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A novel auditory ossicles membrane and the development of conductive hearing loss in *Dmp1*-null mice

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Abstract

Genetic mouse models are widely used for understanding human diseases but we know much less about the anatomical structure of the auditory ossicles in the mouse than we do about human ossicles. Furthermore, current studies have mainly focused on disease conditions such as osteomalacia and rickets in patients with hypophosphatemia rickets, although the reason that these patients develop late-onset hearing loss is unknown. In this study, we first analyzed *Dmp1* lac Z knock-in auditory ossicles (in which the blue reporter is used to trace DMP1 expression in osteocytes) using X-gal staining and discovered a novel bony membrane surrounding the mouse malleus. This finding was further confirmed by 3-D micro-CT, X-ray, and alizarin red stained images. We speculate that this unique structure amplifies and facilitates sound wave transmissions in two ways: increasing the contact surface between the eardrum and malleus and accelerating the sound transmission due to its mineral content. Next, we documented a progressive deterioration in the *Dmp1*-null auditory ossicle structures using multiple imaging techniques. The auditory brainstem response test demonstrated a conductive hearing loss in the adult *Dmp1*-null mice. This finding may help to explain in part why patients with *DMP1* mutations develop late-onset hearing loss, and supports the critical role of DMP1 in maintaining the integrity of the auditory ossicles and its bony membrane.

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Keywords

auditory ossicles membrane; hypophosphatemia rickets; hearing loss; DMP1

INTRODUCTION

Sound signals are detected by hair cells in the cochlea. The distortion of the hair cells via fluid waves generates electrical signals, and finally transduces them to the brain through nerve impulses. The type of motion or attitude detected by a hair cell is based on the signal waves associated with mechanical structures, such as the auditory ossicles and the spiral cochlea. Therefore, any abnormal morphological changes of these bony structures may contribute to hearing problems. Hearing loss, also known as hearing impairment, is defined as the partial or total inability to hear. A collaborative study conducted in 2013 demonstrated that age-related and other types of hearing loss have affected about 1.1 billion people to some degree over their lifetime worldwide [1]. Currently, most studies of these problems have focused on the degenerative loss of neuronal function. Hearing loss usually results in loneliness and disability. With the increasing availability of advanced hearing devices, many deaf people do not have to live in a world of muted indistinct sounds. However, in most types of hearing loss, “normal” hearing cannot be restored [2].

Because of the genetic and pathophysiological similarities of mice to humans, genetically-altered mice have become a powerful model system for bone research. Consequently, the success at producing different transgenic mouse lines to study human diseases has greatly facilitated our understanding of normal and abnormal bone function. However, we know much less about the mouse auditory ossicles compared to what we know about human ossicles in anatomical structure. Furthermore, there exists no detailed comparison of the similarities and differences between mouse and human auditory ossicles in structure and configuration.

Dentin matrix protein 1 (DMP1) was initially identified in dentin and later found to be highly expressed in bone, in which DMP1 controls phosphate homeostasis and mineralization [3, 4]. Interestingly, DMP1 is also expressed in soft tissues (salivary gland, kidney, lung, brain) or tumors, suggesting a role for DMP1 beyond maintaining mineral homeostasis [5–10]. The deletion of murine *Dmp1* has little impact on early development but causes striking defects in tooth and bone during postnatal development [11, 12]. Importantly, we and others have demonstrated that DMP1 mutations result in Autosomal Recessive Hypophosphatemic Rickets in humans, similar to the phenotype of *Dmp1* knockout (KO) mice. This condition is characterized by the presence of large amounts of osteoid in bone (osteomalacia) and is accompanied by elevated circulating fibroblast growth factor 23 (FGF23) [13–16]. Hearing impairment occurs in almost all adult patients with familial X-linked hypophosphatemic rickets (XLH), compared to a minimal incidence of hearing loss in XLH children [17–21]. However, the cause for this difference is largely unknown. Similarly, children with DMP1 mutations showed no apparent defects in hearing but progressively lost their hearing in late life [22].

