1

Proteins Involved in Otoconia Formation and Maintenance

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1. Introduction

The vestibule of the inner ear senses head motion for spatial orientation and bodily balance. In vertebrates, the vestibular system consists of three fluid filled semicircular canals, which detect rotational acceleration, and two gravity receptor organs, the utricle and saccule, which respond to linear acceleration and gravity (**Figure 1**). The utricle and saccule are also referred to as the otolithic organs because they contain bio-crystals called otoconia (otolith in fish). These crystals are partially embedded in a honeycomb layer atop a fibrous meshwork, which are the otoconial complex altogether. This complex rests on the stereociliary bundles of hair cells in the utricular and saccular sensory epithelium (aka macula). When there is head motion, the otoconial complex is displaced against the macula, leading to deflection of the hair bundles. This mechanical stimulus is converted into electrical signals by the macular hair cells and transmitted into the central nervous system (CNS) through the afferent vestibular nerve. In the CNS, these electrical signals, combined with other proprioceptive inputs, are interpreted as position and motion data, which then initiate a series of corresponding neuronal responses to maintain the balance of the body. Electrophysiological and behavioral studies show that the size and density of these tiny biominerals determine the amount of stimulus input to the CNS (Anniko et al. 1988; Jones et al. 1999; Jones et al. 2004; Kozel et al. 1998; Simmler et al. 2000a; Trune and Lim 1983; Zhao et al. 2008b).

Otoconia dislocation, malformation and degeneration can result from congenital and environmental factors, including genetic mutation, aging, head trauma and ototoxic drugs, and can lead to various types of vestibular dysfunction such as dizziness/vertigo and imbalance. In humans, BPPV (benign paroxysmal positional vertigo), the most common cause of dizziness/vertigo, is believed to be caused by dislocation of otoconia from the utricle to the ampulla and further in the semicircular canals (Salvinelli et al. 2004; Schuknecht 1962; Schuknecht 1969; Squires et al. 2004). In animals, otoconial deficiency has been found to produce head tilting, swimming difficulty, and reduction or failure of the air-righting reflexes (Everett et al. 2001; Hurle et al. 2003; Nakano et al. 2008; Paffenholz et al. 2004; Simmler et al. 2000a; Zhao et al. 2008b).

Despite the importance of these biominerals, otoconial research is lagging far behind that of other biomineralized structures, such as bone and teeth, partly due to anatomical and

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methodological constraints. The mechanisms underlying otoconia formation and maintenance are not yet fully understood. In this review, we will summarize the current state of knowledge about otoconia, focusing on the identified compositions and regulatory proteins and their roles in bio-crystal formation and maintenance. Homologs and analogs of these proteins are also found in fish with similar functions but varied relative abundances, but the review will focus on studies using mice as the latter have similar otoconia and inner ear properties as humans.

