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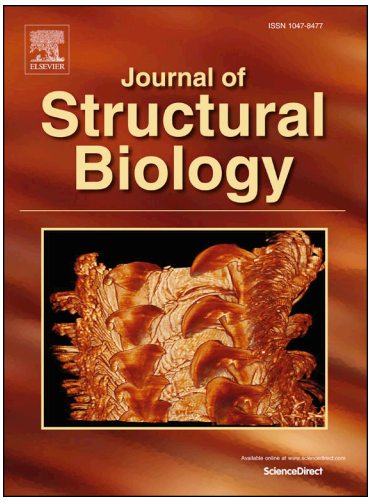

Nanostructure of mouse otoconia

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Nanostructure of mouse otoconia

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Abstract

Mammalian otoconia of the inner ear vestibular apparatus are calcium carbonate-containing mineralized structures critical in maintaining balance and detecting linear acceleration. The mineral phase of otoconia is calcite, which coherently diffracts X-rays much like a single-crystal. Otoconia contain osteopontin (OPN), a mineral-binding protein influencing mineralization processes in bones, teeth and avian eggshells, for example, and in pathologic mineral deposits. Here we describe mineral nanostructure and the distribution of OPN in mouse otoconia. Scanning electron microscopy and atomic force microscopy of intact and cleaved mouse otoconia revealed an internal nanostructure (~50 nm). Transmission electron microscopy and electron tomography of focused ion beam-prepared sections of otoconia confirmed this mineral nanostructure, and identified even smaller (~10 nm) nanograin dimensions. X-ray diffraction of mature otoconia (8-day-old mice) showed crystallite size in a similar range (73 nm and smaller). Raman and X-ray absorption spectroscopy – both methods being sensitive to the detection of crystalline and amorphous forms in the sample – showed no evidence of amorphous calcium carbonate in these mature otoconia. Scanning and transmission electron microscopy combined with colloidal-gold immunolabeling for OPN revealed that this protein was located at the surface of the otoconia, correlating with a site where surface nanostructure was observed. OPN addition to calcite growing *in vitro* produced similar surface nanostructure. Finally, these findings provide details on the composition and nanostructure of mammalian otoconia, and suggest that while OPN may influence surface rounding and surface nanostructure in otoconia, other incorporated proteins (also possibly including OPN) likely participate in creating internal nanostructure.

Keywords: Biomineralization, otoconia, osteopontin, calcite, nanostructure, tomography

1. Introduction

Biomineralization processes have been broadly studied for many mineralized tissues and structures (Lowenstam and Weiner, 1989). Otoconia (and otoliths in fish) are composites of calcium carbonate ($CaCO_3$) mineral and proteins found in the utricle and the saccule of the vestibular apparatus of vertebrate mammals, and in the lagena of nonmammalian vertebrates (Hiatt, 2014; Lundberg et al., 2015). The vestibular system of the inner ear is responsible for head movement detection, for spatial orientation, and for body balance (Hiatt, 2014; Marieb, 1991). In mammals, the vestibular system has three fluid-filled semicircular canals which respond to rotational acceleration, and two receptor organs – the utricle and the saccule – which sense linear acceleration and gravity (Hughes et al., 2006; Lundberg and Xu, 2012; Lundberg et al., 2015).

Otoconia are formed in mammals during the late embryonic stages, and they are considered stable until mid-life where signs of their degeneration can be detected in humans (Anniko et al., 1984). In mice – which share many similar otoconial features to humans – the initial seeding of otoconia is detected at embryonic day 14, with the highest rate of mineralization occurring at embryonic days 15 and 16 (Ornitz et al., 1998). Murine otoconia acquire their final size (a few micrometers) by postnatal day 7, and from there, in health, they generally are preserved throughout life with minimal changes/transitions (House and Honrubia, 2003; Ornitz et al., 1998). Otoconia, surrounded by a low-calcium solution termed the endolymph, are embedded in a membranous structure called the otoconial membrane, which rests on the extremities of hair cell kinocilia and stereocilia in the utricular and saccular sensory epithelium, regions known as the macula (Lundberg and Xu, 2012; Lundberg et al., 2015). With each head movement, the otoconia are displaced by their collective relative inertia as compared to the underlying cells on which they lie, leading to bending of the sensory hair bundles (Lundberg and Xu, 2012). This mechanical impetus

is transformed into electrical signals that are transmitted by the hair cells to the central nervous system of the brain, providing information on the spatial position of the head (Lundberg and Xu, 2012; Marieb, 1991).

Morphologically, mammalian otoconia are barrel-shaped calcitic structures bounded by well-defined rhombohedral facets at both ends, and they diffract as single crystals (Lundberg and Xu, 2012). They are unique in being the only calcium carbonate-containing biomineral found in normal healthy mammals (the main mineral phase of bones and teeth is a calcium phosphate, apatite). However, in some species, calcium phosphate otoconia can be found such as in the *Agnatha* species (jawless fish) where there is negligible or absent crystalline structure (Carlstrom, 1963), or in malformed human inner ears where a mixture of apatite and calcite has been reported (Johnsson et al., 1982). Otoconia can occur as any of the $CaCO_3$ polymorphs depending upon the organism within which they reside (Ross and Pote, 1984). In mammals and birds, the polymorph for otoconia is typically calcite, the most thermodynamically stable calcium carbonate polymorph existing under ambient conditions (Lins et al., 2000). In amphibians and fish, the predominant polymorph is aragonite (Carlstrom, 1963; Ross and Pote, 1984). Vaterite is found in primitive jawfish or in pathologic conditions in the human inner ear (Addadi et al., 2003; Johnsson et al., 1982; Ross and Pote, 1984; Wright et al., 1982). Specific matrix proteins have been shown to promote calcium carbonate polymorph selection in otoconia (Pote and Ross, 1991).

As in other biominerals, and important for the $CaCO_3$ formation in the inner ear, is the presence of an organic matrix consisting mainly of glycoproteins and proteoglycans (Fermin et al., 1995; Lundberg and Xu, 2012; Lundberg et al., 2015; Pote and Ross, 1993; Tachibana and Morioka, 1992). The presence of otoconial proteins is necessary for the formation of otoconia where they sequester and concentrate calcium ions from the endolymph fluid (Lundberg and Xu,

2012), likely amongst other important functions in guiding otoconial growth. Prominent proteins identified in mammalian otoconia are otoconin 90 (OC90), otolin-1 (or otolin), osteopontin (OPN), fetuin-A, SPARC-like protein 1 (SC1), secreted protein acidic and rich in cysteine (SPARC), dentin matrix protein 1 (DMP1) (Lundberg and Xu, 2012; Lundberg et al., 2015; Thalmann et al., 2006; Andrade et al. 2012) and α-tectorin (Xu et al., 2010). OC90 – the main soluble matrix protein of otoconia – modulates the form of calcite crystals *in vitro* (Lu et al., 2010). *In vivo*, in mice, in the absence of OC90, there is either an absence of otoconia, or only a few massive abnormal otoconia that are formed, with overall calcite amounts being decreased roughly by half (Andrade et al., 2012; Xu et al., 2010). Otolin – identified in bony fish (Murayama et al., 2005) – is a member of the collagen X family, and is found in both otoconia and the surrounding otoconial matrix where it might serve as a scaffold protein for biomineralization (Moreland et al., 2014; Yang et al., 2011). Other proteins such as fetuin-A, SPARC, OPN and DMP1, are considered as minor otoconins because of there being a negligible phenotype effect on otoconial formation and vestibular function in transgenic mice lacking these proteins (Xu et al., 2010; Zhao et al., 2007; Zhao et al., 2008; Andrade et al., 2012). Other candidates for mediating otoconial mineralization are the keratan sulfate proteoglycans (KSPGs) (Xu et al., 2010), these being extended biomolecules having strong negative charges for attracting calcium ions and appearing to interact with OC90 and otolin proteins (Lundberg and Xu, 2012).

