



Ultrastructural localization of cochlin in the rat cochlear duct

メタデータ	言語: English 出版者: S. Karger AG 公開日: 2013-08-27 キーワード (Ja): キーワード (En): Hereditary hearing impairment, DFNA9, COCH, Inner ear, Immunogold method 作成者: Hosokawa, Seiji メールアドレス: 所属:
URL	http://hdl.handle.net/10271/2045

Ultrastructural localization of cochlin in the rat cochlear duct

Running head: Cochlin in the cochlea

Seiji Hosokawa^a, Kunihiro Mizuta^a, Hiroshi Nakanishi^a, Yasuyuki Hashimoto^a,
Maki Arai^a, Hiroyuki Mineta^a, Susumu Shindo^b, and Tetsuo Ikezono^b

- a) Department of Otolaryngology, Hamamatsu University School of Medicine,
1-20-1 Handayama, Higashi-ku, Hamamatsu 431-3192, Japan
- b) Department of Otolaryngology, Nippon Medical School, 1-1-5 Sendagi,
Bunkyo-ku, Tokyo 113-8602, Japan

Corresponding author: Seiji Hosokawa, MD

Department of Otolaryngology, Hamamatsu University School of Medicine,
1-20-1 Handayama, Higashi-ku, Hamamatsu 431-3192, Japan

Tel: +81-53-435-2252, Fax: +81-53-435-2253

Email: seijih@hama-med.ac.jp

Key words:

Hereditary hearing impairment, DFNA9, *COCH*, inner ear, immunogold method

Abstract

Cochlin, a product of the *COCH* gene, is a major constituent of the inner ear extracellular matrix. Type II collagen, a protein that contributes to structural stability, is similarly a component of this extracellular matrix. In this study, using the post-embedding immunogold method, we demonstrate the localization of cochlin and type II collagen in the cochlear duct at the ultrastructural level. The immunolabeling of cochlin was observed in the fibrillar substance in the spiral limbus, beneath the inner sulcus cells, and in the basilar membrane, the spiral prominence and the spiral ligament. Immunolabeling of type II collagen was observed in the same fibrillar substance in the extracellular matrix of the cochlear duct. This localization of cochlin is consistent with the expected localization of type II collagen. The localization of cochlin and type II collagen indicates the important roles played by these proteins in the hearing process.

Introduction

Cochlin, a product of the *COCH* gene, is associated with an autosomal dominant sensorineural hearing loss referred to as DFNA9. The symptoms of DFNA9 include not only hearing loss but also vestibular disorders (Bom et al., 1999; de Kok et al., 1999; Fransen et al., 1999; Grabski et al., 2003; Khetarpal, 1993; Manolis et al., 1996; Robertson et al., 1998; Usami et al., 2003; Verhagen et al., 1989; Verhagen et al., 1992). On light microscopic analysis, Khetarpal et al. (1991, 1993) reported severe degeneration of the cochlea and vestibule in association with the deposition of an acidophilic ground substance in the spiral ligament, spiral limbus, spiral lamina and basilar membrane of DFNA9-affected ears. Robertson et al. (2006) also reported the loss of cellularity and the accumulation of an abundant homogeneous acellular eosinophilic deposit in the cochlea and vestibule of DFNA9-affected ears. They suggested that these extracellularly deposited aggregates contain mutated cochlin, and that this mutated cochlin alters the interactions between cochlin and other cochlin-associated proteins. Khetarpal (2000) compared the normal spiral ligament with that in DFNA9-affected ears at the ultrastructural level, and noted the absence of major fibrillar type II collagen bundles. We speculated that the proteins interacting with cochlin might include type II collagen and have previously reported that these two extracellular matrix (ECM) proteins coexist in the same fibrillar substance in the subepithelial area of the semicircular canal (Mizuta, et al., 2008). This localization suggests that cochlin plays a role in structural homeostasis of the vestibule. At present, however, the exact role of cochlin, which accounts for 70% of the inner ear proteins (Ikezono et al., 2001), remains incompletely understood.

Some clues that may help to elucidate the role of cochlin have, nevertheless,

been reported. Since the sensorineural hearing loss observed in DFNA9 has a late onset and progresses slowly, the *COCH* gene has been implicated in this age-related hearing impairment (de Kok et al., 1999). On the other hand, cochlin and type II collagen have been implicated in autoimmune hearing loss in humans (Baek et al., 2006; Yoo et al., 1984). Further, Ikezono et al. (2009) recently reported that a short 16-kDa cochlin isoform (cochlin-tomoprotein) is specific to the perilymph and that this isoform could function as a diagnostic marker of perilymphatic fistula, which is related to hearing loss and vestibular disorder. Thus, we considered that it would be important to investigate the localization of cochlin in the cochlea, and in the present study we accordingly expanded our immuno-electromicroanalysis of cochlin to the cochlea. The role of cochlin in the cochlea, particularly in the basilar membrane, is also briefly discussed.