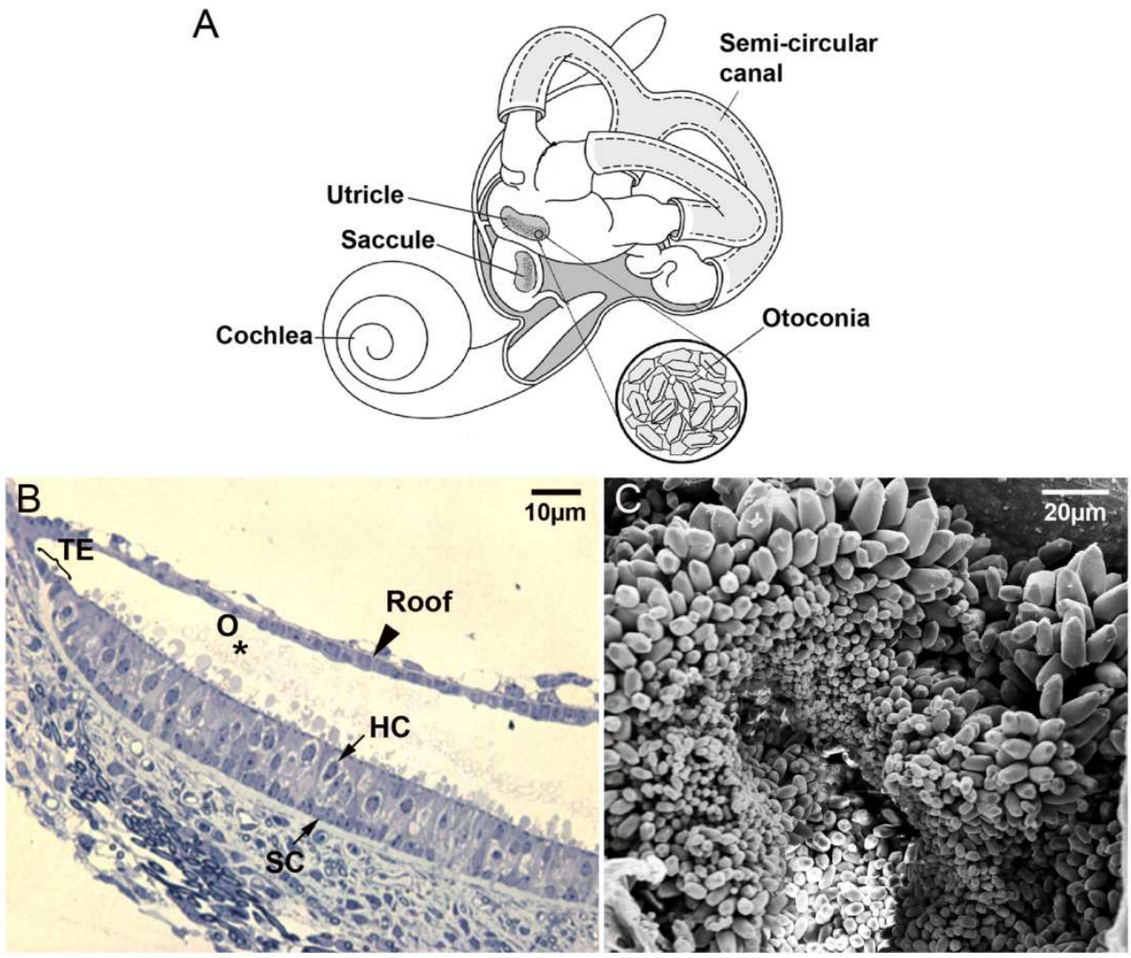

Fig. 1. (**A**) A schematic diagram of the mammalian inner ear. (**B**) A Toluidine blue-stained section of the saccule (P10). (**C**) A scanning electron micrograph of otoconia in the mouse utricle (6.5 months old). HC, hair cells; O, otoconia; SC, supporting cells; TE, transitional epithelium.

2. The roles of otoconial component proteins in crystal formation

Otoconia from higher vertebrates have a barrel-shaped body with triplanar facets at each end (Figure 1C). The core is predominantly organic with a low Ca^{2+} level, and is surrounded by a largely inorganic shell of minute crystallites outlined by the organic matrix (Lim 1984; Lins et al. 2000; Mann et al. 1983; Steyger and Wiederhold 1995; Zhao et al., 2007). Most

primitive fishes have apatite otoliths, more advanced fishes have aragonite otoliths, whereas higher levels of vertebrates have calcite otoconia (Carlstrom D 1963; Ross and Pote 1984). Otoliths in lower vertebrates display a daily growth pattern, whereas otoconia in mammals are formed during late embryonic stages, become mature shortly after birth and may undergo maintenance thereafter (Salamat et al. 1980; Thalmann et al. 2001) (Lundberg, unpublished data). Because otoconia/otoliths from animals of different evolutionary levels all have the common $CaCO_3$ component but have various morphologies and crystalline structures and different protein compositions, otoconins (a collective term for otoconial component proteins) must be important for otoconia formation. More importantly, as the mammalian endolymph has an extremely low Ca^{2+} concentration, otoconins may be essential for $CaCO_3$ crystal seeding.

Indeed, recent studies have demonstrated that the shape, size and organization of $CaCO_3$ crystallites in otoconia and otoliths are strictly controlled by an organic matrix (Kang et al. 2008; Murayama et al., 2005; Sollner et al. 2003; Zhao et al. 2007). The organic components of otoconia primarily consist of glycoproteins and proteoglycans (Endo et al. 1991; Ito et al. 1994; Pisam et al. 2002; Pote and Ross 1991; Verpy et al. 1999; Wang et al. 1998; Xu et al. 2010; Zhao et al., 2007). To date, as many as 8 murine otoconins have been identified (Table 1): the predominant otoconial protein, otoconin-90 (Oc90) and other 'minor' otoconins including otolin-1 (aka otolin) (Zhao et al. 2007), fetuin-A (aka countertrypin) (Thalmann et al. 2006; Zhao et al. 2007), osteopontin (aka Spp1) (Sakagami 2000; Takemura et al. 1994; Zhao et al. 2008a), Sparc-like protein 1 (Sc1, aka hevin and Ecm2)(Thalmann et al. 2006; Xu et al. 2010), possibly secreted protein acidic and rich in cysteine (Sparc, aka BM-40 and osteonectin), and dentin matrix protein 1 (DMP1). Those otoconins are expressed in different cells and secreted into the utricular and saccular endolymph. Most of them are highly glycosylated, which confers thermodynamic stability and other properties (see below) on those proteins. They may interact with each other to form the organic scaffold for efficient and orientated deposition of calcium carbonate, and thus determine the size, shape, crystallographic axes and orientation of individual crystallite.

2.1 Otoconin-90 (Oc90) is the essential organizer of the otoconial matrix

Oc90 is the first identified otoconin, and accounts for nearly 90% of the total protein content of otoconia (Pote and Ross 1991; Verpy et al. 1999; Wang et al. 1998). Subsequent studies have revealed that Oc90 is the essential organizer of the otoconial organic matrix by specifically recruiting other matrix components and Ca^{2+} (Yang et al. 2011; Zhao et al. 2007).

Oc90 is structurally similar to secretory phospholipase A2 (sPLA2). Although it likely does not have the catalytic activity of the enzyme due to the substitutions of a few essential residues in the active site (Pote and Ross 1991; Wang et al. 1998), Oc90 possesses the other features of sPLA2. It is a cysteine-rich secretory protein, and has several glycosylation sites and calcium binding capability. The enriched cysteine residues are likely involved in the formation of higher-order protein structures via intra- and inter-molecular disulfide bonds. The intra-molecular disulfide bonds play an important role in protein folding and the stabilization of the tertiary structure, while the disulfide bonds formed between subunits allow dimerization and oligomerization of the protein.