OPN is a highly phosphorylated, mineral-binding protein having multiple roles in cell adhesion and protein binding in the extracellular matrix of bone and teeth, where the phosphoserine residues and the overall negative charge of this molecule derived from Asp and Glu residues appear to be important for regulating mineralization processes (Fisher et al., 2001; Sodek et al., 2000; Sorensen et al., 1995). OPN is expressed by the sensory hair cells, the nonsensory dark cells, and

the cells of the endolymph sac (Zhao et al., 2008). In recent work pertaining to OPN and biomineralization in another calcium carbonate structure – the avian eggshell – we have shown an association of OPN with the nanostructured texture of the calcitic shell, and we have reproduced similar internal nanostructure in calcite crystals grown *in vitro* in the presence of OPN (Athanasiadou et al., 2018).

Structural studies of mammalian otoconia are sparse, and those that exist mainly describe pathologic circumstances and developmental abnormalities (Johnsson et al., 1982; Wright et al., 1982). In terms of their ultrastructure, human otoconia are known to be composed of three sectors/branches at each end that extend outwards from the central so-called belly region, a site seemingly more susceptible to otoconial degradation (Walther et al., 2014). Despite having both rounded and faceted external morphology at the microscale suggesting perhaps nonclassical crystallization pathways (De Yoreo et al., 2015; Rodriguez-Navarro et al., 2016; Wolf et al., 2016), it remains possible that an internal polycrystalline, coherent nanostructure forms during their formation in the presence of mineral and calcium-binding proteins, as has been shown in numerous calcium-carbonate mineralizing systems, including in eggshells (Athanasiadou et al., 2018; Cuif et al., 2010; Tseng et al., 2014).

Morphological and compositional alterations of otoconia are frequently produced by head trauma, ototoxic drugs, aging, and environmental and genetic factors, and these can lead to balance-related disorders (Lundberg et al., 2015). Benign paroxysmal positional vertigo (BPPV) is a serious disease in which patients suffer from intense nausea and loss of balance (Salvinelli et al., 2004). BPPV occurs when otoconia are dislodged from their initial position and migrate into the semicircular canals (canalithiasis), or when otoconia exist in larger numbers than the active surface area of the utricular cells (utriculithiasis) (Oas, 2001). Aging-related otoconial

degeneration is a high-risk factor for creating free otoconial debris that results in loss of balance in elderly people, consequently leading to falls which frequently cause bone fractures and even death (Agrawal et al., 2009).

Because of the important physiologic functions effected by otoconia, and because otoconial pathologies lead to BPPV, accurate and detailed knowledge of the formation of otoconia and their fine structure is fundamental to understanding the function of the vestibular system. Given the limited understanding of otoconial formation and structure, and to better consider ways to influence their regrowth/regeneration to potentially treat their abnormalities, we describe here the internal structure of mouse otoconia at the nanoscale level. We also describe the localization of OPN at the ultrastructural level in these otoconia, and combine this with an examination of the *in vitro* effects of OPN on growing calcite morphology.

2. Materials and Methods

2.1 Harvesting and embedding of otoconia

Otoconia were processed by manual dissection under a stereomicroscope from C57BL/6 normal (wild-type) and *Opn*$^{-/-}$ (knockout) mice obtained from the Jackson Laboratory (Bar Harbor, ME, USA). All mice used were 8 days old (except for those used for the 2D XRD and EBSD analyses which were 3 months old, Sections 2.3 and 2.9 below). Animals were kept at 24°C in pathogen-free conditions using alternating 12-hour light and 12-hour dark cycles. Mice were fed normal mouse chow (2920X, Teklad global soy protein-free extruded rodent diet, Envigo, Huntingdon, UK), and had access to water *ad libitum*. Mice were sacrificed under isoflurane anesthesia by decapitation, and mouse heads were either fixed with aldehyde and then dissected to isolate the inner ear and dehydrated and embedded in Epon or LR White resin, or the heads were

transferred unfixed immediately into 100% ethanol to prevent any potential dissolution of otoconia from exposure to aqueous solutions. From the latter, otoconia were carefully retrieved through dissection under a stereoscope, and stored in 100% ethanol. Embedded samples were sectioned for histology and mounted on either glass slides for atomic force microscopy or onto grids for scanning electron microscopy (that were also used additionally for transmission electron microscopy). Animal procedures were evaluated and approved by the McGill University Institutional Animal Care and Use Committee following the guidelines of the Canadian Council on Animal Care.

2.2 Light microscopy of the vestibular inner ear system

For the histology of the inner ear vestibular system and for observation of otoconia *in situ*, 1-µm-thick sections from wild-type mouse otoconia were cut from polymerized blocks using a Leica Ultracut E Ultramicrotome (Leica, Wetzlar, Germany). These sections were von Kossa-stained for mineral (black reaction) followed by counter-staining with toluidine blue, with visualization and image recording using an optical microscope (model DMRBE, Leica) equipped with a 3-CCD Sony DXC-950 camera (Sony, Tokyo, Japan).

2.3 X-ray diffraction (XRD), Raman spectroscopy and X-ray absorption (XAS) of the mineral phase of mouse otoconia

The mineral phase of wild-type mouse otoconia was analyzed by powder and single-crystal X-ray diffraction (XRD), Raman spectroscopy and X-ray absorption spectroscopy (XAS). Powder XRD was run on a Bruker D8 Discover diffractometer equipped with a Cu X-ray tube (wavelength 0.154056 nm) and an area detector (GADDS 2D XRD).

For powder XRD, measurements were performed in coupled θ-θ scan mode (500 µm beam spot size) with samples of otoconia spotted as aggregates onto a quartz sample substrate. The determined 2θ scans were refined with software TOPAS v5.0 (Bruker, Germany) and calcite unit

cell parameters and crystallite size were determined. 2D XRD analyses were performed using a Bruker D8 Venture single crystal diffractometer equipped with a PHOTON area detector and a Cu X-ray microsource (200 μm beam size). Measurements were taken in the transmission mode on aggregates of otoconia (dissected from 3-month-old wild-type mice). Frames were recorded while the sample was rotating in φ angle within a 10° angular range using 0.3° steps and an integration time of 60 seconds.

Raman spectroscopy was performed using a Renishaw inVia Raman microscope (Renishaw, Gloucestershire, UK) equipped with a holographic spectrometer and a Leica DM2500 M optical microscope (Leica Microsystems GmbH, Wetzlar, Germany). The excitation source was a 514.5 nm Ar laser with a *ca*. 2 μm laser spot size and a 25 mW excitation power. The laser was focused through a 50× objective (numerical aperture 0.75) on a dense array of otoconia. For each measurement, Raman spectra were acquired for 10 seconds where 3 scans were accumulated for minimizing any noise effects. Spectral reproducibility was confirmed by taking several spot analyses. Measurements were done on 1-μm-thick sections of utricle cut with an ultramicrotome from Epon blocks containing sectioned otoconia and placed on glass coverslips.

