Published in final edited form as:
Hear Res. 2010 June 14; 265(1-2): 83–89. doi:10.1016/j.heares.2010.02.006.

Differences in gene expression between the otic capsule and other bones

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Abstract

Our long term goal is to understand the molecular pathology of otosclerosis and to develop better forms of therapy. Toward this goal, the current study focused on characterizing the molecular factors responsible for the unique biological features of the otic capsule: its minimal rate of remodeling, and lack of healing capacity when fractured. We compared expression levels of 62 genes involved in bone metabolism between the adult murine otic capsule and the tibia and parietal bones; the latter exemplify bones formed by endochondral and intramembranous ossification, respectively. Gene expression levels were measured using real-time quantitative RT-PCR and analyzed using tools of bioinformatics. Expression patterns of key genes were verified with in situ hybridization. The molecular profile of the otic capsule was distinctly different from that of the tibia and parietal bone. Genes found to be most characteristic of the otic capsule were: osteoprotegerin (*opg*), bone morphogenetic protein receptor 1b (*bmpr1b*) and bone morphogenetic protein 3 (*bmp3*). Expression levels were high for *opg* and *bmpr1b*, and minimal for *bmp3* within the otic capsule. We concluded that *opg* and *bmpr1b* likely play important roles in inhibition of remodeling within the otic capsule.

Keywords

Otic capsule; *opg*; *bmpr1b*; otosclerosis

Introduction

The bone surrounding the inner ear, known as the otic capsule, is unique for many reasons. First, its rate of remodeling is minimal compared with other bones, as shown in many

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Conflict of interest: All authors have no conflicts of interest.

species including humans, dogs and pigs (Sorensen et al., 1992; Frisch et al., 1998; Soresen et al., 1990). However, under pathologic conditions such as otosclerosis or Paget's disease, the otic capsule starts to remodel (Schuknecht 1993), which can lead to hearing loss. Second, the otic capsule typically does not heal when fractured, except by fibrous union (Perlman 1939). Third, the otic capsule is the densest bone in the body (Bast and Anson, 1949). Our previous work identified osteoprotegrin, *opg*, as an important factor in the inhibition of remodeling within the otic capsule (Zehnder et al., 2005). We found expression of *opg* in the otic capsule to be much higher than in other bones. We also found that Opg protein is produced in extremely high concentrations within the inner ear and that it, most likely, diffuses into the surrounding otic capsule. We demonstrated that *opg* knockout mice have abnormal otic capsule remodeling that resembles otosclerosis (Zehnder et al., 2006).

We undertook the current study to identify other genes, in addition to *opg*, that provide the molecular basis for the uniqueness of the otic capsule. We focused on genes that are known to play roles in bone remodeling, including inflammatory cytokines (Goldring 2007), and on genes that encode structural proteins. We compared the expression levels of these genes in the murine otic capsule to those in the parietal bone and tibia, two bones produced by developmentally distinct mechanisms. These mechanisms involve the transformation of preexisting mesenchyme into bone, which occurs directly or indirectly (Gilbert 2000). The direct mechanism, or intramembranous ossification, occurs primarily in flat bones of the skull, including the parietal bone. The indirect mechanism, which involves a cartilage intermediate, is known as endochondral ossification, and it gives rise to the axial and appendicular portions of the skeleton, including the tibia, and the otic capsule.

Material and methods

Collection of tissue, extraction of total RNA and cDNA synthesis

Samples of bone were collected from 7 adult C57BL/6J mice obtained from Jackson Laboratories, Maine. The animals, ranging in age from 8–11 weeks, were sacrificed by intraperiotenal injection of urethane (2.5 mg/kg). Otic capsule bone surrounding the cochlea, parietal bone and diaphysis of tibia were microdissected and placed in RNAlater reagent (Ambion). Care was taken to scrape away mucosa of the middle ear and parts of the membranous labyrinth adherent to the otic capsule. However, some contamination with soft tissues adherent to the otic capsule cannot be definitively excluded.