Materials and Methods

Antibodies

Cochlin has von Willebrand factor type A (vWFA)-like domains (Robertson et al., 2003). We used a rabbit polyclonal antibody that recognizes all three cochlin isoforms. This was raised against the vWFA-like domain 1 of cochlin and has been previously described by Ikezono et al. (2004). Briefly, a 19-mer (KADIAFLIDGSFNIGQRRF) peptide corresponding to residues 163–181 in the vWF-A1 domain was used to generate antibodies. The specificity of these antibodies for the corresponding antigenic peptide was confirmed by dot blot analysis and a peptide absorption test (data not shown). Antibodies against type II collagen (Chemicon International acquired by Millipore, Billerica, MA) were purchased commercially.

Tissue processing

Wistar rats (body weight, 100–200 g) were anesthetized with pentobarbital (50 mg/kg body weight, i.p.) according to our institution's ethical regulations for the treatment of animals. A fixative of 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer (PB, pH 7.4) was perfused from the left ventricle, and the temporal bones were then isolated and immediately immersed in the same fixative. Thereafter, the cochleae were dissected under a stereomicroscope and further fixed for 2 h at 4°C.

The preparation of samples for embedding in Lowicryl K4M (Electron Microscopy Sciences, Fort Washington, PA) was performed according to a previously published procedure (Mizuta et al., 2008). Ultra-thin sections were cut using an ultramicrotome and mounted onto nickel grids (400 mesh).

Immunogold labeling

The grid-mounted sections were immersed in a droplet (25 μ l) of 1% bovine serum albumin (BSA; Sigma, St. Louis, MO) 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 overnight at 4°C in a droplet of the optimal concentrations of rabbit antibodies against either cochlin (3.3 μ g/ml in BSA/PBS) or type II collagen (4 μ g/ml in BSA/PBS). After rinsing in PBS, the sections were incubated in colloidal gold conjugated goat anti-rabbit IgG secondary antibody (diameter, 15 nm; 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 counterstained with uranyl acetate for 3 min and lead citrate for 30 s. These sections were then observed under a JEOL JEM-1220

electron microscope. As a negative control, pre-immune rabbit IgG (4 µg/ml in BSA/PBS) was used instead of the primary antibody.

This study protocol was approved by the Hamamatsu University School of Medicine Animal Use Committee.

Results

Immunoreactivity for cochlin and type II collagen was observed in the spiral limbus, beneath the inner sulcus cells and the basilar membrane, beneath the epithelial cells of the spiral prominence and the spiral ligament.

Spiral Limbus

Immunolabeling of cochlin and type II collagen was observed in the fibrous area of the spiral limbus (Figs. 1a, 1b, 1c).

Inner sulcus cells

Immunolabeling of cochlin and type II collagen was observed in the fibrous substance beneath the inner sulcus cells (Figs. 2a, 2b, 2c).

Basilar membrane

The structures that exhibited immunoreactivity for cochlin and type II collagen were the fibrous bundles in the basilar membrane (Figs. 3a, 3b, 3c).

Spiral prominence

The stained fibrils for cochlin and type II collagen beneath the epithelial cells of the spiral prominence were observed to be widely scattered and without orientation (Figs. 4a, 4b, 4c).

Spiral ligament

Immunolabeling of cochlin and type II collagen was observed in the fibrous bundles in the spiral ligament (Figs. 5a, 5b, 5c).

Control

When pre-immune IgG was used as a negative control, the fibrillar substance of the basilar membrane exhibited no immunoreactivity (Fig. 6).

Discussion

Immunoreactivity for cochlin and type II collagen was observed in the same ECM areas of the cochlear duct, particularly in the following sites; the fibrous substance in the spiral limbus, beneath the inner sulcus cells, in the basilar membrane, beneath the epithelial cells of the spiral prominence and in the spiral ligament. The present study is the first report to demonstrate the localization of cochlin in these structures at the ultrastructural level. Localization of cochlin in basilar membrane was not detected in our previous studies using the same anti-cochlin antibody at the light microscopic level (Robertson et al., 2006; Shindo et al., 2008). This might be due to the different tissue processing and staining in the applications of light and electron microscopy. Interestingly, another antibody, which recognizes a different peptide of cochlin (residues 337–355) than the one targeted here (residues 163–181), was reactive to cochlin in this area under light microscopy (Kommareddi et al. 2007).

Our findings for type II collagen at the basilar membrane and the spiral ligament are consistent with previous ultrastructural studies (Dreiling et al., 2002; Kaname et al., 1994). At the electron microscopic level, we previously localized cochlin and type II collagen on the fibrous structures beneath the epithelial cells and supporting cells in the rat semicircular canal (Mizuta et al., 2008). Cochlin appears to localize with type II collagen in the fibrous structures in the extracellular matrix of the cochlea as well as in the vestibule. Nagy et al. (2008) recently demonstrated that the second vWFA-like domain of cochlin has an affinity for type II collagen. This report supports the hypothesis that these

two proteins interact each other.

Several types of collagen have been detected in the ECM of the inner ear (Yoo et al., 1988; Slepecky et al., 1992; Usami et al., 2008). Of these subtypes, type II collagen is responsible for the fibrous structure and appears to play a critical role in maintaining structural stability in the cochlea and vestibule (Slepecky et al., 1992). Localization of these two proteins in the same fibrous substance of ECM in the cochlear duct indicates that cochlin may play a role in the structural homeostasis of the cochlea by cross-linking to the fibrillar type II collagen bundles.