In this study, we first identified a novel bony structure in the mouse between the malleus and the incus, analogous to a non-calcified membrane in the human middle ear. This unique bony structure is directly attached to the eardrum and dramatically increases the contact surface for sound transduction. Next, we showed a progressive defect of both the bony membrane and other auditory ossicles in the *Dmp1* KO mice, leading to a conductive hearing loss. Since no DMP1 expression signal is detected in the hearing hair cells and neuron cells, hearing loss in *Dmp1* KO mice is likely caused by the bony defects in the middle ear, which greatly delay and reduce sound transmission. The current mouse data, in agreement with the clinical case reports, support the critical role of DMP1 in maintaining the integrity of the auditory ossicles and its bony membrane.

Materials and Methods

Mice

In this study, we used *Dmp1*-lacZ knock-in null mice, in which the lacZ reporter gene was inserted to replace the endogenous *Dmp1* gene to provide a powerful tool to study the effects of the loss of *Dmp1* on hearing. At the same time, the expression of the lacZ reporter gene in the heterozygous (HET) mouse was used to trace the endogenous *Dmp1* gene expression pattern [23]. These mice were initially generated using 129 strains of mice and then backcrossed into the C57/B6 background with more than 5 generation crossing. Both male and female mice with 4–6 animals a group were used in the studies. All mice were fed autoclaved Purina rodent chow (5010, Ralston Purina) containing 0.9% calcium, 0.67% phosphorus and 4.4 international units (IU) of vitamin D/gram. The high Pi diet contains 2% phosphorus, 1.1% calcium and 2.2 IU/g vitamin D. The animal protocols were approved by the Institutional Animal Care and Use Committee.

Human auditory ossicles

Human auditory ossicles were dissected from an unknown adult cadaver in the anatomy lab at TX A&M College of Dentistry. Studies of this cadaver were exempted from the College IRB committee.

Histology and Immunohistochemistry

Histological analyses of specimens were performed in paraffin-embedded preparations as previously described [12]. Five μ m sections were cut and used for immunohistochemical staining. Polyclonal antibodies against type-one collagen (COL I), Biglycan, Decorin, and Osteocalcin were obtained from Dr. Larry Fisher (NIDCR, National Institutes of Health) and employed in immunohistochemical staining as previously described [11]. Polyclonal antibodies against DMP1 were obtained from Dr. Chunlin Qin (Texas A&M College of Dentistry) and used as previously described [24].

β -Galactosidase Expression Assay

β -galactosidase staining from a 3-week-old mouse was assessed according to the method described previously [25]. Briefly, the entire head including the brain and the skull was briefly fixed with ice-cold 4% PFA for 30 min before staining overnight in freshly made X-Gal solution (1 mg/mL) at 37°C. The sample was then re-fixed, decalcified, embedded in

wax, sectioned, and counterstained to visualize β -galactosidase activity under the microscope.

PCR genotyping

Genotyping was performed by PCR analysis of genomic DNA extracted from the tail using primers p01 (5'-CTTGACTTCAGGCAAA TAGTGACC-3') and p02 (5'-GCGGAA TTCGATAGCTTGGCTG-3') for the detection of the null *Dmp1* allele (280 bp) and primers p01 (5'-CTTGACTT CAGGCAAATAGTGACC-3') and p03 (5'-CTGTTCCCTCACTCTCACTGTCC-3') for the detection of the wild-type allele (410 bp). The primers p04 (5'-CAGCCGTTCTGAGGAAGACAGTG-3' from *Dmp1* cDNA) and p05 (5'-TGTCCTCAACTCATCAATGTATCT-3', from SV40 poly A) were used to detect the transgene for Col1-*Dmp1*, giving rise to a ~400-bp fragment.

Radiography and Micro-CT

To obtain high-resolution radiographs, both human and mouse auditory ossicles were dissected under a stereomicroscope and then X-rayed on a Faxitron model MX-20 Specimen Radiography System with a digital camera attached (Faxitron X-ray Corp., Buffalo Grove, IL). The entire wild type temporal bone was scanned to obtain Micro-CT images that gauge the integrity of auditory ossicles and provide an overall orientation and configuration of auditory ossicles in the site. Furthermore, the auditory ossicles from the control, *Dmp1* null and the rescued mice were dissected and scanned using a micro-CT imaging system (35, Scanco Medical, Bassersdorf, Switzerland) and analyzed as previously described [26].