After removal of RNAlater, total RNA was purified using RNeasy spin-columns (Qiagen) according to the manufacturer's protocol, with the modification for hypocellular, dense connective tissues (Reno et al., 1997). Specifically, Trizol was used first, followed by chloroform, collection of the aqueous phase, addition of an equal volume of isopropanol, and application of this mixture to RNeasy spin-columns. The RNA quantity and quality was assessed using Agilent 2100 Bioanalyzer (Agilent Technologies) and RNA Pico Kit (Agilent Technologies); samples with RNA integrity number of at least 7, out of possible 10, were used for further analysis. Total RNA that appeared clean and undegraded, based on the Bioanalyzer's electropherograms, was reverse transcribed with Taqman Reverse Transcription Reagents kit (Applied Biosystems). Typical total RNA yields were slightly less than 1 μg for otic capsule samples, 1.5–2 μg for parietal bone, and 2 μg for tibia. Overall, RNA from 5 otic capsules, 4 parietal bones and 6 tibias met our quality criteria. Two of the tibia samples were pooled together to increase RNA yield; results from the pooled and non-pooled sample were in agreement.

Relative quantitation of mRNA using real-time PCR

Real-time quantitative RT-PCR was used to analyze gene expression levels in a sensitive and high throughput fashion. The 62 genes that were studied (supplemental Table 1) were selected based on their known roles in bone metabolism or inflammation, or suspected roles in bone remodeling based on genetic studies of bone diseases. For all genes, 6-FAM linked fluorescent probes and primers were designed and optimized by Applied Biosystems. The measurements were carried out on an Applied Biosystems 7700 Sequence Detector using 96 well plates. For each well, the 25 μl reaction contained: 1.25 l of the 20× probe/primer mix, 1 μl of cDNA template (approximately 15 ng cDNA/μl), 12.5 μl Universal Master Mix (Applied Biosystems), and 10.25 μl distilled water. All cDNA samples were run in triplicate, and each plate was used to test expression of up to 32 genes from a single sample. Fluorescence data were collected over 45 cycles of PCR, which consisted of an initial denaturation step at 95° C for 10 minutes, followed by 45 cycles of 95°C for 15 seconds and 60°C for 1 minute. Gene expression levels were quantified relative to the 18S rRNA gene and compared between bone types using the Comparative threshold cycle (C_T) me thod, i.e. the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001). Statistical significance of the detected changes was determined using the bootstrap method as in Stankovic and Corfas (2003). For a given gene, $\Delta\Delta C_T$ was defined the difference of mean ΔC_T of all samples from one bone type (e.g. otic capsule) and mean ΔC_T of all samples from another bone type (e.g. parietal bone). Data were randomly shuffled 5000 times (using Matlab 5.3, The MathWorks Inc.), and a histogram of "pseudo"$\Delta\Delta C_T$ values was built. The actual $\Delta\Delta C_T$ was considered to be significant at the 0.05 level if it fell further from the mean than 95% of the histogram values. Statistically significant changes in gene expression were at least two fold.

Bioinformatics analysis

Tools of bioinformatics were applied to simultaneously analyze expression patterns of a relatively large number of genes studied in a relatively small number of samples. Genes were ranked according to signal to noise ratio, SNR, defined as the difference of the mean expression levels of each bone type scaled by the sum of standard deviations. Two unsupervised clustering algorithms (implemented in GenePattern 2.0) were used in the analysis of samples from different bone types and genes within a bone type: self-organizing maps (SOM) (Tamayo et al., 1999) and hierarchical clustering (Eisen et al., 1998). The stability of the identified clusters (i.e. sensitivity of the cluster boundaries to sampling variability) was assessed using consensus clustering (Monti et al., 2003). This method simulates perturbations of the original data using resampling techniques, and then applies the clustering algorithm of choice (SOM or hierarchical) to each of the perturbed datasets to assess the agreement, or consensus, among different runs. The results are typically summarized in a consensus matrix, which is depicted as a square heat map. Consensus matrices were generated assuming 2 to 5 clusters, and the best number of clusters determined using visual inspection of the consensus matrices, and the corresponding summary statistics. The increase in the area under the cumulative distribution function corresponding to the histogram of a consensus matrix peaked at the optimal number of clusters.

The class neighbors analysis (Golub et al., 1999) was used to determine which genes serve as good predictors for a bone type (class), and there was *a priori* knowledge of which sample came from what bone type. Genes were ranked based on SNR, and the robustness of the bone type predictors was tested using K-nearest neighbors cross validation (Golub et al., 1999). The cross validation technique is a cyclical procedure in which one sample at a time is withheld and a predictor built on the remaining samples; the predictor then predicts the bone type of the withheld sample.

