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Osteoprotegrin Knockout Mice Demonstrate Abnormal Remodeling of the Otic Capsule and Progressive Hearing Loss

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Abstract

Objectives—The otic capsule, when compared with other bones in the body, is unique in that it undergoes no significant remodeling of bone after development. We previously demonstrated that osteoprotegerin (OPG), which inhibits formation and function of osteoclasts, is produced at high levels in the inner ear of normal mice and secreted into the peril-ymph from where it diffuses into the surrounding otic capsule bone through a lacunocanalicular system. To test our hypothesis that the high level of OPG may be important in the inhibition of otic capsule remodeling, we studied the light microscopic histology of the otic capsule in OPG knockout mice for evidence of abnormal remodeling of bone. We also tested the hearing in OPG knockout mice to determine whether OPG and its influence on surrounding bone is important for auditory function.

Methods—Temporal bone histopathology and pathophysiology were compared in homozygous OPG knockout mice and C57BL/6 (B6) mice, the background strain for the knockouts. Auditory function in age-matched animals from each group was evaluated at approximately 4-week intervals from 8 to 21 weeks using frequency-specific auditory brainstem responses (ABR) and distortion product otoacoustic emissions (DPOAE). After each of the last three evaluations, the cochleae from one mouse of each group were harvested, processed, and examined by light microscopy.

Results—Osteoprotegerin knockout mice demonstrated abnormal remodeling of bone within the otic capsule with multiple foci showing osteoclastic bone resorption and formation of new bone. Such changes were not seen in the age-matched B6 controls. The active bone remodeling process in the knockout animals showed many similarities to otosclerosis seen in human temporal bones. Over the time period that we monitored, auditory function was significantly and progressively compromised in the knockout animals relative to B6 controls. At the earliest age of test (8 wk), the loss was apparent as a mild, high-frequency reduction in sensitivity by ABR. In contrast, DPOAE losses in the knockouts were substantial even at 8 weeks, and by 21 weeks, these losses exceeded our equipment limits. Results of ABR testing showed hearing sensitivity changes in the animals of the background strain were confined largely to the high frequencies, whereas OPG knockouts demonstrated substantial low-frequency shifts in addition to those at high frequencies.

Conclusions—The histopathological and pathophysiological findings in OPG knockout mice support the hypothesis that OPG is important in the inhibition of bone remodeling within the otic capsule and the maintenance of normal auditory function. This mouse may provide a valuable animal model of human otosclerosis.

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Keywords

Bone remodeling; osteoprotegerin (OPG); otic capsule; RANK ligand (RANKL); receptor activator of nuclear factor Kappa B (RANK)

INTRODUCTION

The otic capsule is unique in its composition and pattern of remodeling of bone. It remodels at a very low level compared with other bones in the body. Within the otic capsule, bone turnover varies from practically full inhibition (0.13%/y) in the innermost perilymphatic zone to a gradually increasing, yet still reduced, level at the periphery.[1] The reasons for this low level of bone turnover remain unclear. It has been suggested that this pattern of bone remodeling is a fundamental prerequisite for normal function of the inner ear and has led to the speculation that bone turnover in the otic capsule is controlled by intrinsic factors produced by the inner ear.[1]

Osteoprotegerin (OPG) is a key regulator of bone metabolism together with receptor activator of nuclear factor Kappa B (RANK) and RANK ligand (RANKL).[2] In bone, OPG acts as a soluble, neutralizing antagonist that competes with RANK on preosteoclasts and osteoclasts for RANKL produced by osteoblasts. OPG inhibits the differentiation, survival, and fusion of osteoclastic precursor cells and suppresses activation and promotes apoptosis of osteoclasts[3] (Fig. 1). The physiological balance of bone resorption by osteoclasts and new bone formation by osteoblasts is maintained by the balanced interaction of RANKL, RANK, and OPG. If any one of those three cytokines is dysfunctional, an imbalance in bone remodeling results. For example, OPG knockout mice show severe osteoporosis, whereas overexpression of OPG or RANKL results in osteopetrosis.[3,4]