Type	Protein name	Otoconia phenotype of mutant mice	Reference
Constituent proteins	Oc90	Giant otoconia (few to many)	(Zhao et al. 2007)
	Otolin-1	---	---
	Sc1	---	---
	Sparc?	---	---
	KSPG	---	---
	DMP1	---	---
	α-tectorin	Large otoconia but reduced in number	(Legan et al. 2000)
	Osteopontin	Normal otoconia	(Zhao et al. 2008a)
	Fetuin-A	Normal otoconia?	(Xu et al. 2010)
Regulatory proteins	Otopetrin 1	No otoconia	(Hurle et al. 2003)
	Nox3	No otoconia	(Paffenholz et al. 2004)
	Noxo1	No otoconia	(Kiss et al. 2006)
	Noxa1?	---	---
	p22*phox*	No otoconia	(Nakano et al. 2008)
	PMCA2	No otoconia	(Kozel et al. 1998)
	Pendrin	Large otoconia but reduced in number	(Everett et al. 2001)
	TRPVs	---	---
Anchoring proteins	Otogelin	Detached OM	(Simmler et al. 2000a)
	α-tectorin	Large otoconia but reduced in number	(Legan et al. 2000)
	β-tectorin?	---	---
	Otoancorin?	---	---

Table 1. Identified and validated murine otoconial proteins and their importance in otoconia formation by genetic mutation studies. Shaded ones have no measurable impact on bio-crystal formation. ---, no mutant mice available or unknown otoconia/otolith phenotype.

The Ca^{2+} concentrations of the mammalian endolymph are extremely low at ~20 μM (Ferrary et al. 1988; Salt et al. 1989), with a few reporting much higher in the vestibule (Marcus and Wangemann 2009; Salt et al. 1989). This is much lower than what is necessary for the spontaneous formation of calcite crystals, therefore, otoconial proteins are speculated to sequester Ca^{2+}. Indeed, most of the otoconial proteins have structural features for Ca^{2+} binding. Oc90 has 28 (~6%) Glu and 39 (~8%) Asp out of the total 485 amino acids, endowing the molecule with a calculated acidic isoelectric point (*pI* = 4.5). The measured *pI* of mature Oc90 is even lower (2.9) due to post-translational modifications such as N-linked glycosylation (Lu et al. 2010). This extreme acidic feature may help Oc90 recruit Ca^{2+} and/or interact with the surface of calcium carbonate crystals to modulate crystal growth. Deletion of Oc90 causes dramatic reduction of matrix-bound Ca^{2+} in the macula of the utricle and saccule (Yang et al. 2011). In the absence of Oc90, the efficiency of crystal formation is reduced by at least 50%, and

the organic matrix is greatly reduced, leading to formation of a few giant otoconia with abnormal morphology caused by unordered aggregation of inorganic crystallites (Zhao et al. 2007). A subsequent *in vitro* experiment has also demonstrated that Oc90 can facilitate nucleation, determine the crystal size and morphology in a concentration-dependent manner (Lu et al. 2010). Recent evidence suggests that the formation of otoconia at all in Oc90 null mice may be partially attributed to the compensatory deposition of Sc1 (Xu et al. 2010).

The expression of Oc90 temporally coincides that of otoconia development and growth, also providing evidence for the critical requirement of Oc90 in this unique biomineralization process. Oc90 expression is the earliest among all otolith/otoconia proteins in fish and mice (before embryonic day E9.5 in mice) (Petko et al. 2008; Verpy et al. 1999; Wang et al. 1998), much earlier than the onset of any activities of ion channels/pumps, or the onset of otoconia seeding at around E14.5. Oc90 then recruits other components at the time of their expression to form the organic matrix for calcification (Zhao et al. 2007). When otoconia growth stops at around P7 (postnatal day 7), the expression level of Oc90 significantly decreases in the utricle and saccule (Xu and Lundberg 2012). Although Oc90 has a relatively low abundance in zebrafish otoliths (known as zOtoc1) (Petko et al. 2008), Oc90 morphant fish show more severe phenotypes than morphants for the main otolith matrix protein OMP1 (Murayama et al. 2005; Petko et al. 2008), suggesting that zOc90 (zOtoc1) is essential for the early stages of otolith development (i.e. crystal seeding) whereas OMP regulates crystal growth. Thus, the structure and function of Oc90 is conserved from bony fish to mice (two model systems whose otoconia/otolith are the most studied) regardless of the abundance of the protein in each species.

2.2 Sc1 can partially compensate the function of Oc90

Sc1 was first isolated from a rat brain expression library (Johnston et al. 1990). It is widely expressed in the brain and can be detected from various types of neurons (Lively et al. 2007; McKinnon and Margolskee 1996; Mendis and Brown 1994). As a result, studies of Sc1 have focused on the nervous system. Recently, Thalmann et al. identified Sc1 from mouse otoconia by mass spectrometry (Thalmann et al. 2006). However, Xu et al. (Xu et al. 2010) found that Sc1 was hardly detectable in the wild-type otoconia. Instead, the deposition of Sc1 was drastically increased in otoconia crystals when Oc90 is absent, suggesting a possible role for Sc1 as an alternative process of biomineralization (Xu et al. 2010). *Sc1* knockout mice did not show any obvious phenotypic abnormalities, including vestibular functions (McKinnon et al. 2000)(S. Funk and H. Sage, communication through Thalmann et al. 2006).