In situ hybridization

Mice were anesthetized and fixed by intracardiac perfusion with 4% paraformaldehyde. Specimens were decalcified in 120 mmol/L ethylenediaminetetraacetic acid (EDTA) for one week. The skulls containing temporal bones were embedded in paraffin and serially sectioned in a horizontal plane at a thickness of 10μm. Selected sections were stained with 0.2% azure B, pH 3 to identify cartilage (Lillie, 1965).

A 535 bp HindIII-XhoI fragment of the mouse *opg* cDNA was cloned into pBluescript II SK-vector (courtesy of Dr. Abboud Werner, Heinrich et al., 2005). The mouse *bmpr1b* probe was described in Namiki et al. (1997). To prepare the digoxigenin (DIG)-labeled single stranded antisense and sense RNA probes, the purified fragments were subjected to PCR in the presence of DIG-dUTP (digoxigenin DNA labeling mixture; Roche, Basel, Switzerland) according to the manufacturer's protocol. The efficiency of probe generation was monitored using a standard DIG labeled probe by membrane hybridization. In situ hybridization was performed as in Sassoon and Rosenthal (1993) with minor modifications. Sections were deparaffinized with xylene, washed in 100% ethanol, placed in 4% formaldehyde for 15 min, washed with PBS, digested with proteinase K (10μg/ml) in PBS for 10 min and washed in PBS. The hybridization mixture containing DIG labeled probe was applied to each section and incubated for 16h at 50°C. After application of 5× saline sodium citrate buffer (SSC), sections were washed in RNase buffer solution at 37°C for 30 min. Consecutive washes at 65°C were performed: twice in 2×SSC, twice in 0.1×SSC. To visualize the hybridized *opg* probe, the slides were incubated with alkaline phosphatase-conjugated anti-DIG antibody (Roche) for 60 min after applying the blocking buffer (Roche) for 60 min. The colorimetric reaction was developed with nitro blue tetrazolium salt and bromo-4-chloro-3-indolyl phosphate solution (Roche) in the dark for 2h, and stopped with PBS. For *bmpr1b* probe, fluorescent reaction was used because it gave a better signal than the calorimetric reaction. The slides were exposed to Cy3-conjugated monoclonal anti-DIG-antibody (Jackson Immuno Research, 200-162-156) for 60 min, washed twice with 0.1% Tween, 20mM Tris buffer solution, treated with Cy3-conjugated secondary antibody (AffiniPure Goat Anti-Mouse IgG, Jackson Immuno Research, 1150165-003) for 60 min, washed twice with 0.1% Tween, 20mM Tris buffer solution, and counterstained with nuclear DAPI stain (4', 6-diamidino-2-phenylinodole, ddihydochloride, Sigma, D9542). Slides were mounted with Aquatex (Merck, Darmstadt, Germany), and evaluated by brightfield (*opg*) or fluorescent (*bmpr1b*) microscopy. Serial sections of tissues hybridized with the sense probe served as negative controls.

All animal experiments in this study were approved by the Animal Care and Use Committee of the Massachusetts Eye and Ear Infirmary.

Results

The set of genes we studied was sufficiently rich to allow for robust grouping or clustering of bone types. Clustering was non-overlapping when using hierarchical clustering, and partly overlapping when using SOM clustering; most cross-coupling was present between the otic capsule and parietal bone. Gene expression data are presented in the form of a heat map (Fig. 1) where each column represents a different sample, each row represents a different gene, and color coding reflects row-normalized levels of gene expression with red indicating high, and blue indicating low levels of expression. The otic capsule is compared with tibia (Fig. 1A) and parietal bone (Fig. 1B). The figures illustrate highly reproducible measurements within a bone type as most samples from a given bone type had similar color coding. A majority of the genes was down-regulated (blue in Fig. 1A) in the otic capsule compared to the parietal bone, and up-regulated (red in Fig. 1B) compared to the tibia. Thirty six genes were differentially expressed in the otic capsule compared to parietal bone,

and 38 genes were differentially expressed in the otic capsule compared to tibia. Overall, 56 genes were differentially expressed in the otic capsule compared to parietal bone or tibia.