In a recent study, we found extremely high levels of OPG mRNA in the soft tissue of the cochlea. With immunohistochemistry, we localized the production of OPG as occurring within the spiral ligament, supporting cells of the organ of Corti and interdental cells of the spiral limbus. We also found that OPG was present at high concentrations within the perilymph. Furthermore, we demonstrated the existence of a lacunocanalicular system within the otic capsule as a possible pathway by which OPG can diffuse out from the cochlea into the surrounding bone.[5,6]

Because OPG is such a potent inhibitor of osteoclastogenesis, we hypothesized that the high ratio of OPG to RANKL in the inner ear may be responsible for the nearly complete absence of bone turnover within the surrounding otic capsule. The goal of the present study was to test this hypothesis by light microscopic examination of the otic capsule in homozygous OPG knockout mice. In addition, auditory function was assayed using distortion product otoacoustic emissions (DPOAE) and auditory brainstem responses (ABR). Physiological and histologic results were compared with similar data obtained from age-matched, wild-type mice of the background strain, B6.

MATERIALS AND METHODS

Homozygous OPG knockout mice and B6 wild-type controls were tested at approximately 4-week intervals from 8 to 21 weeks. Functional gene knockouts were generated by targeted gene disruption of exon 2 in the osteoclastogenesis inhibitory factor (OCIF)/OPG locus and backcrossed to the parental B6 strain by Mizuno et al.[7] We obtained five homozygous mutants from CLEA-Japan, Inc., for the following experiments. Because wild-type littermates were not available from those same litters, an equal number of age-matched, commercially available B6 mice were obtained from Jackson Laboratories (Bar Harbor, ME) as controls. Mice from both

groups were housed and treated identically. All animal procedures were approved by the Animal Care and Use Committee of the Massachusetts Eye and Ear Infirmary.

Light Microscopy of Temporal Bones

One animal from each group (i.e., the knockout and wild-type groups) was killed at the ages of 13, 17, and 21 weeks after completion of the audiometric testing (as described subsequently) by intraperitoneal injection of urethane. The chest was opened, the right auricle of the heart was opened, and the animal was perfused with 0.1% $NaNO_3$ in phosphate-buffered saline through the left ventricle. The bulla was opened and filled with fixative consisting of formaldehyde (10% formalin), 0.05% glutaraldehyde, and 1% acetic acid. The temporal bones were then removed and left in the fixative for 7 days. The temporal bones were embedded in paraffin and serially sectioned at a thickness of 10 μm. Every 20^{th} section was stained with hematoxylin & eosin and examined by light microscopy.

Tests of Auditory Function

All procedures were conducted in an acoustically and electrically shielded and heated chamber using our standard techniques. Mice were anesthetized (ketamine HCL, Ketaset, 100 mg/kg, intraperitoneally [IP]; xylazine, Rompun, 10 mg/kg, IP boosters at one third to one half the original dose as required). Body temperature was maintained near 37°C throughout the experiment by control of room air temperature. A small slit was made in the external canal to assist in visualizing the canal and tympanic membrane for possible abnormalities and to optimize placement and coupling of the sound delivery system. There was no evidence of active middle ear pathology on microscopic examination through the intact tympanic membrane in any animal in both knockout and wild-type groups.

Stimuli were created and delivered and responses monitored under computer control using 16-bit A/D, D/A boards (National Instruments, Woburn, MA) controlled in a LabVIEW environment by a PC workstation. Signals used to elicit DPOAEs and ABRs were delivered to the ear using the same custom coupler. The coupler accommodates transducers (Tucker Davis EC1, Alachua, FL) and a Knowles EK3103 Electret microphone (Itasca, IL) used to detect ear canal sound pressure (for DPOAEs) through a probe tube concentric with the 2.54-mm outer diameter sound-delivery tube. Sensitivity versus frequency calibration curves were generated for the monitoring and probe microphones, respectively, enabling conversion from voltage to sound pressure level (SPL; in dB re: 20 μPa). The probe assembly was then placed at the animal's ear canal where “in animal” calibration sweeps were accomplished to determine the actual SPLs generated at the entrance to the bony canal. After calibration, the probe assembly remained in place for the duration of the experiment.