Although Sc1 and Oc90 have no significant sequence similarity, the two proteins share analogous structural features. Murine Sc1 is a secreted, acidic and Cys-rich glycoprotein, and belongs to the Sparc family. Its Sparc-like domain consists of a follistatin-like domain followed by an α-helical domain (EC) containing the collagen-binding domain and 2 calcium-binding EF-hands (Maurer et al. 1995). All of these features likely render Sc1 an ideal alternative candidate for otoconia formation in the absence of Oc90. The high abundance of Glu/Asp residues (52 Glu and 87 Asp out of 634 aa) makes the protein highly acidic (*pI* = 4.2), which, together with the EF-hand motif, provides Sc1 a high affinity for calcium and calcium salts (e.g. calcium carbonate and phosphate). The collagen-binding site in the EC domain can recognize the specific motif of the triple-helical collagen peptide and form a deep 'Phe pocket' upon collagen binding (Hohenester et al. 2008; Sasaki et al. 1998).

The follistatin domain was reported to modulate the process of collagen-binding even though it does not interact with collagen directly (Kaufmann et al. 2004). In addition, the enriched cysteines in the polypeptide backbone of Sc1 may enable the formation of numerous intra- and inter-molecular disulfide bridges, as well as dimerization or even oligomerization of the protein, all of which enable the protein to serve as a rigid and stable framework for inorganic crystal deposition and growth (Chun et al. 2006; Xu et al. 2010).

2.3 Otolin may function similarly to collagen X

Otolin is a secreted glycoprotein present in both otoconial crystals and membranes. The expression level of *otolin* mRNA in the utricle and saccule is much higher than that in the epithelia of non-otolithic inner ear organs (Yang et al. 2011), implicating a potentially critical role of this molecule in otoconia development. In fish, knockdown of otolin led to formation of fused and unstable otoliths (Murayama et al. 2005).

Otolin contains three collagen-like domains in the N-terminal region and a highly conserved globular C1q (gC1q) domain in the C-terminal region, and belongs to the collagen X family and C1q super-family (Deans et al. 2010; Kishore and Reid 1999; Yang et al. 2011). Like collagen X, the N-terminal collagen domains of otolin contain tens of characteristic Gly-X-Y repeats, which can facilitate the formation of collagen triple helix and higher-order structures. Such structural features in otolin may render the protein extremely stable. The C-terminal gC1q domain is more like a target recognition site which may mediate the interaction between otolin and other extracellular proteins. Co-immunoprecipitation experiments demonstrated that Oc90 can interact with both the collagen-like and C1q domains of otolin to form the otoconial matrix framework and to sequester Ca^{2+} for efficient otoconia calcification. Co-expression of Oc90 and otolin in cultured cells leads to significantly increased extracelluar matrix calcification compared with the empty vector, or *Oc90* or *otolin* single transfectants (Yang et al. 2011). Analogously, otolith matrix protein-1 (OMP-1), the main protein in fish otoliths, is required for normal otolith growth and deposition of otolin-1 in the otolith (Murayama et al. 2004; Murayama et al. 2005).

2.4 Keratin sulfate proteoglycan (KSPG) may be critical for otoconia calcification

Proteoglycans are widely distributed at the cell surface and in the extracellular matrix, and are critical for various processes such as cell adhesion, growth, wound healing and fibrosis (Iozzo 1998). A proteoglycan consists of a 'core protein' with covalently attached glycosaminoglycan (GAG) chains. They can interact with other proteoglycans and fibrous matrix proteins, such as collagen, to form a large complex. In addition, proteoglycans have strong negative charges due to the presence of sulfate and uronic acid groups, and can attract positively charged ions, such as Na^{+}, K^{+} and Ca^{2+}. All those features make proteoglycans important players in the extracellular calcification processes. Indeed, both heparan sulfate proteoglycan (HSPG) and chondroitin sulfate proteoglycan (CSPG) are critical for bone and teeth formation. Deletion of those proteins results in various calcification deficiencies (Hassell et al. 2002; Viviano et al. 2005; Xu et al. 1998; Young et al. 2002).

In the inner ear, however, KSPG appears to be the predominant proteoglycan (Xu et al. 2010). KSPG has been detected in chicken and chinchilla otoconia, and shows strong staining in murine otoconia as well (Fermin et al. 1990; Swartz and Santi 1997; Xu et al.

2010). The role of KSPG in otoconia development has not been elucidated yet. It may participate in sequestering and retaining Ca^{2+} for crystal formation because of its strong negative charges. *In vitro* immunoprecipitation results demonstrated that it may interact with Oc90 and otolin to form the matrix framework for the deposition of calcite crystals (Yang et al. 2011).

2.5 Some low abundance otoconins may be dispensable for otoconia formation

Most of the low abundance otoconial proteins play critical roles in bone and/or tooth formation. In contrast, studies by us and other investigators using existing mutant mice have demonstrated that a few of these proteins are dispensable or functionally redundant for otoconia development.