Kommareddi et al. (2007) showed that a prominent 64-kDa band of cochlin co-immunoprecipitated with choline transporter-like protein 2 (CTL2). CTL2 is a multi-transmembrane protein expressed on inner ear supporting cells that was discovered as a target of antibody-induced hearing loss (Nair et al., 2004). The present findings, together with the fact that cochlin co-immunoprecipitates with CTL2, indicate that these proteins may interact with other proteins. Indeed, cochlin and type II collagen have also been implicated as a disease causing antigen in autoimmune hearing loss in humans (Baek et al., 2006; Yoo et al., 1984). The association of these three proteins may therefore have very interesting implications with regard to DFNA9 pathogenesis.

In DFNA9 pathogenesis, Robertson et al. (2006) hypothesized that mutated cochlin accumulates acellular eosinophilic deposits and that this accumulation leads to degeneration of other cochlin-associated proteins. Our findings suggested that type II collagen is one of the candidates of the cochlin-associated proteins. To clarify this hypothesis, it will be interesting to analyze chronologically the ultrastructural pathology and immunohistochemistry of a mutant mouse model of DFNA9 which exhibited progressive age-related hearing loss (Robertson

et al., 2008).

The late-onset progressive sensorineural hearing loss in the DFNA9 ear also suggests a relationship between cochlin and presbycusis, an impairment of hearing characteristic of elderly individuals (de Kok et al., 1999). Robertson et al. (2008) suggested that *COCH* might play important roles in presbycusis, and that cochlin is a major target antigen for autoimmune sensorineural hearing loss. The histopathologic correlates of age-related hearing loss suggest several categories for this type of auditory impairment: sensory-neural, strial, cochlear-conductive, mixed, and indeterminate (Schuknecht and Gacek, 1993). In these categories, cochlear-conductive hearing loss may be related to pathologic change in the basilar membrane. Several studies have shown that thickening of the basilar membrane in aged animals may underlie presbycusis (Ishii et al., 1994; Shimada et al., 1998). The basilar membrane is responsible for the mechano-electrical transduction exhibited by sensory cells, which enables them to absorb stress and withstand traveling waves. This explains why changes in these mechanical properties can cause hearing impairment. Buckiova et al. (2006) have shown a reduction in type II collagen immunoreactivity at the light microscopic level in the spiral ligament, but not in the basilar membrane, of aged Fischer 344 rats (an animal model of strial presbycusis). However, the reduction of type II collagen in the spiral ligament led us to consider that degeneration of type II collagen might occur in the basilar membrane of the aging ear. The pathology of the DFNA9-affected ear includes degeneration of the basilar membrane (Khetarpal et al., 1991, Khetarpal, 1993). At present, however, the relationship between cochlin and the age-related pathology of the inner ear remains unknown. For thin structures such as the basilar membrane, ultrastructural analysis can reveal more detailed pathology than light microscopy, and may be a

better tool for determining age-related pathology. Accordingly, in the near future we plan to conduct an ultrastructural analysis of age-related changes in type II collagen and cochlin in the basilar membrane as well as in the spiral ligament.

In conclusion, the present study suggests that cochlin could cross-link to type II collagen fibers in the spiral limbus, beneath the inner sulcus cells, the basilar membrane, the spiral prominence and the spiral ligament in the cochlear duct, and that it is responsible for the structural integrity of this organ, in particular by enabling the structure to withstand the stress associated with traveling waves. Further studies, however, will be needed in order to determine the nature of the interaction between cochlin and type II collagen.

Acknowledgements

This work was supported by the Acute Profound Deafness Research Committee of the Ministry of Health, Labour and Welfare, Tokyo, Japan.

References

- [1] Baek MJ, Park HM, Johnson JM, Altuntas CZ, Jane-Wit D, Jaini R, Solares CA, Thomas DM, Ball EJ, Robertson NG, Morton CC, Hughes GB, Tuohy VK: Increased frequencies of cochlin-specific T cells in patients with autoimmune sensorineural hearing loss. *J Immunol* 2006;177:4203–4210.
- [2] Bom SJ, Kemperman MH, de Kok YJ, Huygen PL, Verhagen WI, Cremers FP, Cremers CW: Progressive cochleovestibular impairment caused by a point mutation in the *COCH* gene at DFNA9. *Laryngoscope* 1999;109:1525–1530.
- [3] Buckiova D, Popelar J, Syka J: Collagen changes in the cochlea of aged Fischer 344 rats. *Exp Gerontol* 2006;41:296–302.
- [4] de Kok YJ, Bom SJ, Brunt TM, Kemperman MH, van Beusekom E, van der Velde-Visser SD, Robertson NG, Morton CC, Huygen PL, Verhagen WI, Brunner HG, Cremers CW, Cremers FP: A Pro51Ser mutation in the *COCH* gene is associated with late onset autosomal dominant progressive sensorineural hearing loss with vestibular defects. *Hum Mol Genet* 1999;8:361–366.
- [5] Dreiling FJ, Henson MM, Henson OW Jr: The presence and arrangement of type II collagen in the basilar membrane. *Hear Res* 2002;166:166–180.
- [6] Fransen E, Verstreken M, Verhagen WI, Wuyts FL, Huygen PL, D’Haese P, Robertson NG, Morton CC, McGuirt WT, Smith RJ, Declau F, Van de Heyning PH, Van Camp G: High prevalence of symptoms of Meniere’s disease in three families with a mutation in the *COCH* gene. *Hum Mol Genet* 1999;8:1425–1429.
- [7] Grabski R, Szul T, Sasaki T, Timpl R, Mayne R, Hicks B, Sztul E: Mutation in *COCH* that result in non-syndromatic autosomal dominant deafness

(DFNA9) affected matrix deposition of cochlin. *Hum Genet* 2003;113:406-416.