Genes that differentiate bone types

Genes that are most characteristic of a bone type are characterized by a large difference and a small variance between bone types. Two genes were sufficient at each cross validation step to correctly predict 8 out of 9 samples when comparing otic capsules with parietal bones. These two genes were allowed to vary for each step of cross validation, guided by which sample was withheld to build a predictor. Although there were 18 (i.e. 9×2) distinct possibilities for the two genes, 8 genes were used through all steps of the crossvalidation procedure: *bmpr1b* was used 7 times, *bmp7* was used 3 times, *tnni3* was used twice, *col1a2* was used twice, and *lep*, *il6*, *socs1* and *carma1* were each used once.

When comparing otic capsules with tibias, two genes were also sufficient at each cross validation step to correctly predict 9 out of 10 samples. Six distinct genes (out of possible 10×2=20) were used through all steps of the cross-validation procedure: *opg* was used 7 times, *bmp3* was used 5 times, *tnfb1r* was used 3 times, *gjb6* was used 3 times, and *tnni3* and *col1a2* were each used once.

Osteoprotegerin, *bmpr1b* and *bmp3* emerged as key predictors of the otic capsule as they were most frequently used during the cross-validation procedure. Expression of *opg* in the otic capsule, described as mean +/− standard error of the mean, was 97.8 +/−2.5 times higher than in parietal bone (p=0.003) and 9.6+/−2.5 times higher than in tibia (p=0.007). Expression of *bmpr1b* was 6.7 +/−2.0 times higher in the otic capsule than the tibia (p=0.015), and was undetectable in parietal bone (p=0.008). Expression of *bmp3* in the otic capsule was 5.3+/−1.4 times lower than in the tibia (p=0.008), and not different from expression in parietal bone (p=0.32). The expression data in Fig. 1 are suggestive of this particular choice of predictor genes because the ordering of genes in Fig. 1 is according to the sharpness of the transition between any two bone types. The ordering is performed separately for genes with decreased expression level between the two bone types (“red to blue transition”), and for genes with increased expression level (“blue to red transition”). The most prominent genes in the first ranking are displayed at the top of the map, and the most prominent members of the second ranking are displayed starting in the middle of the map. Given that the two ranking sets are of unequal size in general, the least prominent members of the larger set are used to “fill in” the slots in the other set.

Although in situ hybridization detected *opg* mRNA in the otic capsule, even higher levels of expression were found in the soft tissues of the cochlea, i.e. spiral ganglion neurons, root cells of the spiral ligament and interdental cells of the spiral limbus (Fig. 2). The colorimetric reaction used to visualize *opg* mRNA in Fig. 2 produces a specific dark brown signal; diffuse light brown staining, such as in the spiral ligament, is nonspecific, and attributed to electrostatic interaction between the tissue and the probe (Tarenghi, 1998). *Bmpr1b* was expressed in cartilaginous cell rests of the otic capsule and to a lesser degree in fibrocytes of the spiral ligament (Fig. 3). Hybridization with sense probes produced no signal (not shown).

Clustering of genes within the otic capsule

Genes in the otic capsule that share similar patterns of expression are depicted in a square heat map of consensus clustering (Fig. 4). The map illustrates the fraction of instances when any two genes are clustered together across the resampling iterations. When this fraction is 1, genes always cluster, as illustrated by a bright red color in the heat map; when the fraction is 0, genes never cluster, as illustrated by a dark blue color in the heat map. Figure 4 depicts

desirable clustering because the entries of diagonal blocks are close to 1 and off-diagonal blocks are close to 0. The figure depicts 3 clusters, which was optimal for our data and resulted in agreement between SOM and hierarchical clustering. The coupling was strong within clusters and minimal between them.

The mediators of inflammation belonged to a distinct cluster (Fig. 4), which included pro-inflammatory cytokines tumor necrosis factor alpha (*tnfa*), interleukin 1 alpha and beta (*il1a*, *il1b*), interleukin 6 (*il6*), as well as tnfa receptor (*tnfa1r*) and transcription factor nuclear factor of kappa-B subunit 1 (*nfκb1*). All of these pro-inflammatory mediators were downregulated in the otic capsule compared to tibia (Fig. 1A). In contrast, interleukin 11 (*il11*), a cytokine with potent anti-inflammatory activity (Trepicchio and Dorner, 1998) was upregulated in the otic capsule compared to parietal bone (Fig. 1B). Within the otic capsule (Fig. 4), *il11* contributed to a distinct cluster, which included *opg*, *bmp3* and suppressor of cytokine signaling 1 (*socs1*) and 3 (*socs3*). The third cluster included *bmpr1b* and several genes involved with bone morphogenetic protein (BMP) signaling: BMP receptor 2 (*bmpr2*), *bmp4* and *bmp7*.