For example, osteopontin, a multifunctional protein initially identified in osteoblasts, is a prominent non-collagen component of the mineralized extracellular matrices of bone and teeth. Osteopontin belongs to the small integrin-binding N-linked glycoprotein (SIBLING) family. As a SIBLING member, osteopontin has an arginine-glycine-aspartate (RGD) motif, which plays an essential role in bone resorption by promoting osteoclast attachment to the bone matrix through cell surface integrins (Oldberg et al. 1986; Rodan and Rodan 1997). Similar to the role of Oc90 in otoconia development, osteopontin acts as an important organizer in bone mineralization. It modulates the bone crystal sizes by inhibiting the hydroxyapatite formation and growth (Boskey et al. 1993; Hunter et al. 1994; Shapses et al. 2003). Osteopontin null mice have altered organization of bone matrix and weakened bone strength, leading to reduced bone fracture toughness (Duvall et al. 2007; Thurner et al. 2010). However, despite its presence in otoconia and vestibular sensory epithelia, osteopontin is dispensable for otoconia formation, and osteopontin knockout mice show normal vestibular morphology and balance function (Zhao et al. 2008a).

Dentin matrix acidic phosphoprotein 1 (DMP1) is another protein that belongs to the SIBLING family. DMP1 was first cloned from dentin and then found in bone. It plays a critical role in apatite crystal seeding and growth in bone and teeth (George et al. 1993; Hirst et al. 1997; MacDougall et al. 1998). DMP1 null mice show severe defects in bone structure. Lv et al. (Lv et al. 2010) recently found that DMP1 null mice developed circling and head shaking behavior resembling vestibular disorders. They attributed these phenotypes to bone defects in the inner ear. However, it should not be excluded that DMP1 deficiency may affect otoconia as the protein is also present in mouse otoconia at a low level (Xu et al. 2010).

Sparc, aka BM-40 or osteonectin, is generally present in tissues undergoing remodeling such as skeletal remodeling and injury repair (Bolander et al. 1988; Hohenester et al. 1997; Sage and Vernon 1994). The protein is a normal component of osteiod, the newly formed bone matrix critical for the initiation of mineralization during bone development (Bianco et al. 1985; Termine et al. 1981). Sparc has a high affinity for both Ca^{2+} and several types of collagen (Bolander et al. 1988; Hohenester et al. 2008; Maurer et al. 1995). These features likely account for the importance of Sparc in bone formation, and possibly in otoconia formation. Indeed, Sparc is also required for otolith formation in fish (Kang et al. 2008). In the wild-type murine otoconia, however, Sparc is present at an extremely low level (Xu et al. 2010) that it may not play a significant role in crystal formation. Instead, the longer form Sc1 is the preferred scaffold protein when Oc90 is absent (Xu et al. 2010).

Fetuin-A, also known as α2-HS-glycoprotein or countertrypin, is a hepatic secreted protein that promotes bone mineralization. It is among the most abundant non-collagen proteins found in bone (Quelch et al. 1984). Several recent studies demonstrated that fetuin-A can bind calcium and phosphate to form a calciprotein particle and prevent the precipitation of these minerals from serum (Heiss et al. 2003; Price et al. 2002), which may explain the role of fetuin-A in bone calcification and its potent inhibition of ectopic mineralization in soft tissues (Schafer et al. 2003; Westenfeld et al. 2007; Westenfeld et al. 2009). However, fetuin-A null mice have normal bone under regular dietary conditions (Jahnen-Dechent et al. 1997). Fetuin-A is present in otoconia crystals (Zhao et al. 2007), but null mice for the protein do not show balance deficits (Jahnen-Dechent, communication in Thalmann et al., 2006), therefore, it is unlikely that the protein has a major impact on otoconia genesis.

Taken together, findings on these low abundance otoconins indicate similarities and differences between bone and otoconia biomineralization.

3. The roles of regulatory proteins in otoconia formation

Otoconia formation depends on both organic and inorganic components that are secreted into the vestibular endolymph. Non-component regulatory proteins affect otoconia development and maintenance likely by several ways: (1) by influencing the secretion (Sollner et al. 2004), structural and functional modification of the component and anchoring proteins (Lundberg, unpublished data), and (2) by spatially and temporally increasing chemical gradients of Ca^{2+}, HCO_3^-, H^+ and possibly other ions/anions to establish an appropriate micro-environmental condition for crystal seeding and growth.

3.1 NADPH oxidase 3 (Nox3) and associated proteins are essential for otoconia formation

The Noxs are a family of enzymes whose primary function is to produce ROS (reactive oxygen species). These proteins participate in a wide range of pathological and physiological processes. To date, seven Nox family members, Nox1-Nox5, Duox1 and Duox2, have been identified in mammals (Bedard and Krause 2007). Noxs serve as the core catalytic components, and their activities are regulated by cytosolic partners such as $p22^{phox}$, Nox organizers (Noxo1, $p47^{phox}$ and $p40^{phox}$), and Nox activators (Noxa1 and $p67^{phox}$).

Among the identified Nox family members, Nox3 is primarily expressed in the inner ear and is essential for otoconia development (Banfi et al. 2004; Cheng et al. 2001; Paffenholz et al. 2004). It interacts with $p22^{phox}$ and Noxo1 to form a functional NADPH oxidase complex, and all three components are required for otoconia development and normal balance in mice (Kiss et al. 2006; Nakano et al. 2007; Nakano et al. 2008; Paffenholz et al. 2004). However, the mechanisms underlying the requirement of Nox-related proteins for otoconia formation are poorly understood. One possible role of Nox3 is to oxidize otoconial proteins, including Oc90, which then undergo conformational changes to trigger crystal nucleation. Indeed, our recent unpublished data show that Nox3 modifies the structures of a few otoconia proteins (Xu et al. 2012).