XAS synchrotron data collection was performed at the Canadian Light Source (CLS) using the SXRMB beamline. Sections of resin-embedded otoconia cut at 1-μm thickness were placed onto Si wafers. The electron beam was run at 2.9 GeV with a beam current of approximately 200 mA. A set of 5 scans was averaged from three samples to achieve the best signal-to noise ratio. All spectra were obtained at the calcium K-edge and were normalized in reference to the positions of the intrinsic monochromator glitches and of the calcite edge spectrum.

2.4 Scanning electron microscopy (SEM)

To analyze by SEM both the outer surface of otoconia and the internal structure of microtome (diamond knife)-cleaved otoconia, isolated intact otoconia and resin-embedded otoconia were prepared. For external morphology examination, intact isolated whole otoconia were placed on aluminum SEM stubs, coated with a 2-nm thick Pt layer, and examined by SEM at an accelerating voltage of either 2 or 5 kV using an Inspect F-50 FE-SEM (FEI Company). Without sputter coating, ultramicrotome-cut 1-µm-thick microtome sections of otoconia were placed on conductive grids (also for future TEM use) where likewise imaged by SEM under the same conditions.

2.5 Atomic force microscopy (AFM)

To further investigate possible internal nanogranular structure of otoconia, and to correlate this with the nanoscale distribution of organic matrix in the same sample, atomic force microscopy (AFM) was conducted on 1-µm-thick, ultramicrotome-cut sections from wild-type mice. AFM height and amplitude images were taken using a Multimode Nanoscope IIIa atomic force microscope (Veeco, Santa Barbara, CA, USA) operating in the tapping mode in air at room temperature using a nonvertical engage E-scanner and NanoScope version 5.30 software (Veeco/Bruker-AXS Inc., Madison, WI, USA). In the AFM experiments, V-shaped tapping mode probes (typical tip apex radius of approximately 7 nm) with Si cantilevers having a spring constant k = 42 N/m (Bruker-AXS Inc.) were used. The tip force exerted on the surface was optimized by the amplitude set-point being as high as possible to reduce imaging artefacts. The Feret diameters of the units comprising the observed nanostructure were calculated using ImageJ software (1.x version) (Schneider et al., 2012). At least 200 Feret diameters of the otoconial nanostructure domains were calculated from the AFM images (obtained in amplitude mode) after performing the software's high-pass processing to enhance domain boundaries.

2.6 Focused-ion beam (FIB) sample preparation and transmission electron microscopy (TEM)

The TEM investigation was performed on a thin FIB section of an otoconium prepared by a dual-beam FIB-SEM system (FEI Helios 600 NanoLab, FEI, Hillsboro, OR, USA) equipped with a gallium ion source. For this, a nickel TEM grid with otoconia was placed on a flat aluminium SEM stub. A single otoconium was then identified and covered with a protective 2-µm-thick Pt layer from which a 2-µm-thick slab cut from the otoconium was milled/thinned with the FIB. Afterward this initial thinning, the section was transferred onto a Cu TEM half-grid using an EasyLift nanomanipulator for the final thinning at 5 kV and 9.4 nA, from which an 80-nm-thick section was obtained. Bright-field TEM images and selected-area electron diffraction (SAED) patterns were obtained using a Tecnai TF-20 (FEI) microscope operating at 200 kV.

2.7 Electron tomography

The 80-nm-thick FIB-cut section of an otoconium collected on a TEM copper grid was used to collect a series of single-axis tilt images at an accelerating voltage of 200 kV using a Tecnai G2 F20 cryo-S/TEM (FEI) equipped with a Gatan Ultrascan 4000 4k × 4k digital CCD camera system (model 895). Images were taken at a magnification of 62,000X over a tilt range from –40° to +50° for the samples (2° increments in both low tilts and high tilts). The resulting images had pixel sizes of 0.19 nm. For electron tomography in a scanning transmission electron microscope (STEM) (data shown in Supplementary Material), images were recorded using a ThermoFisher 300 kV Titan^3 Themis X-FEG S/TEM at a magnification of 62,000 over a tilt range from –50° to +70° (2° increments in low tilts and 1° high tilt step on the 80-nm-thick sections). The images from the tilt series were aligned, filtered and reconstructed into a tomogram using the IMOD software package (Kremer et al., 1996). The movies for the raw tilt series and reconstruction were done in

IMOD, whereas the movies with 3D volume with solid and surface rendering were generated using UCSF Chimera (version 1.10.1) software.

2.8 3D imaging and reconstruction

Three-dimensional imaging of intact utricular otoconia embedded in an LR White resin block was performed using a Zeiss Xradia 520 Versa (Carl Zeiss Canada Ltd). A series of X-ray frames (totalling 1989 projections) was collected over 360-degrees of rotation at 60 V with a pixel resolution of 0.5 µm. A reconstructed movie of the rotation series was generated using Dragonfly™ v3.6 software (Object Research Systems Inc., Montreal).

2.9 Electron backscatter diffraction (EBSD) and pole figures

Electron backscattering diffraction (EBSD) maps were collected for 20 h from FIB-cut sections of otoconia in the transmission mode using an Auriga Zeiss SEM instrument and a 0.3 µm step-size resolution. All EBSD data were collected and analyzed with AZtec 2.1 software (Oxford Instruments). Data was visualized as crystallographic orientation maps using pseudocolors to represent crystal orientations or pole figures.

2.10 Calcite crystal growth in the presence of OPN

Synthetic calcite crystals were grown *in vitro* in a 10 mM $CaCl_2$ solution with (or without) added full-length phosphorylated bovine milk OPN (0.15, 0.3, 0.45 and 0.9 µM) as provided by Arla Foods and prepared according to the procedure described by Sørensen and Petersen (Sørensen and Petersen, 1993). Calcite crystallization took place for 2 hours on round glass coverslips in small wells placed within a sealed desiccator previously charged with 1 g of $(NH_4)_2CO_3$ powder. For these crystallization experiments, a final volume of 1 ml was used for each solution, with or without protein. The incubation wells and the ammonium carbonate reservoir were not covered for

the total 2 h of the crystallization experiment. The size of the desiccator (Desiccator PC 250 mm/Thermo Scientific) was 33 cm in height and 25 cm in inner diameter. Glass coverslips with calcite crystals were gently removed from solution, and lightly rinsed with distilled water and ethanol, and then, air-dried and placed in a desiccator for storage until further characterization. Triplicates were used for all the crystallization experiments.

2.11 Immunogold labeling for OPN

For immunogold OPN labeling of otoconia *in situ* in normal wild-type mice and OPN-deficient mice (negative control), TEM grid-mounted microtome-cut sections 80 nm in thickness, as well as the respective polymerized block faces themselves from which the sections were cut, were used to localize OPN at the EM level. Otoconia-containing thin sections and block faces were incubated with anti-mouse OPN antibody (R&D Systems Oakville, Canada) for 1 h followed by washing and incubation with protein A-colloidal gold complex (14-nm gold particles, from G. Posthuma, University of Utrecht) for 1 h to detect the immunolabeling reactions. For the sections, conventional staining with uranyl acetate and lead citrate was performed after the immunolabeling protocol. The microtome-cut sections were visualised by TEM as described above, and the block faces were visualized by SEM.