- [8] Ikezono T, Omori A, Ichinose S, Pawanker R, Watanabe A, Yagi T: Identification of the protein product of the *Coch* gene (hereditary deafness gene) as the major component of bovine inner ear protein. *Biochim Biophys Acta* 2001;1535:258–265.
- [9] Ikezono T, Shindo S, Sekiguchi S, Hanprasertpong C, Li L, Pawankar R, Morizane T, Baba S, Koizumi Y, Sekine K, Watanabe A, Komatsuzaki A, Murakami S, Kobayashi T, Miura M, Yagi T: Cochlin-Tomoprotein: A Novel Perilymph-Specific Protein and a Potential Marker for the Diagnosis of Perilymphatic Fistula. *Audiol Neurotol* 2009;14:338–344.
- [10] Ikezono T, Shindo S, Li L, Omori A, Ichinose S, Watanabe A, Kobayashi T, Pawankar R, Yagi T: Identification of a novel Cochlin isoform in the perilymph: insights to Cochlin function and the pathogenesis of DFNA9. *Biochem Biophys Res Commun* 2004;314:440–446.
- [11] Ishii K, Murofushi T, Takeuchi N: Morphological changes in the tectorial and basilar membranes of aged rats. *Eur Arch Otorhinolaryngol* 1994;251:357–360.
- [12] Kaname H, Yoshihara T, Ishii T, Tatsuoka H, Chiba T: Ultrastructural and immunocytochemical study of the subepithelial fiber component of the guinea pig inner ear. *J Electron Microsc* 1994;43:394–397.
- [13] Khetarpal U, Schuknecht HF, Gacek RR, Holmes LB: Autosomal dominant sensorineural hearing loss. Pedigrees, audiologic findings, and temporal bone findings in two kindreds. *Arch Otolaryngol Head Neck Surg* 1991;117:1032–1042.
- [14] Khetarpal U: Autosomal dominant sensorineural hearing loss. Further temporal bone findings. *Arch Otolaryngol Head Neck Surg* 1993;119:106–108.
- [15] Khetarpal U: DFNA9 is a progressive audiovestibular dysfunction with a

- microfibrillar deposit in the inner ear. *Laryngoscope* 2000;110:1379–1384.
- [16] Kommareddi PK, Nair TS, Raphael Y, Telian SA, Kim AH, Arts HA, El-Kashlan HK, Carey TE: Cochlin isoforms and their interaction with CTL2 (SLC44A2) in the inner ear. *J Assoc Res Otolaryngol* 2007;8:435–446.
- [17] Manolis EN, Yandavi N, Nadol Jr. JB, Eavey RD, McKenna M, Rosenbaum S, Khetarpal U, Halpin C, Merchant SN, Duyk GM, MacRae C, Seidman CE: A gene for non-syndromatic autosomal dominant progressive postlingual sensorineural hearing loss maps to chromosome14q12-13. *Hum Mol Genet* 1996;5:1047–1050.
- [18] Mizuta K, Ikezono T, Iwasaki S, Arai M, Hashimoto M, Pawankar R, Watanabe T, Shindo S, Mineta H: Ultrastructural co-localization of cochlin and type II collagen in the rat semicircular canal. *Neurosci Lett* 2008;434:104–107.
- [19] Nagy I, Trexler M, Patthy L: The second von Willebrand type A domain of cochlin has high affinity for type I, type II and type IV collagens. *FEBS Lett* 2008;582:4003–4007.
- [20] Nair TS, Kozma KE, Hoefling NL, Kommareddi PK, Ueda Y, Gong TW, Lomax MI, Lansford CD, Telian SA, Satar B, Arts HA, El-Kashlan HK, Berryhill WE, Raphael Y, Carey TE: Identification and characterization of choline transporter-like protein 2, an inner ear glycoprotein of 68 and 72 kDa that is the target of antibody-induced hearing loss. *J Neurosci* 2004;24:1772–1779.
- [21] Robertson NG, Lu L, Heller S, Merchant SN, Eavey RD, MacKenna M, Nadol Jr. JB, Miyamoto RT, Linthicum Jr. FH, Lubianca Neto JF, Hudspeth AJ, Seidman CE, Morton CC, Seidman JG: Mutations in a novel cochlear gene cause DFNA9, a human nonsyndromatic deafness with vestibular dysfunction. *Nat Genet* 1998;20:299–303.
- [22] Robertson NG, Cremers CW, Huygen PL, Ikezono T, Krastins B, Kremer H,

Kuo SF, Liberman MC, Merchant SN, Miller CE, Nador JB Jr, Sarracino DA, Verhagen WI, Morton CC: Cochlin immunostaining of inner ear pathologic deposits and proteomic analysis in DFNA9 deafness and vestibular dysfunction. *Hum Mol Genet* 2006;15:1071–1085.