ABRs were measured under computer control in response to tone pips (5.6–45.2 kHz; 5-ms duration; 0.5-ms rise/fall; cos2 shaping; 30/s). Response growth with increasing stimulus level was characterized by incrementing level (in 5-dB steps) over the range required to capture threshold to 80 dB SPL. Electrophysiological activity was detected by electrodes (subdermal, needle) placed at the vertex (active), ventrolateral to the ear of test (reference), and midline near tail (common). Responses were amplified (10,000×), filtered (0.3–3 kHz), digitized, and averaged (across 512 discrete samples at each frequency-level combination; artifact reject = 15-μV peak-to-peak). Response values (e.g., peak-to-peak amplitudes) and waveforms were stored to disk. On visual inspection of stacked waveforms, “threshold” was defined as the lowest stimulus level at which response peaks were clearly and repeatably present. These visual detection threshold judgments could be confirmed by offline display and analysis of the stored waveforms.

DPOAEs at 2*f*1-*f*2 were recorded as amplitude versus level functions (L_1 = 80–20 dB; L_2 = L_1 - 10; primaries incremented together in 5-dB steps) spanning the frequency range *f*2 = 5.6–45.2 kHz, selected to equal ABR test frequencies; f_2/f_1 = 1.2. Ear canal sound pressures were amplified, digitally sampled, averaged (25 discrete spectra at each frequency-level combination) and Fast-Fourier transforms were computed from the averaged pressures. DPOAE level at 2*f*1-*f*2 and surrounding noise floor values (±50 Hz of 2*f*1-*f*2) were extracted. Isoresponse contours (L_2 levels required to generate a criterion DPOAE of −5 dB SPL at each frequency) were constructed from amplitude versus sound level data to facilitate comparison with ABRs.[8] For both response metrics, responses absent at the highest level of stimulation were assigned a threshold/isoresponse value 5 dB greater than the stimulus maximum.

RESULTS

Light Microscopy of Temporal Bones

Control Group—None of the ears at any age showed any evidence of remodeling of bone of the otic capsule (Fig. 2A). No osteoclasts were found in any ear. The ossicles, including the stapes footplate, were intact without any areas of bone resorption. All cases showed a layer of otic capsule bone underlying the stapedial artery (Fig. 2A). The cochleae at 21 weeks of age showed losses of outer and inner hair cells in the most basal part of the basal turn, scattered hair cell loss in the apical turn, and mild loss of cochlear neurons in the basal turn. Hair cells and cochlear neurons appeared normal in the remainder of the cochlea. The stria vascularis and other sensory structures were intact and normal in appearance throughout the cochleae (not shown).

Osteoprotegerin Knockout Group—Microdissection of the temporal bones revealed a clear difference in quality of the temporal bones of the two strains. The bullae of the OPG knockout mice were thinner and more fragile compared with the B6 mice at any age. By light microscopy, the otic capsule of all specimens showed significant remodeling of bone. A notable feature of this abnormal remodeling process was the occurrence of sharply defined, focal, hypercellular areas showing bone resorption and deposition (Fig. 2B). Many of these hypercellular areas contained multinucleated osteoclasts (Fig. 2D) in addition to numerous osteoblasts, as well as numerous blood vessels (Fig. 2H). In areas of hypercellularity, the bone was sometimes thicker than the corresponding bone of normal mice (compare area indicated by arrowheads in Figs. 2A and B). In other areas, the petrous bone was abnormally thin, apparently as a result of previous resorption. In some areas, bone resorption traversed the entire width of the otic capsule. This was most apparent at the site between the stapedial artery and the underlying spiral ligament, as shown in Figure 2B. In some instances, bone was eroded so that hypercellular areas of remodeling were continuous with the spiral ligament (Figs. 2F and F′). When these lesions came into contact with the spiral ligament, there was deposition of an eosinophilic staining substance within the ligament and the cavitated bone. The degree of otic capsule remodeling was more pronounced in older animals and appeared to become progressively worse over time. In some areas, resorption of bone had resulted in formation of cavitary lesions, which communicated with the middle ear air space or inner ear fluid space. In areas of bone remodeling, the overlying middle ear mucosa was abnormally thick (Fig. 2B). Accompanying the remodeling of bone was increased vascularization of bone. This was most apparent at the base of the cochlea where the bone is thickest (Fig. 2J). Compare the same area in the wild type (Fig. 2I). The middle ear ossicles also showed remodeling, as evidenced by abnormal areas of hypercellularity in the malleus (Figs. 2F and G) and by erosion of the stapes footplate (Fig. 2E). The soft tissue within the cochlea of OPG knockout mice up to the age of 21 weeks showed cytocochlear losses similar to the control animals with losses of hair cells in the extreme base and apex and mild basal loss of cochlear neurons (not shown). The organ of