3.1 Results

3.2 Optical microscopy and scanning electron microscopy (SEM) – external morphology of otoconia

Figure 1a shows by light microscopy an overview of the location of otoconia in the mouse inner ear from a von Kossa and toluidine blue-stained section of the utricle from a wild-type mouse. Otoconia stained black for mineral (arrow) rest upon a gelatinous mass – the otoconial

membrane – overlying the hair cells of the utricular macula. Figures 1b and 1c show by SEM the external morphology of wild-type mouse otoconia at low and high magnification, respectively, while Movie S1 (Supp. Material) shows the 3D organization of otoconia in the utricle as determined from microcomputed X-ray tomography. The size of the majority of the otoconia generally ranged between 5 and 8 µm, with some smaller otoconia being approximately 2 µm in length being present (Fig. 1b). Higher magnification of the otoconial surface reveals an external nanogranular structure on the barrel-shaped body (Fig. 1c inset), whereas the tri-planar faceted surfaces appear smooth (Fig. 1c).

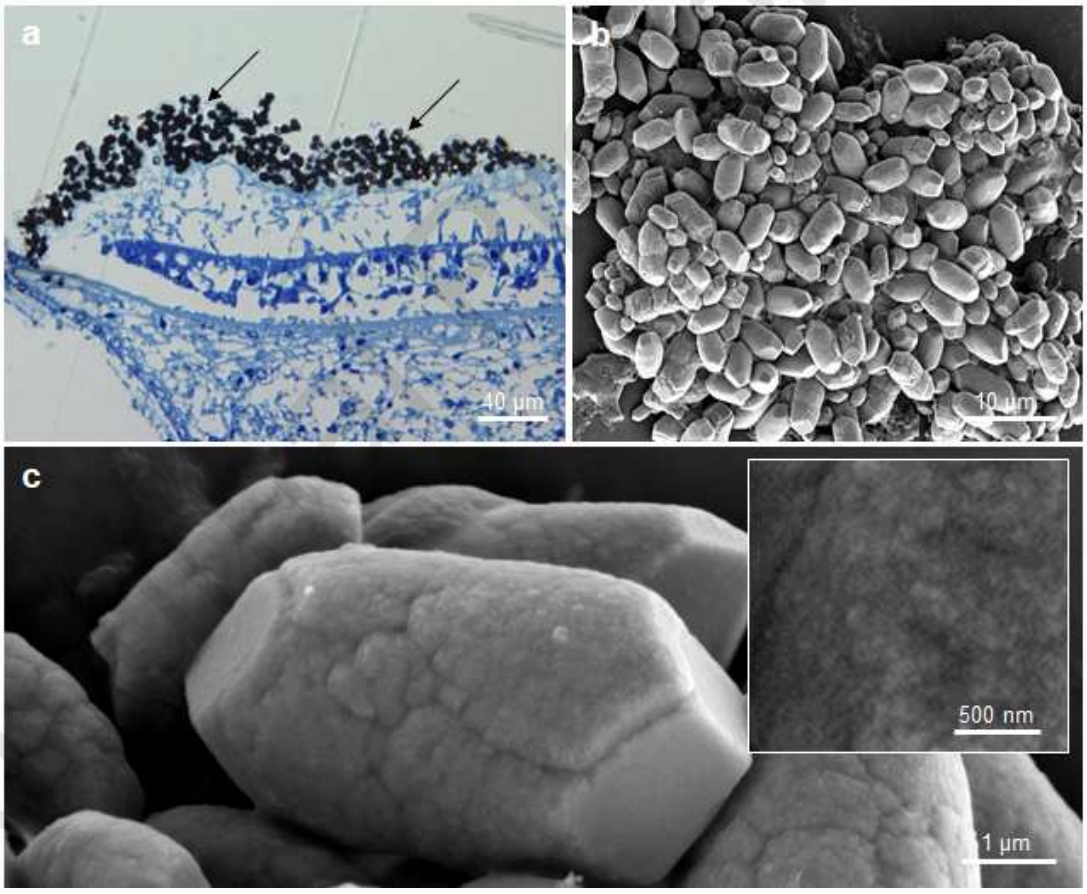

Figure 1. External morphology of wild-type mouse otoconia. (a) Histological staining of a section of the mouse utricle observed by light microscopy after von Kossa staining for mineralized otoconia (black, arrows). (b) Low-magnification SEM image of otoconia. (c) High-magnification SEM image showing details of mature otoconia having surface nanostructure (inset).

3.3 XRD, Raman spectroscopy and XAS – identification of $CaCO_3$ polymorphs and crystallinity of otoconia

As has been observed previously (Pote and Ross, 1986), mouse otoconia are crystallized in the form of calcite. XRD patterns from a cluster of many agglomerated otoconia revealed characteristic peaks corresponding only to calcite (Fig. 2a). The calculated unit cell parameters for otoconia calcite (a = 4.977 Å; c =17.029 Å) are very close to geological calcite (4.990 Å; 17.061 Å) though slightly smaller. The contraction of the unit cell can be explained by a limited substitution of Ca by Mg (2.0%) (Pokroy et al., 2006). Crystallite size, calculated from the major 104 calcite peak was 73 nm, and smaller when all peaks were considered.

Similar to the XRD results, Raman spectra revealed characteristic peaks in the region between 100-1200 cm^{-1} attributable to vibrations arising from the calcite polymorph (Fig. 2b). Particularly demonstrating this, the bands 281 cm^{-1} and 155 cm^{-1} are characteristically attributable to the vibrations of the complete unit cell, referred to as lattice modes. Other bands above 400 cm^{-1} are attributable to the internal modes of the carbonate ions, the symmetric stretching at 1085 cm^{-1} and the in-plane bending at 711 cm^{-1} (Wehrmeister et al., 2011). No evidence of amorphous calcium carbonate was observed from mature otoconia from 8-day-old mice, an age where otoconia are essentially fully formed and have stopped growing. It remains possible that amorphous calcium carbonate is present at earlier stages of otoconial development, which we did not study. The pre-edge peak at 4035.8 eV, corresponding to the electronic transition 1s-3d being characteristic for ACC samples (Politi et al., 2006), was absent from the Ca K-edge XAS spectra of the otoconia (Fig. 2c), and only crystalline forms of Ca (calcite) was present.

