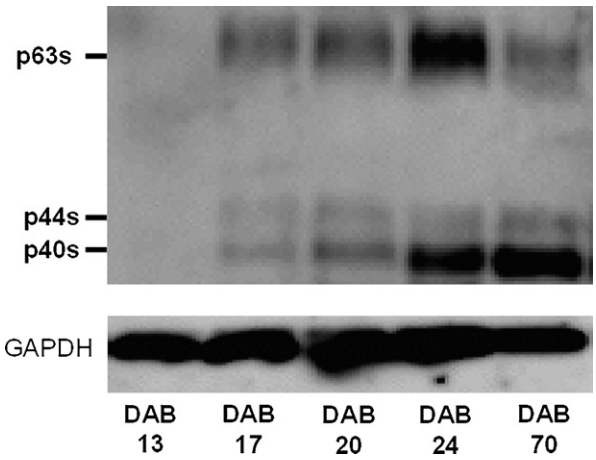

Fig. 3. Representative image of Western blot analysis of the rat inner ear homogenates from DAB13 to DAB70 ($n = 6$ for each group; 0.1 μg per lane). p63s was not expressed at DAB13, but its expression gradually increased up to DAB24. At DAB70, however, expression of p63s was lower than at DAB24. p44s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

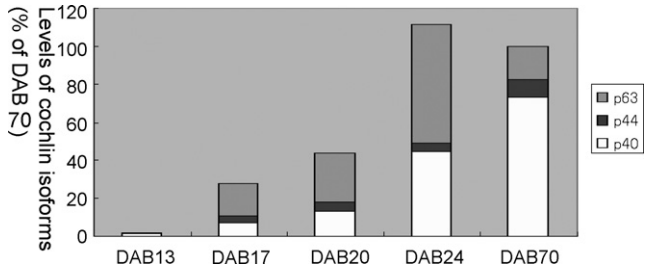

Fig. 4. Levels of expression of the cochlin isoforms in the rat inner ear during postnatal development. We performed 3 independent experiments. The level of expression of each isoform was measured by a densitometer relative to the expression at DAB70 taken as 100%. The data are an average of 3 independent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

and post-translational modification of cochlin expression during development of the inner ear.

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