[23] Robertson NG, Hamaker SA, Patriub V, Aster JC, Morton CC: Subcellular localization, secretion, and post-translational processing of normal cochlin, and of mutants causing the sensorineural deafness and vestibular disorder, DFNA9. *J Med Genet* 2003;40:479–486.

[24] Robertson NG, Jones SM, Sivakumaran TA, Giersch ABS, Jurado SA, Call LM, Miller CE, Maison SF, Liberman MC, Morton CC: A targeted *Coch* missense mutation: a knock-in mouse model for DFNA9 late-onset hearing loss and vestibular dysfunction. *Hum Mol Genet* 2008;17:3426–3434.

[25] Schuknecht HF, Gacek MR: Cochlear pathology in presbycusis. *Ann Otol Rhinol Laryngol* 1993;102:1–16.

[26] Shimada A, Ebisu M, Morita T, Takeuchi T, Umemura T: Age-related changes in the cochlea and cochlear nuclei of dogs. *J Vet Med Sci* 1998;60:41–48.

[27] Shindo S, Ikezono T, Ishizaki M, Sekiguchi S, Mizuta K, Li L, Takumida M, Pawanker R, Yagi T: Spatiotemporal expression of cochlin in the inner ear of rats during postnatal development. *Neurosci Lett* 2008;444:148–152.

[28] Slepecky NB, Savage JE, Yoo TJ: Localization of Type II, IX and V collagen in the inner ear. *Acta Otolaryngol* 1992;112:611–617.

[29] Usami S, Takahashi K, Yuge I, Ohtsuka A, Namba A, Abe S, Fransen E, Pathy L, Otting G, Van Camp G: Mutations in the *COCH* gene are a frequent cause of autosomal dominant progressive cochlea-vestibular dysfunction, but not of Meniere's disease. *Eur J Hum Genet* 2003;11:744–748.

- [30] Usami S, Takumi Y, Suzuki N, Oguchi T, Oshima A, Suzuki H, Kitoh R, Abe S, Sasaki A, Matsubara A: The localization of proteins encoded by *CRYM*, *KIAA1199*, *UBA52*, *COL9A3*, and *COL9A1*, genes highly expressed in the cochlea. *Neuroscience* 2008;154:22–28.
- [31] Verhagen WI, Huygen PL, Theunissen EJ, Joosten EM: Hereditary vestibulo-cochlear dysfunction and vascular disorder. *J Neurol Sci* 1989;92:55–63.
- [32] Verhagen WI, Huygen PL, Bles W: A new autosomal dominant syndrome of idiopathic progressive vestibulo-cochlear dysfunction with middle-age onset. *Acta Otolaryngol* 1992;112:899–906.
- [33] Yoo TJ, Yazawa Y, Floyd R, Tomoda K: Antibody activity in perilymph from rats with type II collagen-induced autoimmune inner ear disease. *Ann Otol Rhinol Laryngol Suppl* 1984;113:1–2.
- [34] Yoo TJ, Tomoda K: Type II collagen distribution in rodents. *Laryngoscope* 1988;98:1255–1260.