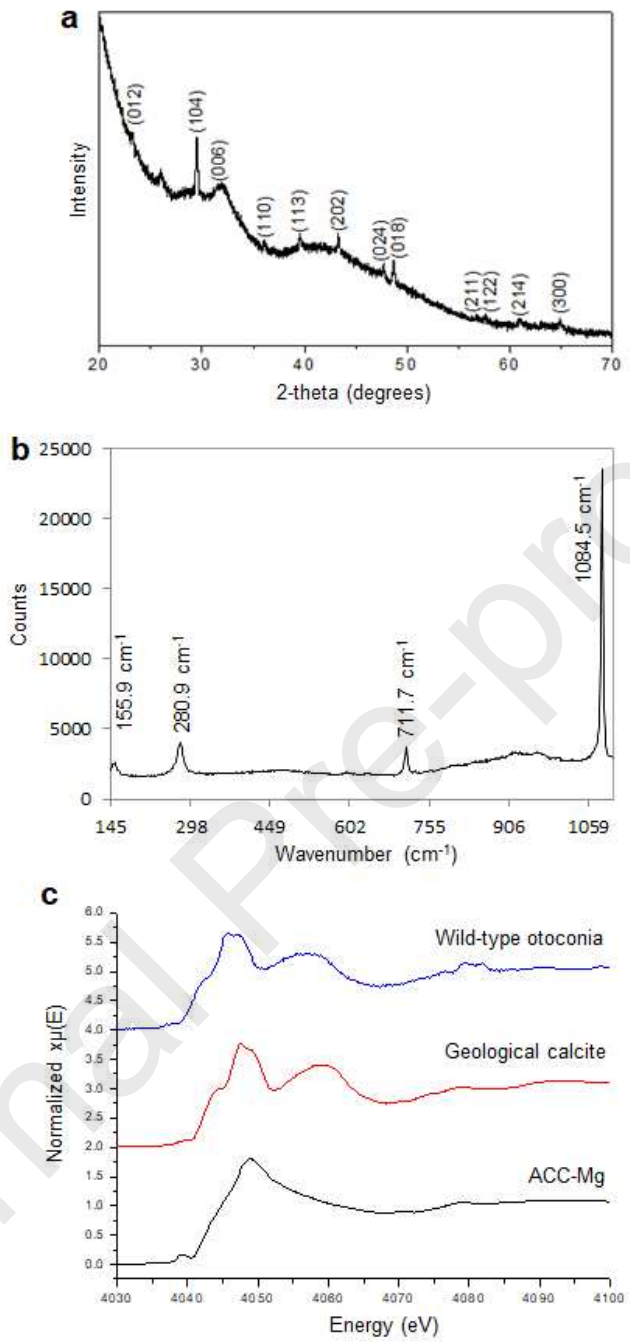

Figure 2. Calcitic mouse otoconia. (a) XRD pattern showing calcite as the mineral phase in mouse otoconia indicating the predominant crystallographic plane reflections. (b) Raman spectrum obtained from a single otoconium. The Raman spectrum was compared with spectra obtained from a Raman database (http://rruff.info/). (c) XAS spectra of wild-type mouse otoconia showing peaks characteristic for calcite. The spectrum was compared with XAS spectra of geological calcite and ACC-Mg obtained under the same experimental imaging conditions.

3.4 Internal structure of otoconia

Figure 3a shows an SEM image of an uncoated, ultramicrotome-cut single otoconium revealing ubiquitous nanostructure throughout its interior. To further analyze and confirm this nanogranular structure, ultramicrotome-cut sections were also examined by AFM. AFM height (Fig. 3b) and amplitude (Fig. 3c) analysis revealed an aligned (inset) otoconial nanogranular structure consisting of about 50 ± 14 nm (SD) in Feret diameter. Phase-mode AFM images, as shown in Figure 3d, revealed delineating margins of different composition at the boundaries of the nanostructure (arrows). Since there was no evidence of ACC in our samples according to the Raman and XAS spectroscopy data, at this mouse age (8-day-old) having mature otoconia, these phase variations are attributed to the presence of organic content delineating the nanostructure, although some potential minor contribution may come from surface topography.

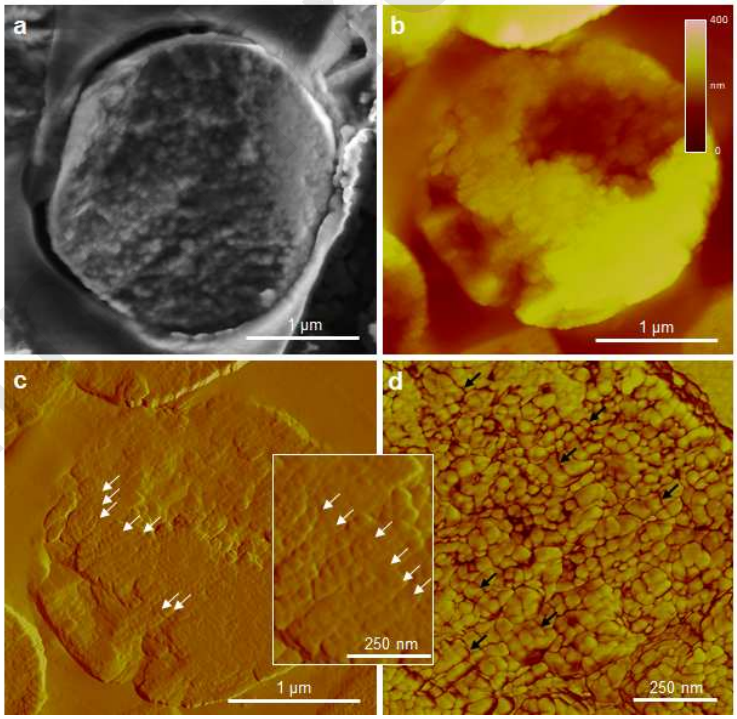

Figure 3. Interior nanostructure of wild-type mouse otoconia. (a) SEM image of the interior of an otoconia from an uncoated otoconium. AFM image in (b) height and (c) amplitude mode showing internal otoconial nanostructure. (d) AFM phase-mode image showing linear arrays of inorganic calcitic nanogranules (yellow, white arrows) presumably surrounded by organic material (red, black arrows). Otoconia were cut/fractured open using a microtome-mounted diamond knife.

As examined by TEM, an even smaller nanostructure size of approximately 10 nm in diameter was detected as seen by higher magnification lattice imaging (Fig. 4a). SAED of these regions showed predominantly single-crystal alignment (Fig. 4a, inset), demonstrating a highly coherent alignment of the nanostructured subunits. Electron tomography 3D reconstructions of mouse otoconia (Figs. 4b-d, and Supp. Material Movies S2, S3) showed abundant and homogeneously dispersed 10-nm-sized nanodomains together with the larger nanostructure initially observed and measured by AFM (Figs. 3b-d). STEM bright and high-angle annular dark-field imaging (HAADF) confirmed the nanostructure of otoconia as shown in Figures S1a,b.

