Phase-contrast X-ray imaging of auditory ossicles in osteopetrotic mice

The three smallest bones of the middle ear, the malleus (hammer), incus (anvil) and stapes (stirrup), may be highly suitable for high-resolution structural analysis using synchrotron radiation. Figure 1 shows conventional microCT images of the auditory ossicles of an adult mouse. The malleus (green) attaches to the tympanic membrane (ear drum) (blue) and transmits sound through the incus (yellow) and stapes (red) to the cochlea in the inner ear (not shown). Using phase contrast X-ray microscopy, Kanzaki *et al.* revealed the internal structure of the semi-spherical protrusion of the malleus called the processus brevis (short process, mb in Fig. 1) for both wild-type and osteopetrotic (*stone-like bones*) mice [1] (Fig. 2). Osteopetrotic mice lack bone resorbing cells called osteoclasts. Osteopetrotic mice lack osteoclasts due to disruption of the gene encoding the transcription factor c-Fos, which is essential for osteoclast formation [2,3].

Most mammalian bones including long bones, vertebrae and auditory ossicles are formed through a process called endochondral (*inside* the *cartilage*) ossification. During development, chondrocytes form cartilage "models" of future bones. Chondrocytes within cartilage terminally differentiate into hypertrophic chondrocytes, which are often paired. Hypertrophic chondrocytes produce the vascular endothelial growth factor (VEGF) to stimulate angiogenesis (new blood vessel formation). In response to the VEGF, endothelial cells begin to invade cartilage to form capillary walls. Concomitantly, both osteoclasts, which resorb cartilage/bone, and osteoblasts, which produce bone matrix, enter into cartilage and replace it with bone, which contains proteins such as collagen fibers and crystals of a calcium phosphate mineral called hydroxyapatite. One question in the field has been whether and how endochondral ossification occurs in the absence of osteoclasts.

More than other bones, the malleal processus brevis of the mouse may be highly advantageous for high resolution analysis of endochondral ossification. It is small in size, which is a prerequisite for high resolution imaging, which requires a narrow field of view. The "diameter" of the processus brevis is approximately 300 μm, which is small enough to fit without processing into the field of view of the X-ray microscope used for analysis. It is also round, unlike femurs, tibias and other long bones in which chondrocytes proliferate and differentiate in columnar organization. By contrast, the malleal processus brevis forms a semi-spherical protrusion that does not grow lengthwise. Thus, its analysis could provide insight into endochondral ossification that is not limited to the growth plate. Finally, auditory ossicles are not buried in soft-tissue or muscle but are sustained largely in the air, making them easy to isolate. In particular, the malleus is easily identifiable at the inner surface of the tympanic membrane.

To visualize auditory ossicles, a synchrotron X-ray phase contrast microscope was constructed with an X-ray Fresnel zone plate (ATN/FZP-S86/416; NTT-AT, Tokyo) and gratings using 9.0 keV

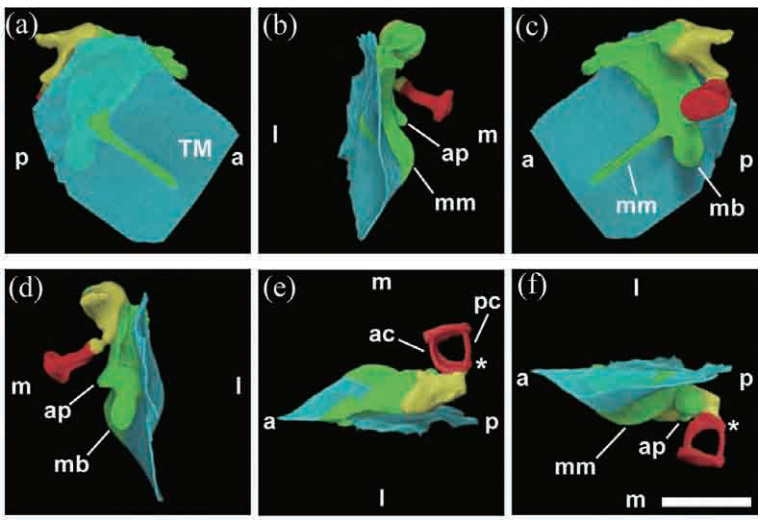

Fig. 1. MicroCT images of right auditory ossicles of an adult mouse. **(a)** lateral [l], **(b)** anterior [a], **(c)** medial [m], **(d)** posterior [p], **(e)** top, and **(f)** bottom views. TM, tympanic membrane; ap, anterior process; mb, malleal processus brevis; mm, malleal manubrium; ac, anterior crus; pc, posterior crus; *, a tubercle for stapedius muscle insertion. Scale bar = 1 mm. [1]

monochromatic X-rays at beamline **BL20XU** [4]. The resulting optical magnification was 20.2. In addition to phase contrast imaging on-focus, we also performed "defocus" imaging in the absence of gratings to enhance edges of osteocyte lacunae and chondrocytes by shifting samples 4 mm downstream from the on-focus position. Reconstructed tomographic images had an effective voxel size of 0.22 μm and the field of view was approximately 300 μm in diameter.