Figure Legends

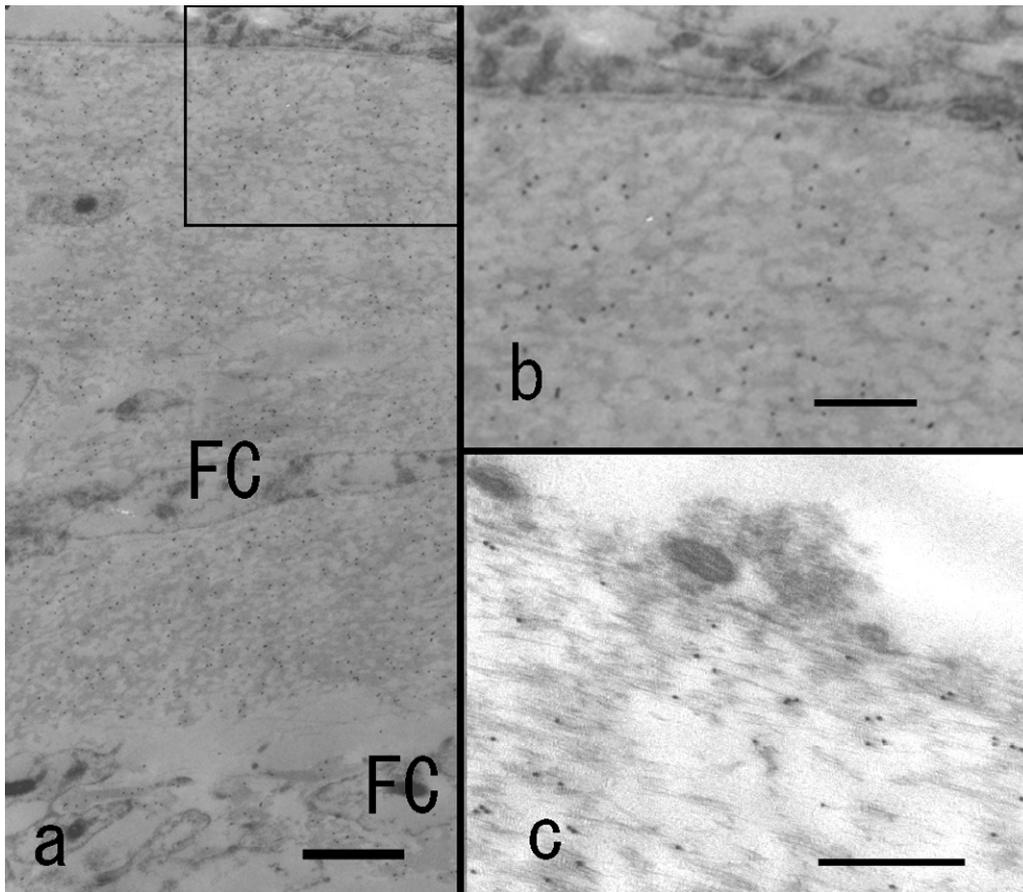


Fig.1. Immuno-electronmicroscopy for cochlin and type II collagen expression in the spiral limbus.

- a) Cochlin: Gold particles in the fibrous substance in the spiral limbus. Bar = 1 μm .
- b) Cochlin: Higher magnification of the open square area in Fig.1a. Bar = 0.5 μm .
- c) Type II collagen: Gold particles were seen in the fibrous substance in the spiral limbus. Bar = 0.5 μm .

FC=fibrocytes.

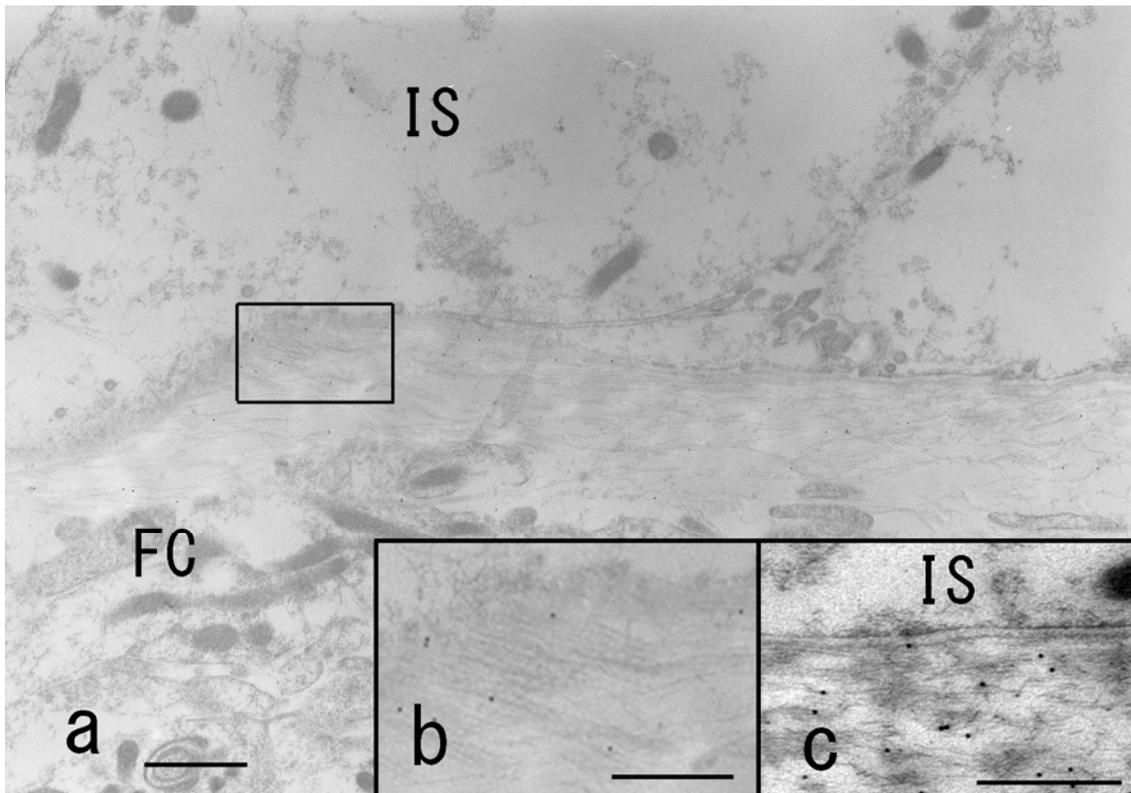


Fig. 2. Immuno-electronmicroscopy for cochlin and type II collagen expression beneath the inner sulcus cells.

- a) Cochlin: Immunolabeling of cochlin was seen in the fibrous substance beneath the inner sulcus cells (IS). Bar = 1 μ m.
- b) Cochlin: Higher magnification of the open square area in Fig.2a. Bar = 0.5 μ m.
- c) Type II collagen: Gold particles were seen in the fibrous substance beneath the inner sulcus cells (IS). Bar = 0.5 μ m.

FC = fibrocytes.