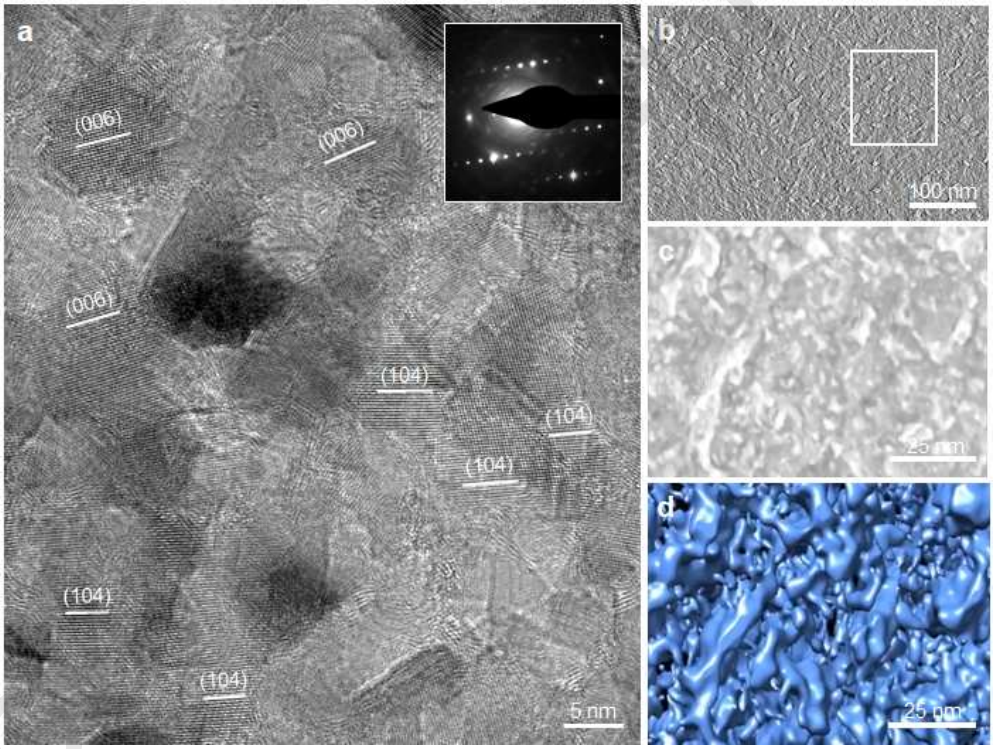

Figure 4. Nanostructure of otoconia by TEM after FIB sectioning. (a) High-resolution TEM lattice imaging of nanodomains, with SAED indicating predominantly single crystal alignment. (b) Bright-field TEM image from a tilt series of a nanostructured branch region. (c, d) Three-dimensional tomographic reconstructions of the nanodomain branch region indicated by the boxed area in (b) [solid and surface rendering in (c) and (d), respectively].

Single-crystal 2D XRD of otoconia showed calcite diffraction rings with rounded spots that were similar to those produced by ground geological calcite crystal (optical-quality Iceland spar)

which indicate that aggregates of otoconia are generally randomly oriented with respect to each other, and that the crystalline phase of individual otoconia behaves like highly pure calcite single crystals (Figs. 5a,b). Despite this single crystal behaviour in diffraction, we observed by SEM, AFM and electron tomography, an internal granular nanostructure similar to that observed in other calcium carbonate biomineralization systems. Thus, the nanograins forming the otoconia calcite crystals must have a nearly perfect coherent orientation as seen in the SAED patterns (Fig. 4a, inset). These nanograin results are supported by the data in the 001 pole figures and their contour plots obtained after whole-otoconia EBSD data analysis where the angular spread values for geological calcite and otoconia are quite low and quite similar (0.58 deg and 0.47 degrees, respectively) (Figs. 5c-f).

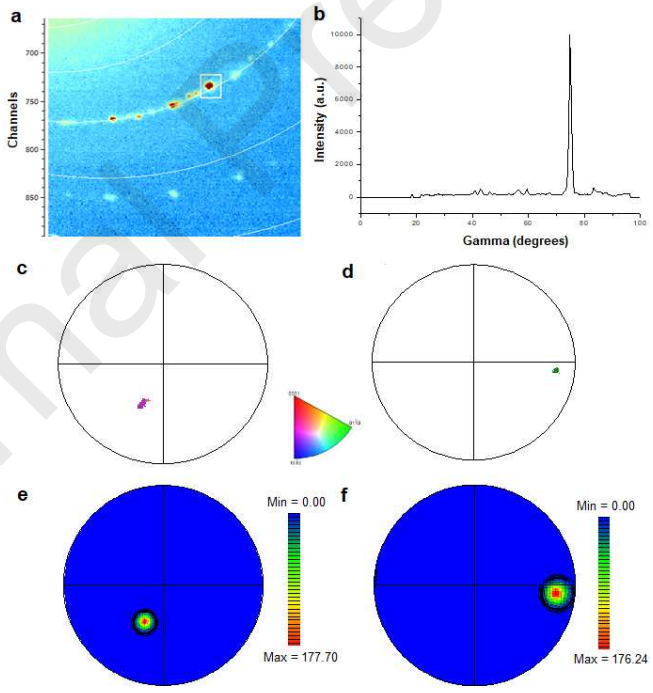

Figure 5. 2D XRD and EBSD analysis of otoconia. (a) 2D X-ray diffraction pattern produced by otoconia showing single-crystal diffraction spots (white box). (b) Intensity profile along the 104 calcite ring as a function of the γ angle. (c, d) 001 pole figures showing almost identical scattering of the *c*-axis within geological calcite and otoconia, respectively left and right panels. (e, f) Contour plots of the same data shown in c and d, respectively.

3.5 Immunogold labeling for OPN in mouse otoconia

The colloidal-gold immunolabeling imaging approach was used to provide a means for high-resolution localization and mapping of proteins in mineralized tissues by electron microscopy (McKee and Nanci, 1995). Furthermore, if samples can be cut with microtome, postembedding immunogold labeling allows protein localization within the interior of biomineralized specimens. Here, we were able to detect OPN in otoconia by both TEM and SEM in wild-type and *Opn*$^{-/-}$ mice, the latter being used as a negative control where the transgenic mice have been designed to lack entirely OPN in all their tissues as induced by a gene ablation approach.

OPN at the surface of the otoconia was readily detected, with Figures 6a-d showing TEM and SEM immunogold labeling for OPN as indicated in wild-type mice. As expected, no labeling was observed in negative-control mice lacking OPN (*Opn*$^{-/-}$) (Figs. 6e-h). A limiting difficulty with this approach is that the aqueous procedures necessary to do antibody labeling and other protocol steps particularly for relatively soluble calcite-containing samples, produces essentially a total decalcification of the otoconia and release of mineral-bound proteins into the solution (indeed, this is how otoconial proteins can be extracted for biochemical analyses). In the present study, this aqueous dissolution unfortunately resulted in voids in the prepared samples (all panels of Fig. 6) which precluded potential labeling of proteins in the interior of the otoconia, but allowed OPN localization at the surface of the otoconia where OPN molecules are trapped locally at the surface by the infiltrated embedding resin and are not released by the dissolution of the mineral.

