3D imaging of auditory osteoblasts using a Talbot phase-sensitive X-ray tomographic microscope

In many mammals, hearing-related bones of the middle and inner ear are among the densest bones. Three auditory ossicles in the middle ear—namely the malleus, incus, and stapes—for a chain that transmits vibration from the tympanic membrane to the inner ear. The cochlea and semicircular canals, the delicate organs of the inner ear, are surrounded by a highly calcified bony labyrinth. Density of the ossicles and the bony labyrinth is functionally important, as insufficient mineralization seen in conditions such as osteogenesis imperfecta, cleidocranial dysplasia, or X-linked hypophosphatemia can lead to hearing loss.

During development, bone formation by osteoblasts occurs via either endochondral or intramembranous ossification. In intramembranous ossification, bone develops directly from sheets of osteoblasts, while endochondral ossification requires a cartilaginous template (anlage). Hearing-related bones mainly undergo endochondral ossification, like long bones. Osteoblasts produce matrix vesicles containing calcium and phosphate ions, which are crystallized into biological apatite, the major inorganic component of bone material. Bone mineralization is initiated by nucleation and growth of apatite crystals in fibrillar collagen secreted by osteoblasts. Orientation and arrangement of collagen fibrils and biological apatite have been analyzed using various imaging techniques, all of which confirm that collagen fibrils are the major determinant of bone matrix mineralization. We previously identified “osteogenic capillaries” composed of endothelial cells and pericytic osteoblasts during endochondral ossification of mouse ear bones [2]. However, cellular mechanisms underlying formation of dense ear bones by osteoblasts during development remain largely unknown.

In this study [1], we found that in young mice, hearing-related bones are highly mineralized immediately after they are produced (Figs. 1(a) and 1(b)) and that pericytic osteoblasts, a structural element of osteogenic capillary, in hearing-related bones produce both type I and type II collagens as bone matrix, while conventional osteoblasts and chondrocytes primarily produce type I and type II collagens, respectively (Fig. 1(c)). Type II collagen-producing pericytic osteoblasts were genuine osteoblasts as they express osteocalcin, localize along alizarin-labeled osteoid, and form osteocyte lacunae and canaliculi, as do conventional osteoblasts. Analysis using quantitative backscattered electron imaging (qBEI) revealed that calcium concentration (weight%) in newly formed bone was much higher in ear bones (malleus) than in femur in the same mouse at the same time after new bone formation (Fig. 1(d)). Furthermore, ear bones exhibit not only greater mineral content, but better arranged collagen fibrils than that found in conventional osteoblasts (Fig. 1(e)). Based on these results, we conclude that organic and inorganic components of hypermineralized ear bones exhibit properties distinct from conventional bones and that these type II collagen-producing hypermineralizing osteoblasts (termed auditory osteoblasts) represent a new osteoblast subtype [1].

Vascular networks also develop during endochondral ossification of conventional bone, whereas osteogenic capillaries associated with hypermineralizing osteoblasts are unique to ear bones. To define cellular structure

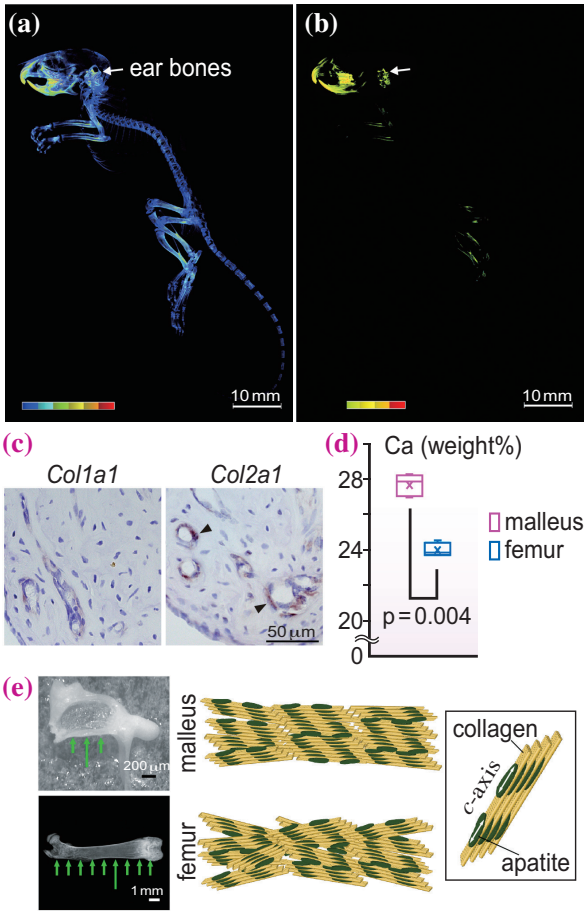

Fig. 1. Identified features of ear bones. **(a)** Heat map of tissue mineral density (TMD) (100–1500 mg/cm^3) in a microCT image of a P21 mouse. **(b)** High TMD regions (500–1500 mg/cm^3) of (a). **(c)** *In situ* hybridization of *Col1a1* and *Col2a1* in the malleus of P21 mouse. Arrowheads: *Col2a1*-positive cells. **(d)** Calcium concentration (Ca weight%) in malleus and femur of P21 mice. **(e)** Left: Representative images of malleus and femur showing points (green arrows) assessed using a microbeam X-ray diffraction system. Right: Schematic diagram constructed based on that analysis showing alignment of the apatite *c*-axis on collagen fibrils in malleus and femur. [1]