Corti and cochlear neurons were normal in the remainder of the cochlea. The stria vascularis and other sensory structures were normal throughout the cochlea.

Auditory Function

ABR and DPOAE thresholds are shown for both OPG knockout and B6 groups spanning the range of ages tested (Fig. 3). At the youngest age of test (8 wk; n = 5 in each group), knockouts showed high-frequency ABR threshold elevations when compared with those of age-matched animals from the background B6 strain. Low- to midfrequency responses were normal for both strains. At the same age, DPOAEs recorded from knockouts are already elevated to near equipment limits at both low and high frequencies. At the final test age (21 wk), a substantial low- to midfrequency loss of sensitivity was present by ABR testing in the knockouts, and DPOAEs were largely absent or near equipment limits. Both groups showed progression of high-frequency hearing loss by both metrics of function.

DISCUSSION

The strain of OPG knockout used in these experiments has an osteoporotic phenotype that has been studied previously. Skeletal abnormalities are closely similar to human osteoporosis. They are otherwise fertile with no other known histologic abnormalities.[7] The otic capsule has never been examined.

In the present study, we found that the otic capsule of OPG knockout mice demonstrated clear evidence of a progressive and abnormal remodeling process that was not observed in the controls. The findings of this study in combination with our previous work[5] indicate that production of OPG within the cochlea may play an important role in the inhibition of bone remodeling within the otic capsule and in the maintenance of normal cochlear physiology. The evidence indicates that OPG diffuses from the cochlea into the surrounding bone through a lacunocanalicular system to maintain the otic capsule in an inactive stage. Our data fit very well with the findings of Sorensen et al.,[1] who have demonstrated that inhibition of otic capsule remodeling is most pronounced immediately adjacent to the cochlea and diminishes in a centrifugal manner as one moves away from the cochlea.

The active remodeling process in the OPG knockout mice has many similarities to otosclerosis seen in human temporal bones. A notable feature of the abnormal remodeling process in the OPG knockout mice was the occur-rence of sharply defined, focal, hypercellular areas of the otic capsule showing bone resorption and deposition, which resembles the lesions of active otosclerosis.[9,10] When the remodeling foci in the OPG knockouts reached the spiral ligament, they appeared to induce the deposition of an eosinophilic staining substance within the spiral ligament (so-called “hyalinization”), which is also a feature of otosclerosis when it makes contact with the spiral ligament.[11,12] In addition, the observed thickening of middle ear mucosa overlying these remodeling foci and the occurrence of cavitation within some foci in the knockouts are also features that are observed in human temporal bones with otosclerosis.[12] OPG knockout mice clearly differ from clinical otosclerosis in several ways. In OPG knockout mice, active remodeling occurs throughout the entire skeleton and diffusely within the otic capsule. This remodeling also involves the incus and malleus, which is rare in otosclerosis. There is no histologic evidence of stapes fixation in OPG knockout mice. Although strikingly similar in many ways to otosclerosis, these profound differences suggest that otosclerosis is a fundamentally different process than that which is observed in OPG knockout mice. Additional studies may prove valuable in better understanding the molecular pathways that are important in the inhibition of otic capsule remodeling and initiation of abnormal remodeling processes within the otic capsule such as otosclerosis and Paget's disease of bone.