Backscattered scanning electron microscopy (SEM)

The dissected auditory ossicles were fixed immediately in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1M cacodylate buffer solution (pH 7.4) at room temperature for 4 h and then transferred to 0.1 M cacodylate buffer solution. The tissue specimens were dehydrated in ascending concentrations of ethanol (from 70% to 100%), embedded in methyl-methacrylate (MMA, Buehler, Lake Bluff, IL) and then the surfaces were polished using 1 μ m and 0.3 μ m alumina alpha micropolish II solution (Buehler) on a soft cloth rotating wheel. The bony surface was acid-etched with 37% phosphoric acid for 2–10 s, followed by 5% sodium hypochlorite for 5 min. The samples were then coated with gold and palladium as described previously and examined using an FEI/Philips XL30 Field emission environmental scanning electron microscope (Hillsboro, OR) [27].

Auditory brainstem response (ABR) tests

The 4.5 month old mice were anesthetized with xylazine (4 mg/kg) and ketamine (40 mg/kg) (i.m.) prior to all procedures [28]. These animals were placed in a sound-isolated, electrically shielded booth, and needle electrodes were subcutaneously placed below the tested ear (reference electrode), in the vertex (active electrode), and below the contralateral ear (ground electrode). The sound stimulus consisted of 15 ms tone bursts (rise-fall time 1 ms) at 2, 4, 8, and 16 kHz, and were generated by Tucker-Davis hardware. The sound stimuli were specifically delivered into the ear canal from an encased, shielded Etymotic ER2 earphone through a 13 mm tube. Response waveforms (100,000 gain, filtered from 0.3–3.0

kHz) were averaged (1024 epochs) using a Tucker-Davis data acquisition system. The response threshold was defined as the “interpolated value between the last level at which no response was observed.” The sound delivery system was calibrated with a 1/4 inch ACO Pacific condenser microphone (Belmont, USA) in a volume approximating the mouse external ear canal and expressed as dB SPL.

RESULTS

Sharp differences of auditory ossicles between human and mouse specimens in the anatomical structure

For better comparison of mouse and human data, we first documented the gross structure and organization of three auditory ossicles in humans (Fig. 1a), in which there are two non-calcified membranes surrounding the malleus and stapes with the malleus head upward in the photograph taken under the dissection microscope (left) and in radiographic (right) images. Next, we examined the gross structure and organization of 14-day-old mouse auditory ossicles using multiple imaging techniques (Fig. 1b–d). The photographic image revealed a massive contact between the eardrum and malleus/incus bones (1b). Of note, the malleus head does not articulate with the incus as it does in humans (Fig. 1a). The x-gal-stained (blue) image obtained from the hetero lacZ knockin (which replaces the endogenous *Dmp1* gene) skull displayed blue colored ossicles and a bony membrane with an approximately two-cell-layer thickness, in which numerous blue osteocytes (Ocys) were revealed (1c). The bony membrane structure was further confirmed by the 3-D uCT image (1d). Furthermore, we analyzed the thickness of this bony membrane at ages of 14 days, 4.5 months and 6-months and showed no apparent difference, indicating that the bone membrane remains thin during the early and adult mouse lifespan. Together, these data revealed a novel bony membrane structure and a different configuration of the rodent ossicles compared to the human auditory ossicles (see Supplementary Fig. 1 for a side by side comparison diagram), as well as a high expression level of DMP1 in the auditory ossicles (indicating the potential role of DMP1 in auditory ossicles).