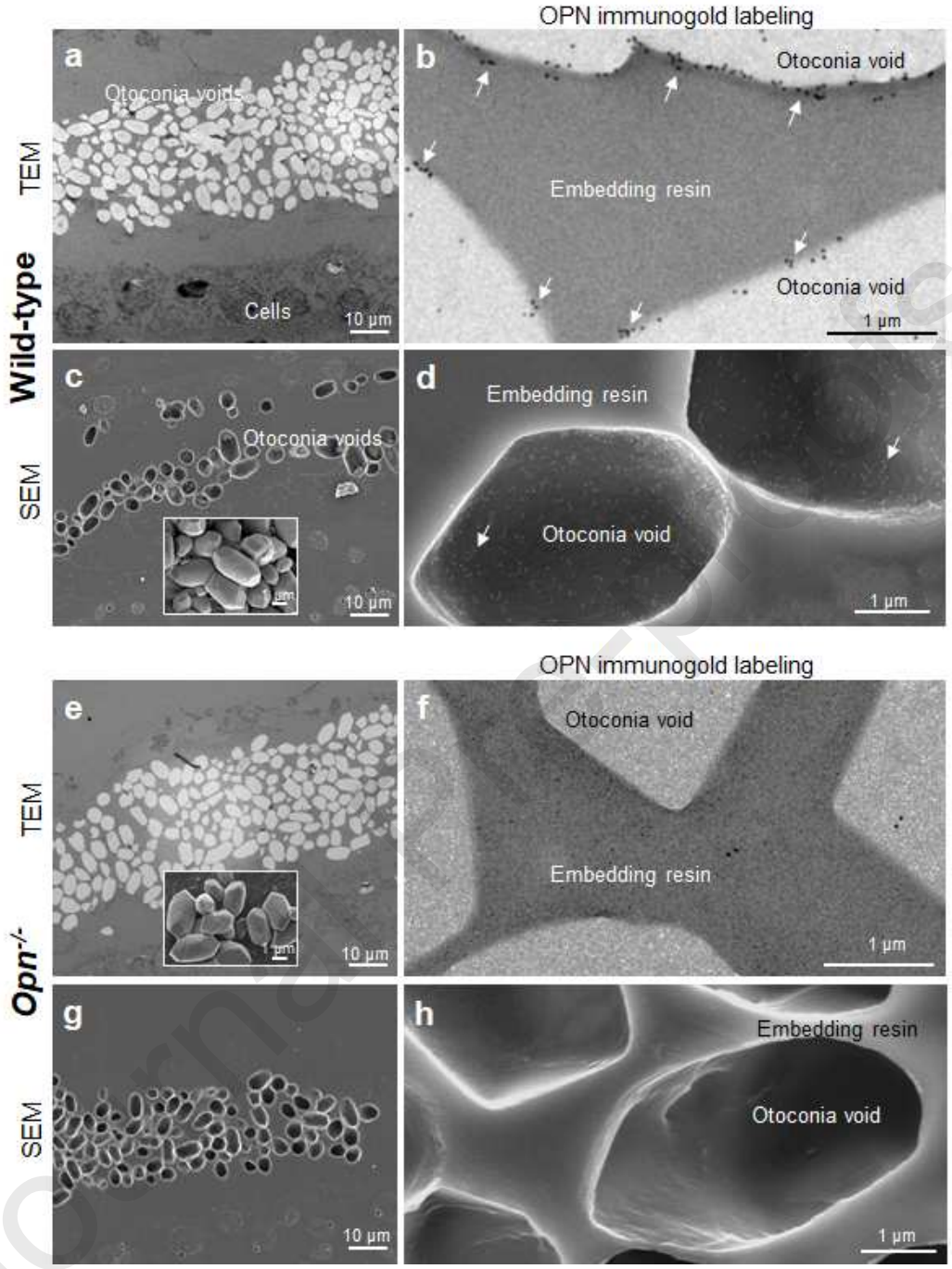

Figure 6. Immunogold labeling of OPN in otoconia. (a, c) In wild-type mice, otoconial voids shown by TEM and SEM, respectively, after immunolabeling for OPN (aqueous procedures dissolve the calcitic otoconia). Inset: Intact wild-type mouse otoconia (without aqueous exposure) shown by SEM. By TEM (b) and SEM (d), immunogold labeling for OPN shows gold particles (arrows) at the surface of otoconial voids. (e, g) In OPN-deficient mice, otoconial voids shown by TEM and SEM, respectively, after immunolabeling for OPN. Inset: Intact otoconia from OPN-deficient mice (without aqueous exposure) shown by SEM. By TEM (f) and SEM (h), as expected in this negative control, immunogold labeling for OPN was absent.

3.6 Effect of OPN on calcite crystal growth *in vitro*

To investigate a possible contribution of OPN protein to the production of nanostructure at the surface of otoconia, we examined the effects of OPN on calcite crystal growth *in vitro* as shown in Figure 6. At a low concentration of OPN (0.15 µM), calcite crystals retained their {104} rhombohedral morphology (Fig. 7a), but after increasing the OPN concentration to 0.3 µM and 0.45 µM, morphological changes became more evident to produce altered rounded growth step edges (Figs. 7b,c, respectively). At the highest OPN concentration used (0.9 µM), calcite crystals start developing aggregates of {104} rhombohedra (Fig. 6d), all with rough surfaces and apparent surface nanostructure similar to that seen at the surface of otoconia (Fig. 1c).

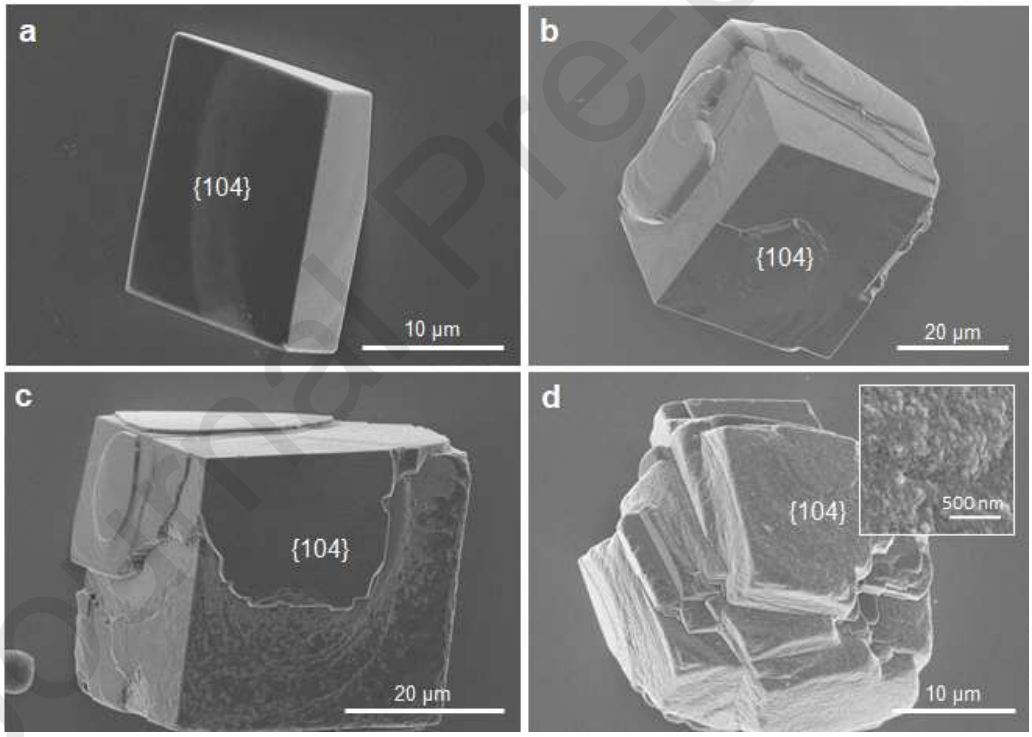

Figure 7. Effect of full-length purified OPN protein on calcite growth *in vitro*. Increasing concentration of OPN (a) 0.15 µM, (b) 0.3 µM, (c) 0.45 µM and (d) 0.9 µM gradually alters the external morphology of synthetically grown calcite crystals, producing evident surface nanostructure (inset).

4. Discussion

The structural and functional relationship between the organic matrix and the mineral component of various biomineralized tissues and structures – including otoconia – is a topic of considerable interest in the field of biomineralization. In recent years, reports on amorphous mineral phase precursors and mineral nanostructure have increasingly provided new insights into mechanistic processes for biomineralization at the molecular and atomic level. In biomineralized tissues and structures, mineral nanostructure has emerged as a common theme shared by many organisms where nanostructured morphology and mechanical properties can be attributed to the presence of incorporated organic matrix biomolecules (Wolf et al., 2016).