Our study also demonstrated that the OPG knockouts have a progressive and severe hearing loss (HL), as determined by ABR and DPOAE testing when compared with controls. The occurrence of this HL also points to the importance of OPG and lack of otic capsule remodeling in the maintenance of normal cochlear physiology. The HL was first apparent in the high frequencies and spread to involve the mid and lower frequencies at later ages. Both the controls and the knockouts showed hair cell loss in the basal turn, which can explain the high frequency loss, similar to what has been described in C57BL/6 animals by others.[13] However, we did not find any histopathologic correlates for the substantial mid- and low-frequency HL observed in the knockout mice. We speculate that the active remodeling of bone adjacent to the spiral ligament may have affected cochlear physiology in a manner similar to what is believed to occur in cases of active otosclerosis that results in sensorineural HL.[14] Another explanation may be that the HL in the middle and lower frequencies may have been conductive or middle ear in origin, given that there was significant resorption of ossicles in some ears. We note that DPOAE responses were absent, although outer hair cells were intact, a finding that could have resulted from either middle ear problems or cochlear pathology (for example, loss of endolymphatic potential resulting from spiral ligament dysfunction). Further insight into the source of the loss in hearing sensitivity could be gained by future studies incorporating measurements of endolymphatic potential, stapes velocity, and round window velocity.

CONCLUSIONS

The histopathophysiological and pathophysiological findings in OPG knockout mice support the hypothesis that OPG is important in the inhibition of bone remodeling within the otic capsule and the maintenance of normal auditory function. This mouse may provide a valuable animal model of human otosclerosis.

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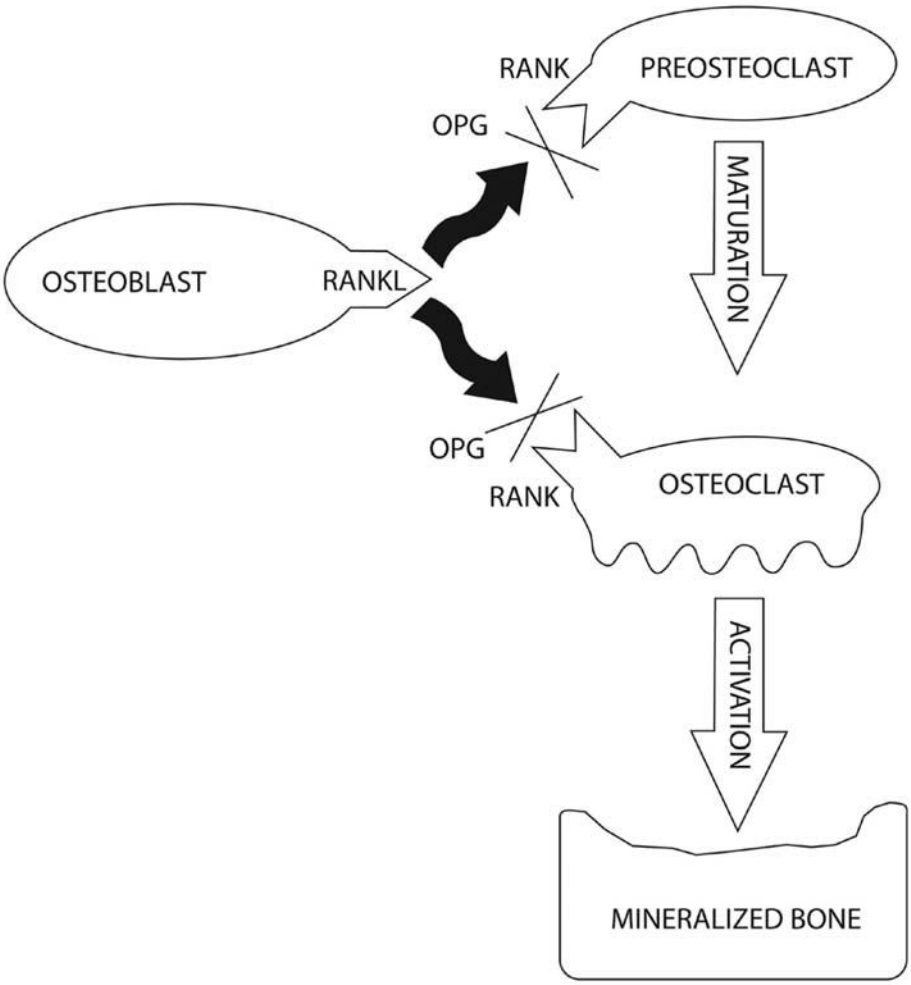