A novel mechanism proposed by Nakano et al. (Nakano et al. 2008) states that while the Nox3-complex passes electrons from intracellular NADPH to extracellular oxygen, the plasma membrane becomes depolarized. Such depolarization of the apical membrane would elevate

endolymphatic Ca^{2+} concentration by preventing cellular Ca^{2+} uptake from endolymph, and by increasing paracellular ion permeability to allow Ca^{2+} influx from perilymph to endolymph. In addition, Nox3-derived superoxide may react with endolymphatic protons and thereby elevate the pH so that $CaCO_3$ can form and be maintained.

3.2 Otopetrin 1 may mobilize Ca^{2+} for $CaCO_3$ formation

Otopetrin (Otop1), a protein with multiple transmembrane domains, is essential for the formation of otoconia/otolith in the inner ear (Hughes et al. 2004; Hurle et al. 2003; Sollner et al., 2004). The protein is conserved in all vertebrates, and its biochemical function was first revealed by studying the phenotypes of two mutants, the *tilted* (*tlt*) and *mergulhador* (*mlh*) mice, which carry single-point mutations in the predicted transmembrane (TM) domains (*tlt*, Ala_{151}→Glu in TM3; *mlh*, Leu_{408}→Gln in TM9) of the *Otop1* gene. Both *tlt* and *mlh* homozygous mutant mice show non-syndromic vestibular disorders caused by the absence of otoconia crystals in the utricle and saccule (Hurle et al. 2003; Zhao et al. 2008b). Those mutations in *Otop1* do not appear to affect other inner ear organs, making *tlt* and *mlh* excellent tools to investigate how Otop1 participates in the development of otoconia and in what aspects the absence of otoconia impacts balance functions.

In fish, expression of *Otop1* is in both hair cells and supporting cells before otolith seeding, but is restricted in hair cells during otolith growth (Hurle et al. 2003; Sollner et al. 2004). In mice, *Otop1* exhibits complementary mRNA expression pattern with Oc90 in the developing otocyst, and high Otop1 protein level is visible in the gelatinous membrane overlying the sensory epithelium, suggesting that it may be integral to the membrane vesicles released into the gelatinous layer (Hurle et al. 2003). However, a more recent study by Kim and colleagues using a different antibody (Kim et al. 2010) demonstrated that Otop1 is expressed in the extrastriolar epithelia of the utricle and saccule, and is specifically localized in the apical end of the supporting cells and a subset of transitional cells. They also found that the *tlt* and *mlh* mutations of Otop1 change the subcelluar localization of the mutant protein, and may underlie its function in otoconia development (Kim et al. 2011).

Both *in vitro* and *ex vivo* studies demonstrated that one of the functions of Otop1 is to modulate intra- and extracellular Ca^{2+} concentrations by specifically inhibiting purinergic receptor P2Y, depleting of endoplasmic reticulum Ca^{2+} stores and mediating influx of extracellular Ca^{2+} (Hughes et al. 2007; Kim et al. 2010). Under normal conditions, the concentration of Ca^{2+} in the mammalian endolymph is much lower than that in the perilymph and other extracellular fluids, and is insufficient to support normal growth of otoconia. Hence, Otop1 may serve as the indispensible Ca^{2+} source that supports otoconia mineralization.

Moreover, Otop1 may also regulate the secretion of components required for otoconia formation. In zebrafish, Otop1 was shown to affect the secretion of *starmaker*, a protein essential for otolith formation, in the sensory epithelia (Sollner et al. 2004).

3.3 PMCA2 is a critical source of Ca^{2+} for $CaCO_3$ formation

Calmodulin-sensitive plasma membrane Ca^{2+}-ATPases (PMCAs) are vital regulators of otoconia formation by extruding Ca^{2+} from hair cells and thereby maintaining the appropriate Ca^{2+} concentration near the plasma membrane. There are four isoforms of mammalian PMCA (PMCA1-4) encoded by four distinct genes and each of them undergoes

alternative exon splicing in two regions (Keeton et al. 1993). All four PMCAs are expressed in the mammalian cochlea and extrude Ca^{2+} from hair cell stereocilia, whereas PMCA2a, a protein encoded by *Atp2b2* gene, is the only PMCA isoform present in vestibular hair bundles (Crouch and Schulte 1996; Dumont et al. 2001; Furuta et al. 1998; Yamoah et al. 1998). Null mutation in *Atp2b2* results in the absence of otoconia and subsequent balance deficits (Kozel et al. 1998), underpinning the importance of PMCA2 in otoconial genesis.