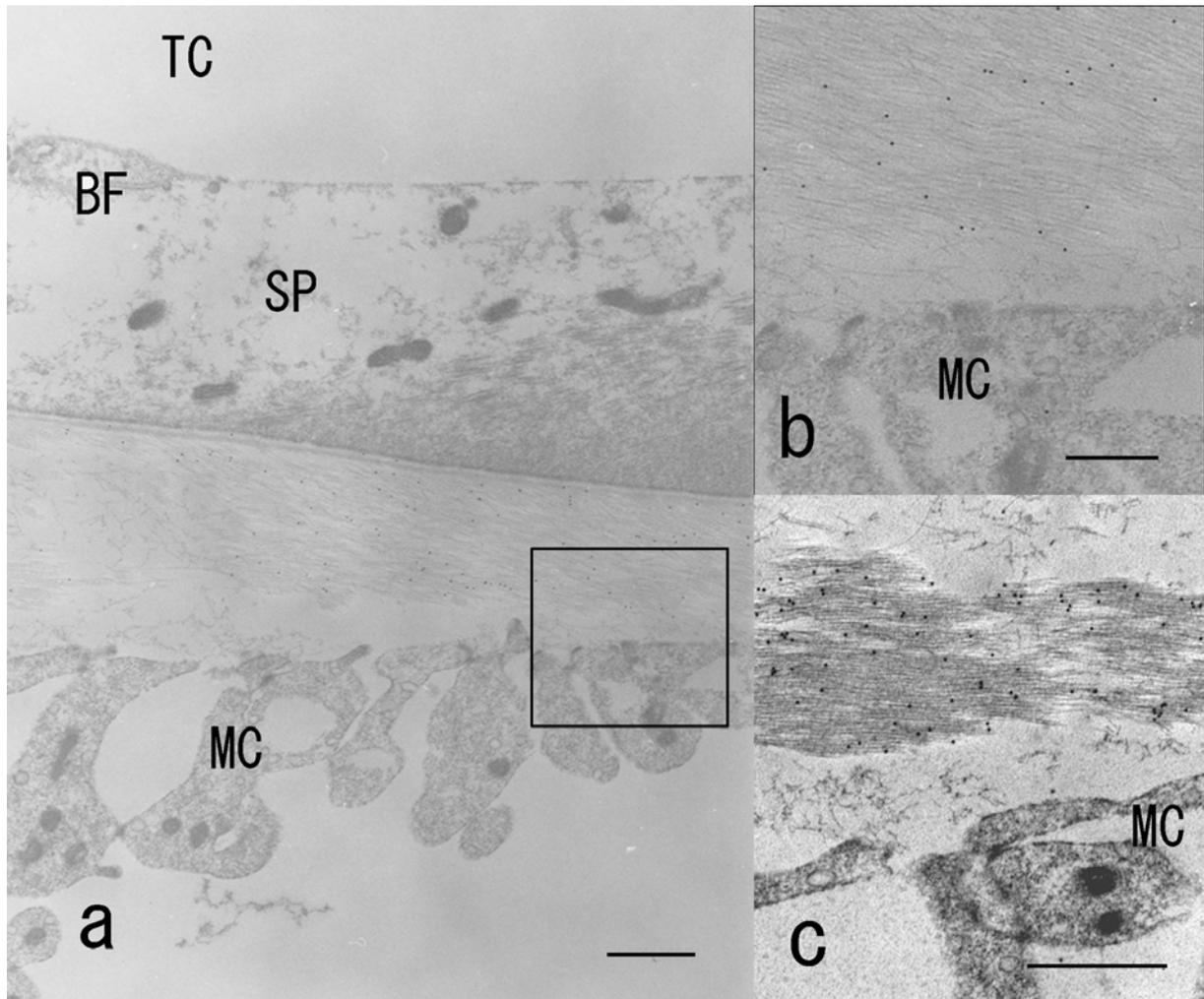


Fig.3. Immuno-electronmicroscopy for cochlin and type II collagen expression in the basilar membrane.

a) Cochlin: The fibrillar bundles in the basilar membrane were immunoreactive for cochlin. Bar = 1 μm .

b) Cochlin: Higher magnification of the open square area in Fig. 3a. Bar = 0.5 μm .

c) Type II collagen: Gold particles were seen in the fibrillar bundles in the basilar membrane. Bar = 0.5 μm .

TC = tunnel of Corti. BF=tunnel basilar fiber. SP = supporting cells of the sensory cells. MC = mesothelial cells.

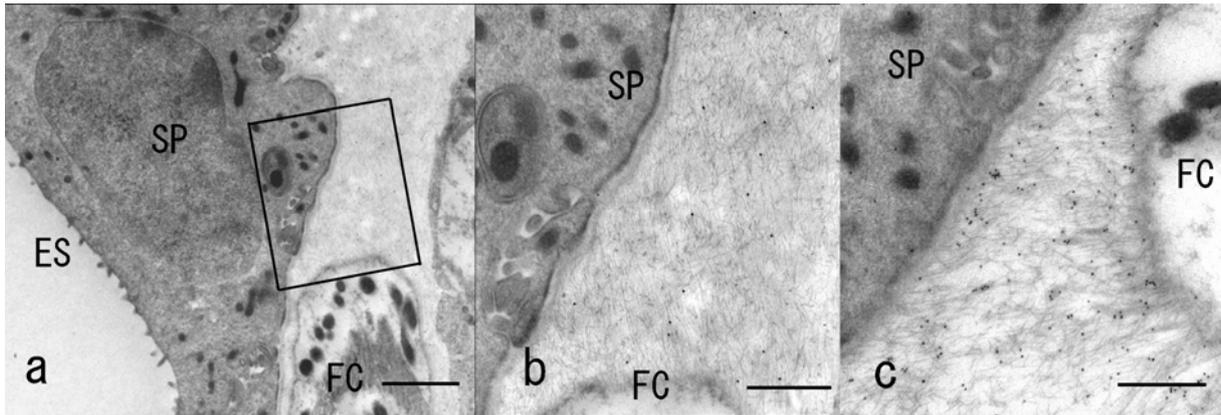


Fig. 4. Immuno-electronmicroscopy for cochlin and type II collagen expression beneath the epithelial cells of the spiral prominence.