Deformity of auditory ossicles in *Dmp1*-null mice

To define the impact of *Dmp1* deletion, we first stained the 14-day-old ossicles using alizarin red, which showed a more intense red color and a bumpier structure under the stereomicroscope (Fig. 2a, *right panel*) compared to the control animal (2a, *left panel*). The radiographic image revealed a more porous bony structure with a less intensity of minerals in the KO mouse (2b, *right panel*). The uCT image demonstrated a reduced mineral content in the porous KO bony membrane (2c, *right panel*) compared to the smooth bony membrane in the control (2c, *left panel*). Furthermore, the x-gal stained KO ossicles displayed an uneven blue color, indicating a structural change (2d, *right panel*). By the age of 4.5-months, the overall structure of the ossicles had greatly enlarged with an uneven bony structure in the KO visible under the stereo microscope (Fig. 2e, *right panel*). The radiographic images further confirmed the abnormal change in the KO ossicle structure (Fig. 2f; *right panel*). An uneven distribution of minerals in the KO stapes was also demonstrated by the backscattered SEM image (Supplementary Fig. 2, *right panel*). In summary, the data indicated a progressive change in the structure of the KO auditory ossicles, which became worse with

aging. The poorly formed auditory ossicles and bony membrane in *Dmp1*KO mice may have affected the conversion efficiency of sound waves to motion in the ossicles.

A sharp change in extracellular matrix (ECM) proteins in the KO auditory ossicles

To understand what causes the bony structural changes in the *Dmp1* KO ossicles, we first examined the poorly formed ECM in the KO malleus using H&E staining (Fig. 3a). The immunostained images displayed no apparent changes in the expressions of COL I (Fig 3b) and osteocalcin (Fig 3c) in the KO mice. However, there was a great increase in biglycan (Fig 3d) and decorin (Fig 3e) compared to the controls, reflecting a sign of mineralization defects. Similar changes occurred in the KO incus (Supplementary Fig 2). These changes suggest that DMP1 is required for ECM protein maturation and mineralization in the auditory ossicles, which is consistent with the overall skeletal osteomalacia phenotype and the changes in ECM protein expression patterns in the DMP1null mice [29–31].

Moderate hearing loss in *Dmp1* KO mice

To further investigate if these changes in the KO auditory ossicles impair hearing, auditory brainstem response (ABR) analyses, a neurological test of auditory brainstem function in response to auditory stimuli, were carried out in both the adult control and the KO mice (4.5-month-old). Compared to the age-matched controls, the KO mice did not respond to weak intensity sound stimuli (<45 dB SPL at 16 kHz and <55 dB SPL at 8 kHz separately; Fig. 4a). Furthermore, there was an approximately 0.5 msec delay for the same stimulus intensity from 40 to 100 dB SPL, separately. Next, we tested the threshold change in three different frequencies (4000 Hz, 8000 Hz and 16000 Hz), which showed a much higher threshold in the KO mice at all three frequencies (Fig 4b). Lastly, we tested their response wave curves at different stimuli and observed moderate changes in the KO mice compared to the control animals (Fig 4c). Taken together, the data described above demonstrated a moderate conduction hearing defect in the KO mice (as reflected by the parallel shift in latency relative to the control group).

Re-expression of DMP1 or restoration of Pi levels in KO mice rescued auditory ossicles phenotype

Previously, we showed that the re-expression of DMP1 in bone cells using the 3.6 Col 1 promoter fully rescues the *Dmp1* KO phenotype [29–31]. We also demonstrated that restoring the Pi levels in *Dmp1* KO mice greatly improves but does not fully rescue the KO phenotype [13, 26]. To explore the direct and indirect (via regulation of Pi levels) roles of DMP1 in maintaining the auditory ossicles we analyzed these null specimens that were collected from our previous studies using the 3D Micro-CT analysis approaches. As shown in Fig. 5, the re-expression of DMP1 in bone cells fully rescues the *Dmp1* KO auditory ossicles (5a), and the Pi levels in the *Dmp1* KO mice via a high Pi diet gradually improves the KO auditory ossicles (5b). Furthermore, we showed that DMP1 was expressed in bone cells but not in hearing cells, and re-expressing DMP1 in bone cells or providing a high Pi diet greatly improved the bone phenotype (Fig 6a). However, all these alternations had no apparent impact on the morphology of the organ of corti, supporting the notion that the auditory ossicles defect is responsible for hearing loss in *Dmp1* KO mice (Fig 6b).