Discussion

The otic capsule has long been recognized as a histologically unique bone (Bast and Anson, 1949) and the current study unravels a part of the molecular basis for such uniqueness. The genes that we identified as being most characteristic of the otic capsule may not be unique because we did not study the whole genome.

Genes most characteristic of the otic capsule

The emergence of *opg* as the most characteristic gene of the otic capsule is reassuring because our previous work established the importance of that gene in the inhibition of otic capsule remodeling (Zehnder et al., 2005; Zehnder et al., 2006). The current in situ hybridization data are consistent with our reported immunohistochemical data for Opg protein, suggesting that cells most abundantly expressing *opg* mRNA also produce the highest levels of the secreted Opg protein. We additionally describe expression of *opg* in spiral ganglion neurons; functional significance of this finding remains to be determined. Higher levels of *opg* expression in the cochlear membranous labyrinth than the bony otic capsule (Fig. 2) is consistent with our earlier work (Zehnder et al., 2005). Taken together, high levels of *opg* expression in the otic capsule detected by quantitative RT-PCR may, at least in part, reflect contamination from the surrounding cochlear tissue or blood.

Another characteristic gene, *bmpr1b*, encodes for a receptor for BMPs that play essential roles in skeletal development and repair (Chen et al., 2004; Wozney and Rosen, 1998). The importance of BMP pathways for the formation of the otic capsule and maintenance of the membranous labyrinth was established in chicken embryos (Chang et al., 1999; Gerlach et al., 2000; Chang et al., 2002). Ectopic expression of the constitutively active form of *bmpr1b* caused cartilage overgrowth, whereas the dominant negative form of *bmpr1b* caused loss of cartilage (Chang et al., 2002). Inhibition of BMP signaling by Noggin leads to a loss of not only the otic capsule but also the membranous epithelium (Gerlach et al., 2000). Our results complement earlier work in embryonic avian otic capsule and suggest that *bmpr1b* continues to play an important role in the adult mammalian otic capsule. Expression of *bmpr1b* in cartilaginous rests is intriguing because these rests are unique histologic features of the otic endochondral bone, and they have been implicated in the development of otosclerosis (Bast and Anson, 1949). Specificity of *bmpr1b* expression in the otic capsule is corroborated by lack of *bmpr1b* expression in the parietal bone – an intramembranous bone whose development does not involve a cartilage intermediate. Transgenic and knockout mice have established that *bmpr1b* is required for limb chondrogenesis (Yi et al., 2000; Baur

et al., 2000) and bone formation (Zhao et al., 2002); otic capsules of these mice have not been studied. The described human mutations in *bmpr1b* (Lehmann et al., 2003; Demirhan et al., 2005) are typically not associated with hearing loss.

A final gene highly characteristic of the otic capsule was *bmp3* by virtue of its low expression in the otic capsule. The product of this gene is a negative regulator of bone density (Daluiski et al., 2001) and the most abundant BMP in the adult trabecular bone. Given that the otic capsule is the densest bone in the body, its low level of expression of a major negative regulator of bone density is not surprising. We analyzed inner ears of *bmp3* knockout mice (Daluiski et al., 2001) and *bmp3* over-expressor mice (Gamer et al., 2009) and found no gross abnormalities within the otic capsule or cochlear membranous labyrinth. This suggests a minimal role for *bmp3* in controlling remodeling of the otic capsule.

Inflammatory mediators are downregulated in the otic capsule

We found pro-inflammatory cytokines to be downregulated and anti-inflammatory cytokines to be upregulated in the otic capsule compared with tibia and parietal bone, respectively. Inhibition of inflammation in the otic capsule may be important for maintenance of normal hearing because elevated levels of pro-inflammatory cytokines in the cochlea are associated with hearing loss (Brodie and Yeung, 2004), and there is anatomic communication between the otic capsule and cochlear perilymph (Zehnder et al., 2005). Pro-inflammatory cytokines promote pathologic formation of new bone within the membranous labyrinth, known as labyrinthitis ossificans, which is associated with profound deafness and loss of vestibular function. Prompt suppression of inflammatory response by steroids can reduce the incidence of labyrinthitis ossificans and the accompanying hearing loss (Hartnick et al., 2001).