Fig. 1.

Schematic diagram showing the relation between osteoblasts and osteoclasts. RANKL expressed by osteoblasts stimulates its specific receptor RANK on preosteoclasts and osteoclasts to initiate the differentiation, activation, and survival of osteoclasts. Osteoprotegerin acts as soluble neutralizing factor and blocks the effect of RANKL.

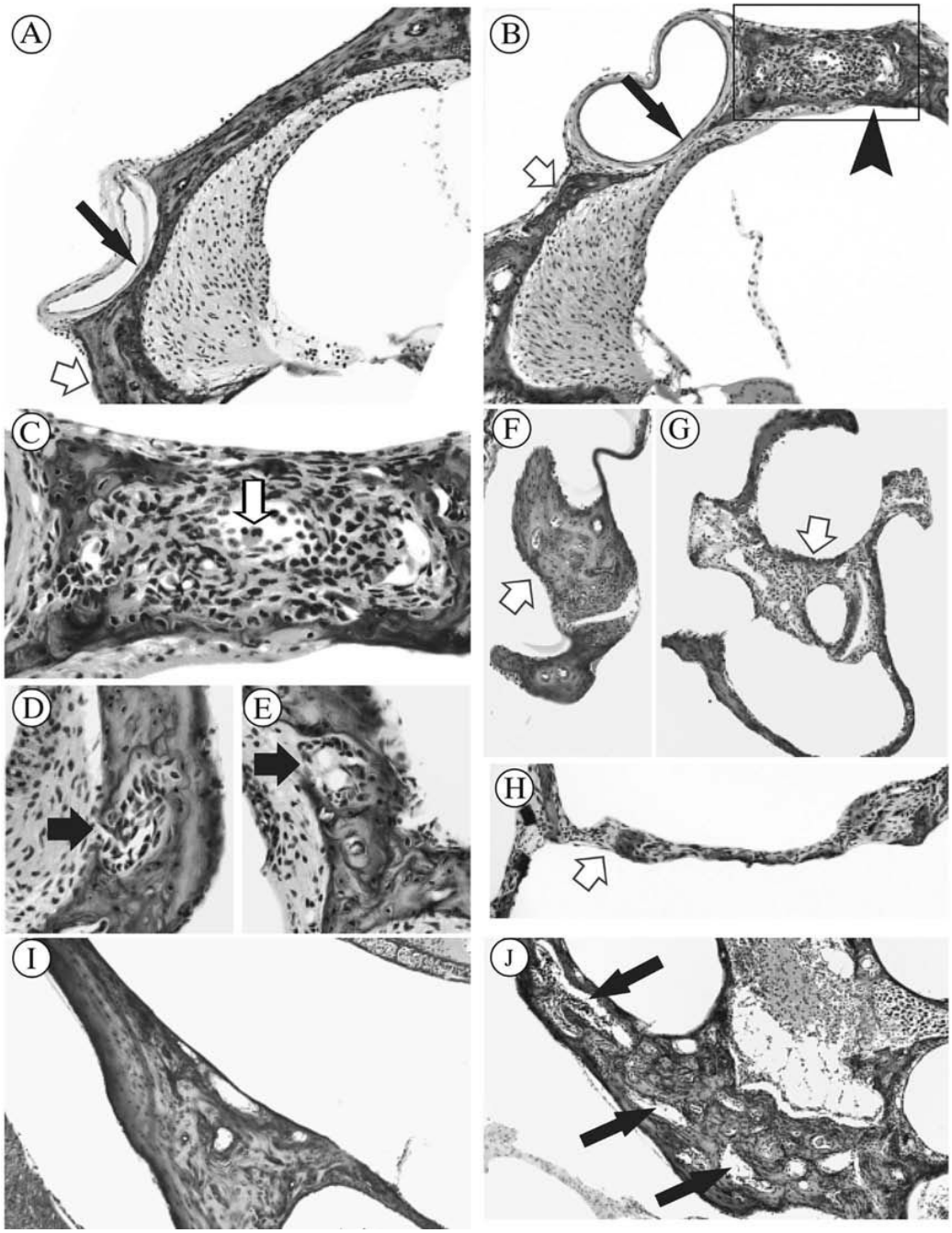

Fig. 2.

All examples are from 13-week-old mice. (A) Lower basal turn of normal C57 control mouse. Open arrow indicates the squamous middle ear epithelium. Filled arrow indicates the otic capsule separating the stapedial artery and the underlying spiral ligament. (B) OPG −/− basal turn. The middle ear mucosa (open arrow) is thickened. The bony shell between the stapedial artery and spiral ligament (closed arrow) has been eroded and the bone superior to the artery is thickened and filled with cells (arrowhead). (C) Higher magnification of the highly cellular otic capsule indicated by the boxed area in B. The