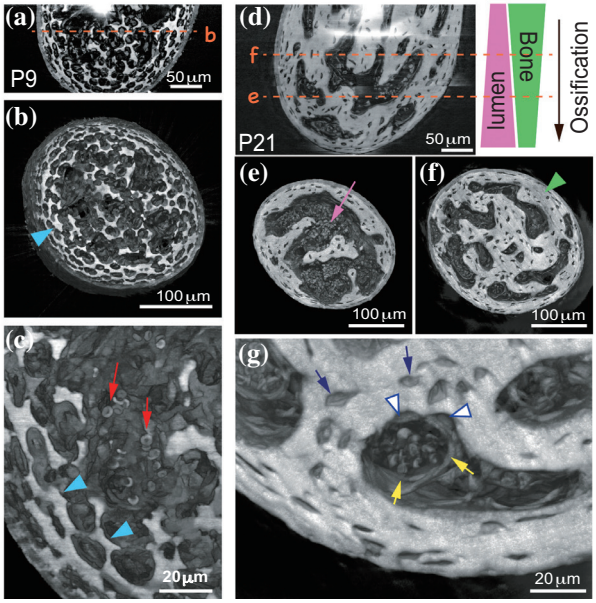

Fig. 2. Synchrotron X-ray phase images of osteogenic capillaries during endochondral ossification. **(a)** Mineralized lattice-like cartilaginous matrix in the malleus of P9 mice. Vertical section. Dotted line **b** indicates the horizontal cutting plane shown in (b). **(b)** Horizontal section showing partially resorbed cartilaginous matrix (arrowhead) at P9. **(c)** Magnified view of (a) showing capillary invasion. Arrows: red blood cells; arrowheads: cartilaginous matrix. **(d)** Mineralized bone matrix in the malleus of P21 mice. Vertical section. Dotted lines **e** and **f** indicate horizontal cutting planes shown in (e) and (f), respectively. **(e)** Horizontal section near the tip of the malleus, showing enlarged osteogenic capillary lumen (arrow) at P21. **(f)** Horizontal section of the malleus, showing osteogenic capillaries with narrower lumens surrounded by increased regions of bone matrix (arrowhead) as compared to those seen in (e). **(g)** Magnified view of osteogenic capillaries in (f). Yellow arrows: endothelial cells; open arrowheads: auditory osteoblasts; blue arrows: osteocytes. Cell types were identified by morphology and proximity to red blood cells. [1]

of osteogenic capillaries accompanied by auditory osteoblasts within hard tissues, we performed Talbot phase-sensitive X-ray tomographic microscope imaging [3] at SPring-8 **BL37XU** using a monochromatic X-ray beam (9.0 keV). At the initial stage of endochondral ossification of the malleus (postnatal day 9, P9), mineralized lattice-like cartilaginous matrix was partially resorbed (Figs. 2(a) and 2(b)). Higher magnification revealed that capillary vessels, which contained red blood cells, had invaded cartilaginous matrix (Fig. 2(c)). Based on analysis of the ossification progress, the entire malleus had been replaced by bone matrix by P21 (Fig. 2(d)) and osteogenic capillaries narrowed as bone rapidly formed (Figs. 2(e) and 2(f)). We also observed that auditory osteoblasts adjacent to endothelial cells were in contact with bone matrix and that osteocytes were embedded in bone matrix around osteogenic capillaries (Fig. 2(g)). Such close association of auditory osteoblasts with endothelial cells in osteogenic capillaries likely supports efficient bone matrix production and mineralization during and after endochondral ossification of hearing-related bones.

In summary, we found that ear bones exhibit a higher tissue mineral density (TMD) as well as higher degree of apatite orientation (reflecting collagen fibril orientation) than do long bones (Fig. 3). The higher the TMD and anisotropic arrangement of apatite crystallites, the more rapid the speed of sound in a bone [4]. These data collectively suggest that hypermineralization of hearing-related bones likely optimizes the material properties required for conductive and sensorineural hearing function. Overall, our study reveals that auditory osteoblasts, a new osteoblast subtype, form the densest hearing-related bones using collagen types distinct from those found in conventional bone.

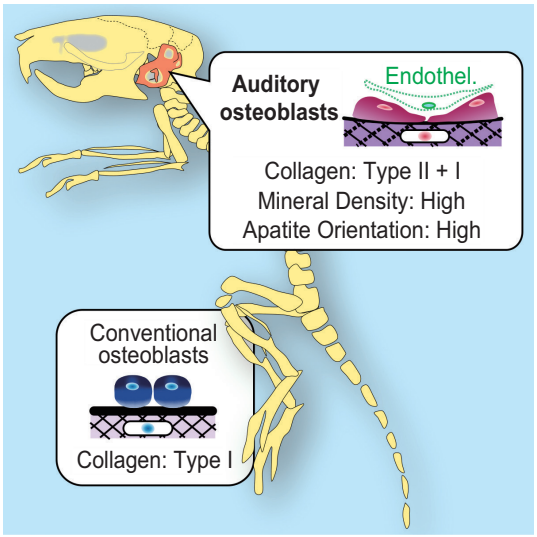

Fig. 3. Summary of properties of auditory osteoblasts. Endothel, endothelial cell. [1]

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References

[1] *Y. Kuroda, K. Kawaai, N. Hatano, Y. Wu, H. Takano, A. Momose, T. Ishimoto, T. Nakano, P. Roschger, S. Blouin, K. Matsuo: JBMR.* ***36*** *(2021) 1535.*
[2] K. Matsuo *et al.*: Development **142** (2015) 3912.
[3] A. Momose *et al.*: Proc SPIE **10391** (2017) 103910Y.
[4] T. Ishimoto *et al.*: Bone **127** (2019) 82.