For calcitic mammalian otoconia – where in humans there exist major recognized debilitating health consequences linked to otoconial abnormalities and displacement – little is known about the structure of otoconia at the nanoscale. Using high-resolution, nanostructure-determining 2D and 3D characterization techniques, here we describe that there is an internal calcitic nanostructure in mouse otoconia consisting of domains ranging around approximately 50 nm in size with interwoven organic material surrounding individual inorganic calcitic nanostructures.

AFM in the tapping mode and using the phase function can discriminate between inorganic and organic phases within complex biomineral structures, being highly sensitive to sample inhomogeneity and compositional variations (Mass et al., 2014). Using this approach to study the interior of otoconia exposed by various means, we observed that the mineral phase consisted of closely packed and highly aligned nanogranules throughout the otoconial interior, a structural arrangement consistent with other descriptions of similar features in numerous calcareous

biominerals such as, for example, the sea urchin spicule (Seto et al., 2012), mature tablets of nacre (Hovden et al., 2015), and chicken and guinea fowl eggshells (Athanasiadou et al., 2018; Perez-Huerta and Dauphin, 2016). Commonly it seems in these cases, the nanogranules are aligned and surrounded by a fine organic matrix, or by a combination of organics and amorphous calcium carbonate (ACC) (Cuif et al., 2010). Notably, most observations on such biomineralized tissues/structures have revealed a nanostructure similar to what we report here for mammalian otoconia, this being roughly spheroidal fields of nanogranules ranging in size from between 50 to 100 nm (Athanasiadou et al., 2018; Perez-Huerta and Dauphin, 2016). Such results are consistent with there being a nonclassical crystallization pathway to develop this highly aligned nanostructure that deviates from the classical crystallization pathway (Rodriguez-Navarro et al., 2016; Wolf et al., 2016; Yang et al., 2011, Yang et al., 2011), but additional work under cryo-conditions is needed to confirm this. Previous model studies have indicated that organic additives, *e.g*. acidic biomacromolecules, could favor a nonclassical aggregation-based crystal growth mechanism by stabilizing ACC particles (Cölfen and Antonietti, 2008; Wolf et al., 2016). In the present study in 8-day-old mice having mature otoconia, ACC was not detected in the otoconia from the Ca-K edge XAS and Raman spectroscopy, but this could be attributed to its loss during sample preparation, or to the relatively mature state of the examined otoconia, where ACC could in fact be present at earlier prenatal or perinatal developmental stages of mouse otoconial development.

The smallest nanodomain size that we observed in the otoconia was approximately 10 nm, as observed by HRTEM. Differences in these dimensions could be related to regional variations in organic content (likely type or amount of proteins) acting as inhibitors/regulators of mineralization as has been proposed for the calcitic nanostructure of chicken eggshell *G. gallus domesticus* (Athanasiadou et al., 2018). Previously, *in vitro* crystal growth work by others has

shown that OC90, fetuin-A and OPN act as inhibitors of calcite crystal growth, primarily attributable to their having high-binding affinity to calcium (Hong et al., 2015). These results are in agreement with our own calcite crystal growth experiments in which the presence of OPN modifies the typical rhombohedral shape of calcite in a concentration-dependent manner. With increasing OPN concentration, the {104} crystal faces of calcite have abundant growth islands and rounded step edges, and evident surface nanostructure, disclosing changes in the growth mechanism of these crystal faces attributable to the added OPN. Even at higher OPN concentrations, the synthetic calcite crystals tend to create aggregates with rough surfaces of slightly mismatched {104} rhombohedra. Although this reflects surface activity, we previously have shown that beyond the surface, additional incorporation of OPN occurs into the crystal interior to generate internal nanostructure (Athanasiadou et al., 2018).

In previous *in vitro* calcite growth experiments by others, it was shown that the addition of either OC90 or otolin protein alone could not produce the external shape of mature otoconia, but when both proteins were added in combination, they could to some degree produce calcitic structures that resembled otoconial morphology (Moreland et al., 2014). Similar to this, calcite-gelatin composites grown by a double-diffusion method in gelatin gel matrices revealed similar morphological features to mammalian otoconia (having branch and belly regions), but these were much larger in size (Huang et al., 2008). Focused-ion beam-cut sections of these artificial composites demonstrated a dense crystalline branch region and a poorly crystalline belly region (Simon et al., 2011).

Occluded OPN into synthetic calcite crystals can induce a nanostructure similar to the nanostructure observed in calcitic chicken eggshell, where higher OPN concentration creates smaller nanostructure and increases hardness (Athanasiadou et al., 2018). Our finding showing

that OPN is concentrated at the surface of the otoconia as detected by high-resolution, postembedding immunogold labeling is novel at the ultrastructural level, and is consistent with previous studies using conventional immunohistochemistry and light microscopy (Sakagami, 2000; Takemura et al., 1994). However, because otoconial calcite is readily soluble under aqueous conditions, dissolution of the mineral rapidly occurs during the required aqueous immunolabeling incubation and washing steps, and we were not able to associate OPN with interior structure (only with the surface region where the embedding medium retained resident components). Thus, while likely affecting surface mineralization of the otoconia, it remains possible that OPN is also present within the interior of otoconia and contributes to the nanostructure that we have observed here, and which can be reproduced *in vitro* using OPN. Indeed, otoconins such as OC90 could likewise participate in this nanostructuring process. OPN is co-expressed locally in the inner ear with OC90 by the vestibular dark cells and in the endolymphatic sac (Ignatova et al., 2004; Sakagami, 2000; Verpy et al., 1999), meaning that OC90 and OPN could have similar functions in some regard, even partially compensate for each other during otoconial formation. In particular, OC90 has a strong effect on otoconial formation; OC90-knockout mice have rod-like large calcitic aggregates that are susceptible to dissolution (Zhao et al., 2007). In contrast, in OPN-null mice where Oc90 levels are presumably not altered, there is normal balance behavior and normal otoconial morphology (Zhao et al., 2008). Since OPN has now been localized to the very periphery of otoconia in mice (our work) and in rats (Takemura et al., 1994), and given its role in creating mineral nanotexture as we have recently shown for calcite crystals in the chicken eggshell, OPN could indeed be a protein involved in controlling surface growth and nanostructure in otoconia. However, the interplay between OPN and OC90, if any, along with interactions amongst other otoconial proteins in this biomineralization process, remain to be determined.

5. Conclusions

In summary, the internal structure of mouse otoconia at the nanoscale has been determined. Within the interior of otoconia, using a combination of advanced imaging techniques, we show that otoconia consist of densely packed and highly aligned calcite nanodomains likely surrounded by organic material, with OPN being present at the very surface of the otoconia. The surface localization of OPN suggests involvement in the growth process and/or termination of otoconial growth via the mineralization-inhibiting activity of this protein on crystal growth. This study provides detailed findings on the internal and external structure of otoconia, information that may lead to a better understanding of what goes wrong with otoconia in diseases such as vertigo.

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Highlights

- Calcitic mouse otoconia have a nanostructure ranging in tens of nanometres
- Diffracting as single crystals, the nanostructure of otoconia is coherently aligned
- The 3D nanostructure of otoconia is determined by electron tomography
- Immunogold labeling shows mineral-binding osteopontin at the surface of otoconia