DISCUSSION

Genetically-engineered mouse models are widely used in skeletal research due to the similarity in the overall long bone structure between humans and mice. However, the configuration of the auditory ossicles of humans and mice is dramatically different. Furthermore, the cause for late onset hearing loss in patients with hypophosphatemic rickets is largely unknown. In this study, we analyzed and compared the structure and configurations of the auditory ossicles of humans compared to mice using multiple *in vivo* approaches and investigated the progression of the auditory ossicle defects in the *Dmp1* KO from postnatal day 14 to month 6. Our main findings were: 1) There is a unique, thin bony membrane with an approximately two cell thickness in the mouse malleus compared to a non-calcified thin membrane in the human malleus; 2) There are drastic changes in the morphology of the *Dmp1* KO ossicles that progress with age and are likely responsible for the conductive hearing loss in adult mice; and 3) the targeted expression of DMP1 in bone cells fully prevents the above phenotype from occurring, and a high Pi diet greatly improve the KO auditory ossicle phenotype.

When the eardrum (tympanic membrane) in humans is caused to vibrate by sound waves, it moves the malleus from side to side like a lever to amplify the sound. To our surprise, there are distinct differences between mouse and human auditory ossicles in their configuration and connection to the eardrum. In humans, the eardrum is mainly connected to the handle of the malleus. The sound-induced motion of the malleus head is then relayed to the incus (Fig 1a). In contrast, the mouse eardrum is attached to both the malleus and incus with a much broader contact area, and the head of the malleus is free from any connection to the rest of the ossicles, as is the human malleus head (Fig 1b). Apparently, this very different configuration in mice is less efficient at amplifying sound transduction from the eardrum to the stapes. Interestingly, the thin membrane surrounding the malleus and stapes in humans is made of soft tissue, which may have little impact on the sound transduction. On the other hand, the mouse membrane surrounding the malleus is a two-cell-layer bony membrane structure (Fig 1b–c). Currently, we do not know exactly what the biological significance of this bony membrane is in mice. However, we reason that it may facilitate the sound transduction and amplification in mice in two ways. First, it greatly increases the direct contact surface area between the eardrum and malleus, which can transmit more concentrated energy on the eardrum via three auditory ossicles over a smaller surface area of the oval window of the cochlea. Secondly, it is highly likely that the bony membrane transmits sound waves much more efficiently than the soft membrane in humans. We further speculate that walking and running upright on two feet like humans required specialized adaptations of the entire skeleton, including auditory ossicles. This adaptation process makes human ossicles more efficient in amplifying the sound via a better lever between the eardrum and the stapes due to a favorable configuration of the auditory ossicles in humans (Fig. 1 and Supplementary Fig 1). It is also likely that a lack of the bony membrane structure in humans may make the sound recognition more precise, since we have much more complex languages but mice do not. All of these speculations may inspire more studies in this area in future.

Most studies on hypophosphatemic rickets, in which an X-linked dominant disorder (XLH) due to *PHEX* mutations is the most common form, have focused on delayed walking, waddling gait, leg bowing, enlarged cartilages, bone pain, craniosynostosis, spontaneous dental abscesses, and growth failure. Hearing loss in these patients is largely ignored, as a majority of children with this disease have normal hearing but develop hearing loss in adult life [17–21]. Similarly, patients with *DMP1* mutations do not develop hearing impairment until later in life [22]. In this study, we attempted to understand why and how late-onset hearing loss occurs using *Dmp1* KO mice as a model, which develop extremely similar phenotypes to those in humans [13]. Our studies revealed a progressive change of the KO ossicle phenotype with age from relatively mild to more severe alterations such as the development of an uneven, patchy bony membrane. This observation agrees with the other bone phenotypes that progress with age [11]. As a result, the efficiency of the conversion of sound energy to mechanical energy by vibration of the auditory ossicles may be reduced due to the poorly formed auditory ossicles in the KO mice. Because both *Dmp1* KO and *Phex* KO mice display almost identical phenotypes [32, 33], the current studies may be helpful in understanding the cause of late-onset hearing loss in patients with XLH.

