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Research report

Immunohistochemical localization of megalin and cubilin in the human inner ear

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HIGHLIGHTS

- Megalin and cubilin immunoreactivity (IR) was found in the human cochlea and vestibule.
- Megalin-IR and cubilin-IR was seen in epithelial cells of the Reissner's membrane and spiral prominence.
- Megalin-IR and cubilin-IR was seen in the transitional and dark cells of vestibular end organs.
- Both megalin and cubilin may play an important role in inner ear endocytic transport.

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ABSTRACT

Megalin and cubilin are endocytic receptors expressed in many absorptive polarized epithelia. These receptors have been implicated in the transport of gentamicin in the inner ear as possible contributors to ototoxic damage. Megalin and cubilin have been characterized in detail in the mouse and rat inner ear, but not in the human inner ear. In this study, megalin and cubilin were localized by immunohistochemistry using affinity-purified antibodies in formalin fixed frozen cryostat and celloidin embedded sections of the human inner ear. In the cochlea megalin and cubilin were localized in marginal cells of the stria vascularis, epithelial cells of the spiral prominence and the Reissner's membrane. In the macula utricle and cristae ampullaris, megalin and cubilin were localized in transitional and dark cells, but not in vestibular hair cells and supporting cells. In the endolymphatic duct megalin and cubilin were localized in the epithelial cells. The localization of megalin and cubilin in the human inner ear is consistent with previous reports in the inner ear of animal models and suggest that these receptors may play an important role in the inner ear endocytic transport, and maybe potential targets for prevention of ototoxic damage or the delivery of medications.

1. Introduction

Cellular compartmentalization in the inner ear allows for proper hearing and balance function. In the cochlea, the scala vestibuli and the scala tympani contain perilymph and the scala media contains endolymph, with highly different potassium and sodium concentrations and protein composition (Wangemann, 2002; Zdebik et al., 2009). The transport of proteins and other molecules in and out of these compartments is likely to be mediated by megalin and cubilin two multi ligand endocytic receptors found in the apical part of epithelial cells of the kidney and inner ear (Arai et al., 2008; Christensen and Birn, 2002; Eshbach and Weisz, 2017; Gbuker et al., 2002; Ishida et al., 2006; Knipper et al., 2006; Konig et al., 2008; Kozyraki et al., 1998; Mizuta et al., 1999; Moestrup and Verroust, 2001; Tauris et al., 2009; Verroust and Christensen, 2002) and other absorptive epithelia (Nielsen et al., 2016). In the mouse and rat cochlea megalin and cubilin are located in epithelial cells of the Reissner's membrane, stria vascularis, spiral prominence and the endolymphatic sac *and* in transitional and dark cells of vestibular end organs (Arai et al., 2008; Ishida et al., 2006; Konig et al., 2008; Mizuta et al., 1999; Tauris et al., 2009).

Megalin, formerly called gp330, is a 600-kDa transmembrane protein belonging to the low-density lipoprotein (LDL) receptor-related family (Christensen et al., 1992, 2002). Megalin is encoded by *LRP2* (Farquhar et al., 1994; Raychowdhury et al., 1989; Tauris et al., 2009). Megalin was originally identified as the pathogenic autoantigen in Heymann nephritis, a rat model of human membranous nephropathy

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(Farquhar et al., 1994). Complete cloning and sequencing of megalin identified this molecule as the largest member of the LDL receptor-related protein family (Saito et al., 1994).

Megalin is expressed in several absorptive epithelial cells, including kidney proximal tubules, visceral yolk sac, epididymis, and female reproductive tracts (Christensen and Birn, 2002; Moestrup and Verroust, 2001), and the rat inner ear (Mizuta et al., 1999). Megalin serves as a scavenger receptor and Ca^{2+}-binding receptor (Christensen et al., 1992), and functions to regulate hormone metabolism and vitamin D absorption in cooperation with another receptor, cubilin (Christensen and Birn, 2002). Megalin has also been implicated in the binding of aminoglycosides in the kidney (McWilliam et al., 2017), and inner ear, and maybe involved in ototoxicity. Biallelic pathogenic variants in *LRP2* are associated with the autosomal recessive disorder Donnai-Barrow syndrome and facial-ocular-acoustic renal syndrome (DBS/FOAR) that includes sensorineural hearing loss among other phenotypes (Nielsen et al., 2016).

Cubilin (CUBN) acts as a receptor for intrinsic factor-vitamin B12 complexes, it is also referred as gp280. Cubilin is a 460-kDa peripheral membrane encoded by the *CUBN* (Moestrup et al., 1998). The complete cDNA sequence of cubilin have been characterized in the rat (Moestrup et al., 1998), dog (Xu et al., 1999), and human (Kozyraki et al., 1998). Biallelic pathogenic variants have been associated with megaloblastic anemia that could lead to sensory impairment (Aminoff et al., 1999).

Megalin and cubilin endocytic receptors and their ligands provide epithelial cells with important nutrients (Verroust and Christensen, 2002). Identification of these endocytic receptors and their potential to transport ligands in the human inner ear may have important clinical application in the development of novel treatment of several inner ear diseases. Given the tightly controlled drug transport of medications through the elaborate blood labyrinthine barrier (Ishiyama et al., 2017; Shi, 2016) and epithelial barriers, the identification of megalin and cubilin in the human inner ear may be relevant for the design and administration of drugs that can be delivered via endocytosis in the treatment of human otopathologies.