- a) Cochlin: Gold particles in the fibrous substance beneath the epithelial cells of the spiral prominence. Bar = 1 μ m.
- b) Cochlin: Higher magnification of the open square area in Fig.4a. Bar = 0.5 μ m.
- c) Type II collagen: Gold particles were seen beneath the epithelial cells of the spiral prominence. Bar = 0.5 μ m.

SP=epithelial cells of the spiral prominence. FC=fibrocytes. ES=endolymphatic space.

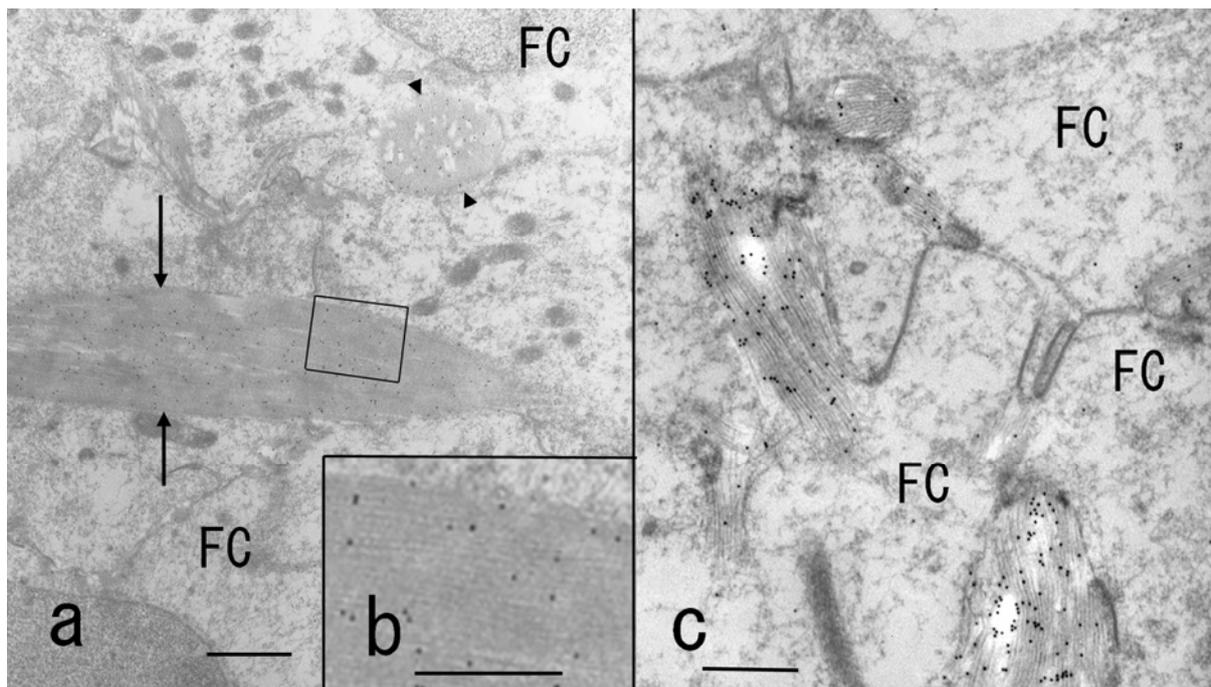


Fig. 5. Immuno-electronmicroscopy for cochlin and type II collagen expression in the spiral ligament.

- a) Cochlin: Gold particles were also observed in the banded bundles, which have a parallel array in the area of the fibrocytes in the spiral ligament. Parallel cut of the fibrous bundles (arrows). Vertical cut of the fibrous bundles (arrow heads). Bar = 1 μm .
- b) Cochlin: Higher magnification of the open square area in Fig.5a. Bar = 0.5 μm .
- c) Type II collagen: Gold particles were seen on the fibrillar bundles in the spiral ligament. Bar = 0.5 μm .

FC=fibrocytes.

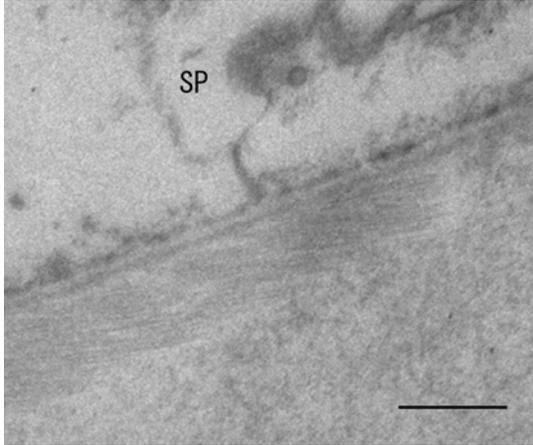


Fig. 6. Control. No gold particles were observed in the fibrillar substance in the basilar membrane. SP = supporting cells of the sensory cells. Bar = 0.5 μm .