Kanzaki *et al.* compared the processus brevis structure in wild-type and c-Fos null osteopetrotic mice [1]. In adult wild-type mice, the malleal processus brevis contained osteocyte lacunae [1] (Fig. 2(a)) and blood vessels, indicating that endochondral ossification was complete. Curiously, in c-Fos null osteopetrotic mice the malleal processes brevis was filled with chondrocytes and lacked blood vessel invasion (Fig. 2(b)). Persistent chondrocytes and a lack of blood vessels have also been seen in other mouse models of osteopetrosis, which lack RANKL or TRAF6 [1]. Therefore, without osteoclasts, endochondral ossification is impaired and blood vessel invasion is blocked. Why blood vessels fail to invade the malleal processus brevis in the absence of osteoclasts is currently unclear. One possibility is that osteoclasts precede and lead blood vessel invasion. Alternatively, osteoclasts might stimulate hypertrophic chondrocytes to secrete VEGF, which induces angiogenesis. It is worth noting that, at the metaphyseal side of growth plates of long bones in osteopetrotic mice, numerous blood vessels invade cartilage presumably by secreting metalloproteinases such as metalloproteinase-9. Further three-dimensional and biochemical analyses will be necessary to analyze roles of osteoclasts in endochondral ossification.

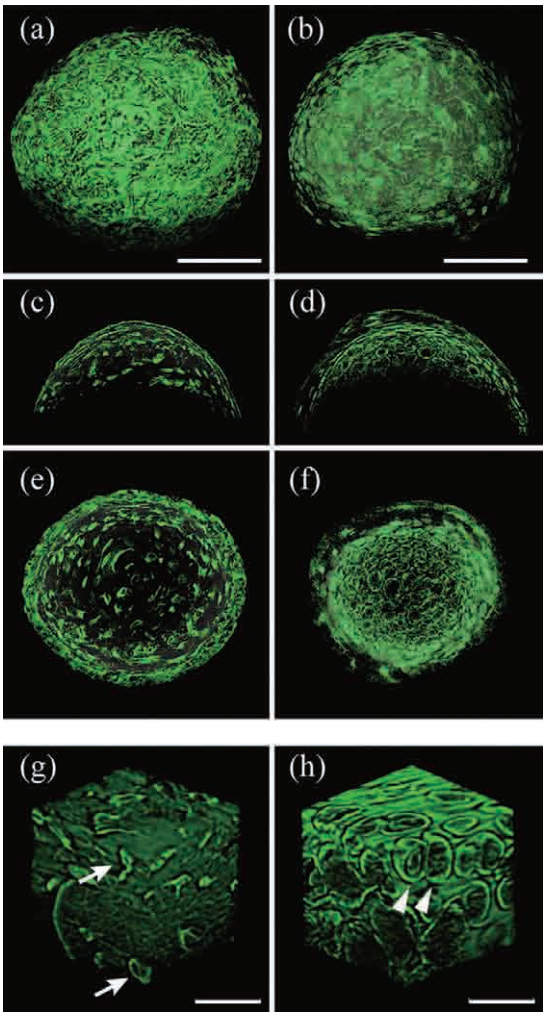

Fig. 2. Synchrotron tomographic microscopic images of the malleal processus brevis with defocus phase contrast. **(a, b)** 3D images of the processus brevis. **(c, d)** vertical sections. **(e, f)** horizontal sections. Scale bar = 100 μm. **(g, h)** Internal 30-μm cubes generated *in silico*. Scale bar = 20 μm. **(a, c, e, g)** wild-type adult mice. **(b, d, f, h)** adult osteopetrotic mice lacking c-Fos. Arrows indicate osteocyte lacunae in the bone matrix, while arrowheads indicate paired chondrocytes. Voxel size was 0.22 μm. [1]

Kanzaki *et al.* also found that mice made osteopetrotic either by c-Fos or RANKL knockout, exhibit impaired hearing based on the auditory brainstem response, which revealed that a higher threshold (louder sound) was required to generate brain-waves at the brain stem level [1]. Persistent chondrocytes seen in osteopetrosis may contribute to inefficient sound conductance. Surprisingly, Kanzaki *et al.* found that the size of auditory ossicles was larger and the middle ear cavity containing the ossicles was smaller than that of wild-type mice, causing direct contact between malleus and the middle ear cavity wall and apparently decreasing ossicle vibration. Therefore, osteoclasts are required for both endochondral ossification inside cartilage/bone and sculpting of the outer surface of bones.

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References

[1] *S. Kanzaki, Y. Takada, S. Niida, Y. Takeda, N. Udagawa, K. Ogawa, N. Nango, A. Momose and K. Matsuo: Am. J. Pathol.* ***178*** *(2011) 1270.*
[2] Z.Q. Wang *et al.*: Nature **360** (1992) 741.
[3] K. Matsuo *et al.*: Nat. Gent. **24** (2000) 184.
[4] Y. Takeda *et al.*: Appl. Phys. Exp. **1** (2008) 117002.