In the present study megalin and cubilin localization in the human inner ear was investigated by immunohistochemistry using formalin fixed cryostat sections and celloidin embedded sections of the human inner ear. We found that their localization in the human inner ear closely resembled the one found in the inner ear of rodents and suggest the existence of a tightly regulated homeostatic mechanism for endocytic transport mediated by megalin and cubilin.

2. Results

2.1. Megalin and cubilin localization in the human cochlea

Megalin and cubilin were localized by immunofluorescence (-IF) in formalin fixed cryostat sections of the human cochlea microdissected from normal temporal bones obtained at autopsy (no audio-vestibular disorders, Table 1). Megalin and cubilin colocalized in epithelial cells of the Reissner's membrane (Fig. 1). The epithelial cells of the Reissner's membrane form a continuous layer. Megalin-IF (green) was seen in the cytoplasm of epithelial cells (scala media) (Fig. 1a). Cubilin-IF (red) was also seen in these epithelial cells (Fig. 1b). Merged image, shows that both megalin and cubilin colocalized in Reissner's membrane epithelial cells (yellow color), few cells were cubilin-IF only (red color) (Fig. 1c). Higher magnification view shows the punctated megalin and cubilin immunofluorescence signal (Fig. 1d), yellow color indicated colocalization. DAPI (in blue) allowed the identification of cell nuclei. Mesenchymal cells located at the scala vestibuli were non-IF for megalin and cubilin.

Megalin-IF was also seen in epithelial cells of the spiral prominence and marginal cells of the stria vascularis (Fig. 2a, green color) and cubilin-IF (Fig. 2b, red color). Merged image (from Fig. 2a and b) shows colocalization of megalin and cubilin (Fig. 2c, yellow color). Higher magnification view of the spiral prominence (from Fig. 2c), shows in detail the localization of both proteins, cells in the spiral ligament were non immunoreactive (Fig. 2d). Inner and outer hair cells and supporting cells in the organ of Corti and spiral ganglia neurons were non-IF to both megalin and cubilin.

Table 1
Temporal bones used in this study.

Specimen #	Age (years)/ gender	PMT	Type of section	Staining type	Figure
1/R	70/F	13	FFF	IF	1a–d, 4b3
2/L	80/M	12	FFF	IF	2a–d, 4b, 4b1, 4b2
3/L	82/M	14	FFF	IF	4a, 4a1, 4a2
4/L	91/F	10	FFF	IF	4a3, 7d1, 7d2
5/L	54/F	12	celloidin	HRP-DAB	3a
6/R	79/M	14	celloidin	HRP-DAB	3b
7/R	67/F	12	celloidin	HRP-DAB	5a, 5a1
8/R	71/M	9	celloidin	HRP-DAB	5b, 5b1
9/R	76/F	12	celloidin	HRP-DAB	6a, 7d
10/L	84/F	9	celloidin	HRP-DAB	6b

Abbreviations: R: right side, L: Left side; M: male, F: Female, PMT: post mortem time in hours. All inner ear sections used in this study were from temporal bone donors with normal hearing and balance. FFF: Formalin (10% buffered) fixed frozen cryostat sections. HRP-DAB: immunohistochemistry using horseradish peroxidase and diamino-benzidine, IF: immunofluorescence. Figure: indicates specimen used for these micrographs.

Megalin and cubilin immunolocalization was also investigated in formalin fixed celloidin embedded sections of the human cochlea using secondary antibodies labeled with horseradish peroxidase and visualized with diaminobenzidine (HRP-DAB) (Fig. 3). Megalin-IR and cubilin-IR were found in the Reissner's membrane, marginal cells of the stria vascularis and spiral prominence (Fig. 3a and b respectively). Intermediate and basal cells of the stria vascularis and cells in the spiral ligament were non-immunoreactive for both megalin and cubilin. All human cochleas immunostained using fluorescent or HRP labeled secondary antibodies exhibited a similar megalin and cubilin distribution (Table 1).

2.2. Megalin and cubilin immunofluorescence (IF) in the human vestibular end organs

Megalin-IF and cubilin-IF was found in formalin fixed cryostat sections of the human cristae ampullaris and macula utricle microdissected from temporal bones (Table 1). Megalin-IF (green color) was seen in the apical portion of transition and dark epithelial cells located outside the crista sensory epithelia (Fig. 4a). Cubilin-IF (red color) was seen in the cytoplasm of transitional and dark cells (Fig. 4a1). The crista hair cells and supporting cells were non-IF for both megalin and cubilin; this also was true for the stromal cells, nerve fibers and terminals and vascular tissue located underneath the crista sensory epithelia. Colocalization of megalin and cubilin is seen in Fig. 4a2 (merged image from Fig. 4a and a1). Higher magnification view shows the specific localization (yellow color) of both megalin and cubilin in the apical portion of transitional and dark epithelial cells (Fig. 4a3). A similar pattern of megalin and cubilin immunofluorescence was seen in the macula utricle. Megalin (green color) and cubilin (red color) were found in the transitional and dark cells (Fig. 4b and b1, respectively). Merged images show colocalization of both proteins (Fig. 4b2). Higher magnification view of transitional cells showed specific immunofluorescence for both megalin and cubilin at the apical portion of transitional epithelial cells (Fig. 4b3). Like in the cristae vestibular hair cells, supporting cells and stromal cells underneath the utricle sensory epithelia were non-IF for both megalin and cubilin.

Megalin and cubilin immunoreactivity (-IR) was also investigated in celloidin embedded sections of the crista ampullaris and macula utricle