In summary, we analyzed *Dmp1* lac Z knock-in auditory ossicles (in which the blue reporter is used to trace the osteocyte expression in bone) using X-gal staining and showed a novel bony membrane surrounding the mouse malleus. This finding was further confirmed by 3-D micro-CT, X-ray, and Alizarin Red stain images. We speculate that this unique structure amplifies and facilitates sound wave transmissions in two ways: by increasing the contact surface between the eardrum and malleus and accelerating the sound transmission due to its mineral content. Next, we documented a progressive deterioration in the *Dmp1*-null auditory ossicle structures using multiple imaging techniques. The auditory brainstem response test demonstrated a conductive hearing loss in the adult *Dmp1*-null mice. This finding may explain in part why patients with *DMP1* mutations develop late-onset hearing loss, and may also support the critical role of DMP1 in maintaining the integrity of the auditory ossicles and its bony membrane.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- A novel bony membrane surrounding the mouse malleus for sound transmissions;
- A progressive deterioration in the *Dmp1*-null auditory ossicle structures with aging;
- A conductive hearing loss in the adult *Dmp1*-null mice.

Figure 1. Striking differences between auditory ossicles of humans and mice

A unique bony membrane in the mouse. **a)** The photograph (*left panel*) and radiograph (*left panel*) of adult human auditory ossicles with a figure in the middle showing the relationship of the eardrum, malleus, incus, and stapes; **b).** Photograph showing the eardrum, which is attached to the entire malleus; **c).** An x-gal stained *Dmp1-lacZ*-knock-in skull, in which three auditory ossicles and a thin transverse bony membrane are apparent. The blue color reflects the DMP1 expression in osteocytes (Ocy); and **d).** Micro-CT image displaying the

relationship among the external auditory canal, eardrum (with a pseudo-color), auditory ossicles, and bony membrane.

Figure 2. Progressive changes of the *Dmp1* null auditory ossicles at the gross level (right panels) compared to the age-matched control (left panels). a–d)

The 14-day-old auditory ossicles were revealed by Alizarin red stain (a), radiographs (b), micro-CT images (c), and x-gal stained images (d); and e–f) The 4-month-old auditory ossicles displayed an uneven bony membrane in the photograph (e) with a low mineral content indicated by the radiograph (f) in the *Dmp1* knockout (KO) mice.

Figure 3. Changes in extracellular matrix proteins in the *Dmp1* null malleus at the cellular and molecular levels: 4.5-month-old KO (right panels) compared to age-matched control (left panels) (a) The H&E stain shows “porous areas”; (b–e) The IHC images of COL 1 (b) and osteocalcin (c) show no apparent differences between the KO and the age-matched control; The decorin (c) and biglycan (d) IHC images show increased but patchy immunoreactivity signals compared to the control.

Figure 4. Comparison of brainstem auditory evoked responses between *Dmp1* null mice and the control

(a) The latency-intensity function curves were recorded at 16 KHz (top) and 8KHz (bottom) with the control at the lower part of the curve and KO at the upper part of the curve; (b). The threshold curves were significantly different between the KO and the control at all frequencies (4, 8 and 16 kHz) tested; and (c). Representative latency responses to different stimuli were compared between the control (left) and KO. The parallel shift in latency in the KO group relative to the control group reflects a conductive hearing loss.

Figure 5. Targeted expression of DMP1 in bone cells or feeding a high Pi diet prevents or greatly improves the *Dmp1* KO auditory ossicle phenotype

a) The 3-D micro-CT images showed a porous malleus in the 4.5-month-old *Dmp1* KO mouse (*middle panel*) compared to the age-matched control (*left panel*) and the rescued KO mouse (*right panel*); and **b)** Feeding a high Pi diet to one month-old *Dmp1* KO mice for two months (*upper panel*) or four months (*lower panel*) greatly improved the porous bone phenotype, including the bony membrane.

Figure 6.

DMP1 is restricted in bone cells and the Dmp1 null inner ear showed porous bone at cellular and molecular levels: 4.5-month-old KO (right panels) compared to age-matched control (left panels). (a) The DMP1 IHC stain images display a restricted expression of DMP1 in bone (left and right panels); and (b) The H&E stain images showed “porous bone” with little changes in the KO organ of corti (middle panels) and the targeted re-expression of DMP1 rescued Dmp1 KO bone phenotype (right panel).