3.4 Pendrin regulates endolymph pH, composition and volume

Pendrin, encoded by *Slc26a4*, is an anion transporter which mediates the exchange of Cl^-, I^-, OH^-, HCO_3^-, or formate, across a variety of epithelia (Scott et al. 1999; Scott and Karniski 2000). In the inner ear, pendrin is primarily expressed in the endolymphatic duct and sac, the transitional epithelia adjacent to the macula of the utricle and saccule, and the external sulcus of the cochlea (Everett et al. 1999). Pendrin is critical for maintaining the appropriate anionic and ionic composition and volume of the endolymphatic fluid, presumably due to HCO_3^- secretion. Mutations in human *SLC26A4* are responsible for Pendred syndrome, a genetic disorder which causes early hearing loss in children (Dai et al. 2009; Luxon et al. 2003). Studies using an *Slc26a4* knockout mouse model have revealed that pendrin dysfunction can cause an enlargement and acidification of inner ear membrane labyrinth and thyroid at embryonic stages, leading to deafness, balance disorders and goiter similar to the symptoms of human Pendred syndrome (Everett et al. 2001; Kim and Wangemann 2010; Kim and Wangemann 2011). The mice have much lower endolymphatic pH, resulting in the formation of giant crystals with reduced numbers in both the utricle and saccule (Everett et al. 2001; Nakaya et al. 2007). Recently, Dror et al. have also demonstrated that a recessive missense mutation within the highly conserved region of *slc26a4* results in a mutant pendrin protein with impaired transport activity. This mutant mouse has severely abnormal mineral composition, size and shape of otoconia, i.e., giant $CaCO_3$ crystals in the utricle at all ages, giant CaOx crystals in the saccule of older adults, and ectopic giant stones in the crista (Dror et al. 2010). Therefore, pendrin participates in otoconia formation through providing HCO_3^-, which is essential for forming $CaCO_3$ crystals and for buffering the endolymphatic pH. Pendrin can also buffer pH through other anions such as formate.

3.5 Carbonic anhydrase (CA) provides HCO_3^- and maintains appropriate pH for otoconia formation and maintenance

CA catalyzes the hydration of CO_2 to yield HCO_3^- and related species, and is thus thought to be important for otoconia formation by producing HCO_3^- and keeping appropriate endolymph pH. CA is widely present in the sensory and non-sensory epithelia of the inner ear (Lim et al. 1983; Pedrozo et al. 1997), especially the developing endolymphatic sac of mammalian embryos contain high levels of CA. Administration of acetazolamide, a CA inhibitor, in the latter tissue can decrease the luminal pH and HCO_3^- concentration (Kido et al. 1991; Tsujikawa et al. 1993). Injection of acetazolamide into the yolk sac of developing chick embryos alters and inhibits normal otoconial morphogenesis (Kido et al. 1991). Activation/deactivation of macular CA under different gravity is associated with changes in otolith sizes in fish (Anken et al. 2004). Immunohistochemstry shows that CAII is co-expressed with pendrin in the same cells in the endolymphatic sac, suggesting that those two proteins may cooperate in maintaining the normal function of the endolymphatic sac (Dou et al. 2004), which is an important tissue for endolymph production.

In addition to CA, HCO_3^--ATPase and Cl^-/HCO_3^--exchangers are involved in the transepithelial transport of bicarbonate ions to the endolymph, and affect carbon incorporation into otoliths (Tohse and Mugiya 2001).

3.6 Transient receptor potential vanilloids (TRPVs) may also regulate endolymph homeostasis

Studies suggest that TRPVs may also play an important part in fluid homeostasis of the inner ear. All TRPVs (TRPV1-6) are expressed in vestibular and cochlear sensory epithelia (Ishibashi et al. 2008; Takumida et al. 2009). In addition, TRPV4 is also present in the endolymphatic sac and presumably acts as an osmoreceptor in cell and fluid volume regulation (Kumagami et al. 2009). Both TRPV5 and TRPV6 are found in vestibular semi-circular canal ducts (Yamauchi et al. 2010). In pendrin-deficient mice, the acidic vestibular endolymphatic pH is thought to inhibit the acid-sensitive TRPV5/6 calcium channels and lead to a significantly higher Ca^{2+} concentration in the endolymph, which may be another factor causing the formation of abnormal otoconia crystals (Nakaya et al. 2007). However, direct evidence has yet to be presented on whether TRPV-deficiency will lead to otoconia abnormalities.

4. The roles of anchoring proteins in the pathogenesis of otoconia-related imbalance and dizziness/vertigo

The inner ear acellular membranes, namely the otoconial membranes in the utricule and saccule, the cupula in the ampulla, and the tectorial membrane in the cochlea, cover their corresponding sensory epithelia, have contact with the stereocilia of hair cells and thus play crutial role in mechanotransduction. In the utricle and saccule, otoconia crystals are attached to and partially embedded in a honeycomb layer above a fibrous meshwork, which are collectively called otoconial membranes, and are responsible for the site-specific anchoring of otoconia. Disruption of the otoconial membrane structure may cause the detachment and dislocation of otoconia and thus vestibular disorders.

The acellular structures of the inner ear consist of collagenous and non-collagenous glycoproteins and proteoglycans. Several types of collagen, including type II, IV, V and IX, have been identified in the mammalian tectorial membrane (Richardson et al. 1987; Slepecky et al. 1992). In the otoconial membranes, however, otolin is likely the main collagenous component. As to the noncollagenous constituents, three glycoproteins, otogelin, α-tectorin and β-tectorin, have been identified in the inner ear acellular membranes in mice to date (Cohen-Salmon et al. 1997; Legan et al. 1997). The proteoglycan in mouse otoconia is keratin sulfate proteoglycan (KSPG) (Xu et al. 2010).