arrow indicates a multinucleated osteoclast. (D and E) Closed arrows indicate places where the otic capsule of the upper turn of the cochlea has been eroded in a −/− animal to create a large opening between the spiral ligament and the

interior of the capsule. (F and G) Open arrows show a comparison of the normal (F) and OPG −/− (G) at the incudomalleal joint (H) The footplate of the stapes of an OPG −/− case showing only connective tissue (arrow) at one point along the eroded bone. (I) The otic capsule beneath the basal turn of a control mouse with few cells and few open spaces. (J) The corresponding region of an OPG −/− animal showing hypercellularity and numerous large vascular channels (arrows).

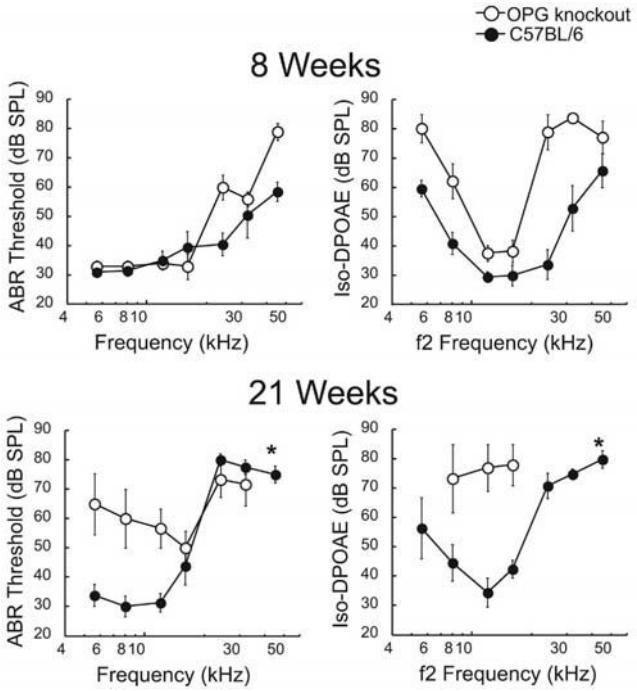

Fig. 3.

Auditory function in osteoprotegerin knockout and control (B6) groups of mice at 8 weeks and 21 weeks, as determined by auditory brainstem response and distortion product otoacoustic emissions testing. Data shown are mean ± standard error.