Otogelin is a glycoprotein that is present and restricted to all acellular membranes of the inner ear (Cohen-Salmon et al. 1997). At early embryonic stages, otogelin is produced by the supporting cells of the sensory epithelia of the developing vestibule and cochlea, and presents a complementary distribution pattern with Myosin VIIA, a marker of hair cells and precursors (El-Amraoui et al. 2001). At adult stages, otogelin is still expressed in the vestibular supporting cells, but become undetectable in the cochlear cells. Otogelin may be required for the attachment of the otoconial membranes and consequently site-specific anchoring of otoconia crystals. Dysfunction of otogelin in either the *Otog* knockout mice or

the twister mutant mice leads to severe vestibular deficits, which is postulated to be caused by displaced otoconial membranes in the utricle and saccule (Simmler et al. 2000a; Simmler et al. 2000b).

α-tectorin and β-tectorin, named with reference to their localization, are major non-collagenous glycoproteins of the mammalian tectorial membrane (Legan et al. 1997). In addition, these two proteins are abundant constituents of the otoconial membranes, but are not present in the cupula (Goodyear and Richardson 2002; Xu et al. 2010). In the mouse vestibule, α-tectorin is mainly expressed between E12.5 and P15 in the transitional zone, as well as in a region that is producing the accessory membranes of the utricle and saccule, but absent in the ampullae of semicircular canals (Rau et al. 1999). Mice with targeted deletion of α-tectorin display reduced otoconial membranes and a few scattered giant otoconia (Legan et al. 2000).

β-tectorin has a spatial and temporal expression pattern distinct from that of α-tectorin in the vestibule. It is expressed in the striolar region of the utricule and saccule from E14.5 until at least P150 (Legan et al. 1997; Rau et al. 1999), suggesting that the striolar and extrastriolar region of the otoconial membranes may have different composition. *Tectb* null mice show structural disruption of the tectorial membrane and hearing loss at low frequencies (Russell et al. 2007). However, no vestibular defects have been reported.

Interestingly, both otogelin and α-tectorin possess several von Willebrand factor type D (VWFD) domains containing the multimerization consensus site CGLC (Mayadas and Wagner 1992). This structural feature is probably essential for the multimer assembly of those proteins to form filament and higher order structures.

Otoancorin is a glycosylphosphatidylinositol (GPI)-anchored protein specific to the interface between the sensory epithelia and their overlying acellular membranes of the inner ear (Zwaenepoel et al. 2002). In the vestibule, otoancorin is expressed on the apical surface of the supporting cells in the utricle, saccule and crista. Although the function of otoancorin has not been elucidated, the C-terminal GPI anchor motif of this protein likely facilitates the otoancorin-cell surface adhesion. It is proposed that otoancorin may interact with the other components of the otoconial membranes, such as otogelin and tectorins, and with the epithelial surface, thus mediating the attachment of otoconial membranes to the underlying sensory epithelia (Zwaenepoel et al. 2002).

5. Summary and future direction

Like other biominerals such as bone and teeth, otoconia primarily differ from their non-biological counterparts by their protein-mediated nucleation, growth and maintenance processes. With only $CaCO_3$ crystallites and less than a dozen glycoprotein/proteoglycan components, otoconia are seemingly simple biological structures compared to other tissues. Yet, the processes governing otoconia formation are multiple and involve many more molecules and much complicated cellular and extracellular events including matrix assembly, endolymph homeostasis and proper function of ion channels/pumps. Expression of the involved genes is well orchestrated temporally and spatially, and the functions of their proteins are finely coordinated for optimal crystal formation. Some of these proteins also play vital roles in normal cellular activities (e.g. hair cell stimulation) and other vestibular function. Some other proteins (e.g. otolin, tectorins and otoancorin) still need to be further investigated

of their functions. Animal models with targeted disruption of otolin and otoancorin are not yet available, and animal models with double mutant genes (e.g. Oc90 and Sc1) have not been studied but can yield more information on the precise role of the organic matrix in $CaCO_3$ nucleation and growth. Additional studies are needed to further uncover the mechanisms underlying the spatial specific formation of otoconia. The high prevalence and debilitating nature of otoconia-related dizziness/vertigo and balance disorders necessitate these types of studies as they are the foundation required to uncover the molecular etiology.

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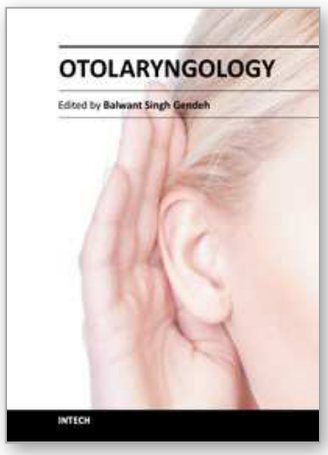

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This book emphasizes on different aspects of otolaryngology - the medical sciences of diagnosis and treatment of ENT disorders. "Otolaryngology" is divided into various clinical sub-specialities, namely otology, rhinology, laryngology, and head and neck. This book incorporates new developments, as well as future perspectives in otolaryngology. I would like to dedicate this book to those of you who will pick up the torch and by continued research, close clinical observation and the highest quality of clinical care, as well as by publication and selfless teaching, further advance knowledge in otolaryngology from this point forward. It is intended to be a guide to other books to follow. Otolaryngologists, researches, specialists, trainees, and general practitioners with interest in otolaryngology will find this book interesting and useful.

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