Clustering methods suggest mechanistic relatedness among genes

Although expression levels of individual mRNAs are not always predictive of protein expression and function, co-regulation of genes can predict function (Zhang et al., 2004). Our clustering analysis was based on the assumption that all genes are independent, although many genes that we studied share common biologic pathways. The assumption of independence allowed us to not only confirm that genes within the same biologic pathway cluster together, but also to suggest new functional relatedness among genes that were previously not studied in the otic capsule. For example, clustering of *bmpr1b* with BMPs (Fig. 4) is consistent with prior description of BMPs and their receptors in the otic capsule where they are thought to promote chondrocyte differentiation (Chang et al, 2002). Moreover, variants in human *BMP2* and *BMP4* genes have been associated with susceptibility to otosclerosis (Schrauwen et al., 2008). Other genes that cluster with *bmpr1b* include transforming growth factor beta 2 (*tgfb2*), which has been shown to stimulate chondrogenic differentiation in cultured periotic mesenchyme (Frenz, 2001), and collagen type II, alpha 1 (*col2a1*), which encodes for type II collagen in cartilaginous cell rests within the endochondral layer (Ishibe and Yoo, 1990). The remainder of the genes that cluster with *bmpr1b* were not previously described in the otic capsule. However, they are known to play important roles in cartilage and chondrocyte function.

We found that *tgfb1* gene, which has been associated with human otosclerosis (Thys et al., 2009), clustered with pro-inflammatory mediators, consistent with known pro-inflammatory effects of *tgfb1* in some settings (Blobe et al., 2000). Our data corroborate the view that otosclerosis involves an inflammatory stage, such as due to a host response to an inciting event (Harris and Keithley, 1993).

Implications for otosclerosis

Otosclerosis, a disease of the otic capsule characterized by abnormal bone remodeling, is influenced by genetic and environmental factors (reviewed by Ealy and Smith, 2009). Linkage studies have identified 8 loci that contribute to familial cases of otosclerosis. Specific causative genes within these loci, which contain many genes, remain to be identified. Genetic association studies, however, have implicated specific genes in pathogenesis of otosclerosis: COL1A1 (McKenna et al, 1998), TGFB1 (Thys et al, 2009), BMP2 (Schrauwen et al, 2008), BMP4 (Schrauwen et al, 2008) angiotensin I AGT (Imauchi et al, 2008), angiotensin I converting enzyme (ACE, Imauchi et al, 2008), and reelin (RELN, Schrauwen et al, 2009; Schrauwen et al, 2009b). Only COL1A1 was known at the time we began the current st udy. We found *col1a1* to be expressed at lower levels in the otic capsule than other bones (Fig. 1). However, *col1a2* – a binding partner of *col1a1* to form functional type I collagen – emerged as a more robust predictor of the otic capsule than *col1a1* in the current study. Studies have not found association of COL1A2 with otosclerosis (McKenna et al., 1998; Rodriguez et al., 2004).

We found *tgfb1* to be expressed at higher levels in the otic capsule than in parietal bone, and not significantly different than in tibia (Fig. 1). Clustering of *tgfb1* with pro-inflammatory mediators, as well as *col1a1* and *col1a2* (Fig. 4), suggests that future studies of pro-inflammatory cytokines in the otic capsule may provide mechanistic insights into pathogenesis of otosclerosis.

It is interesting that BMP2 and BMP4 have recently been associated with otosclerosis, and they bind to BMPR1B, which we have identified to be characteristic of the otic capsule. This suggests that understanding BMP signaling in the human otic capsule may be relevant for understand unique predisposition of this bone to otosclerosis. Mechanisms of AGT, ACE and RELN action in the otic capsule await further characterization.

Summary

We have shown that the molecular profile of the otic capsule is distinctly different from that of the tibia and parietal bone. The genes that we identified as being most characteristic of the otic capsule play a role in inhibition of bone remodeling. Dysregulation of these genes may contribute to the pathogenesis of otosclerosis, which is characterized by pathologic remodeling of the otic capsule.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Saumil Merchant for helpful comments on earlier versions of the manuscript. This work was supported by NIDCD Grant 5RO1 DC03401-06 (MJM), American Otological Association (KMS) and Mr. Lakshmi Mittal (MJM).

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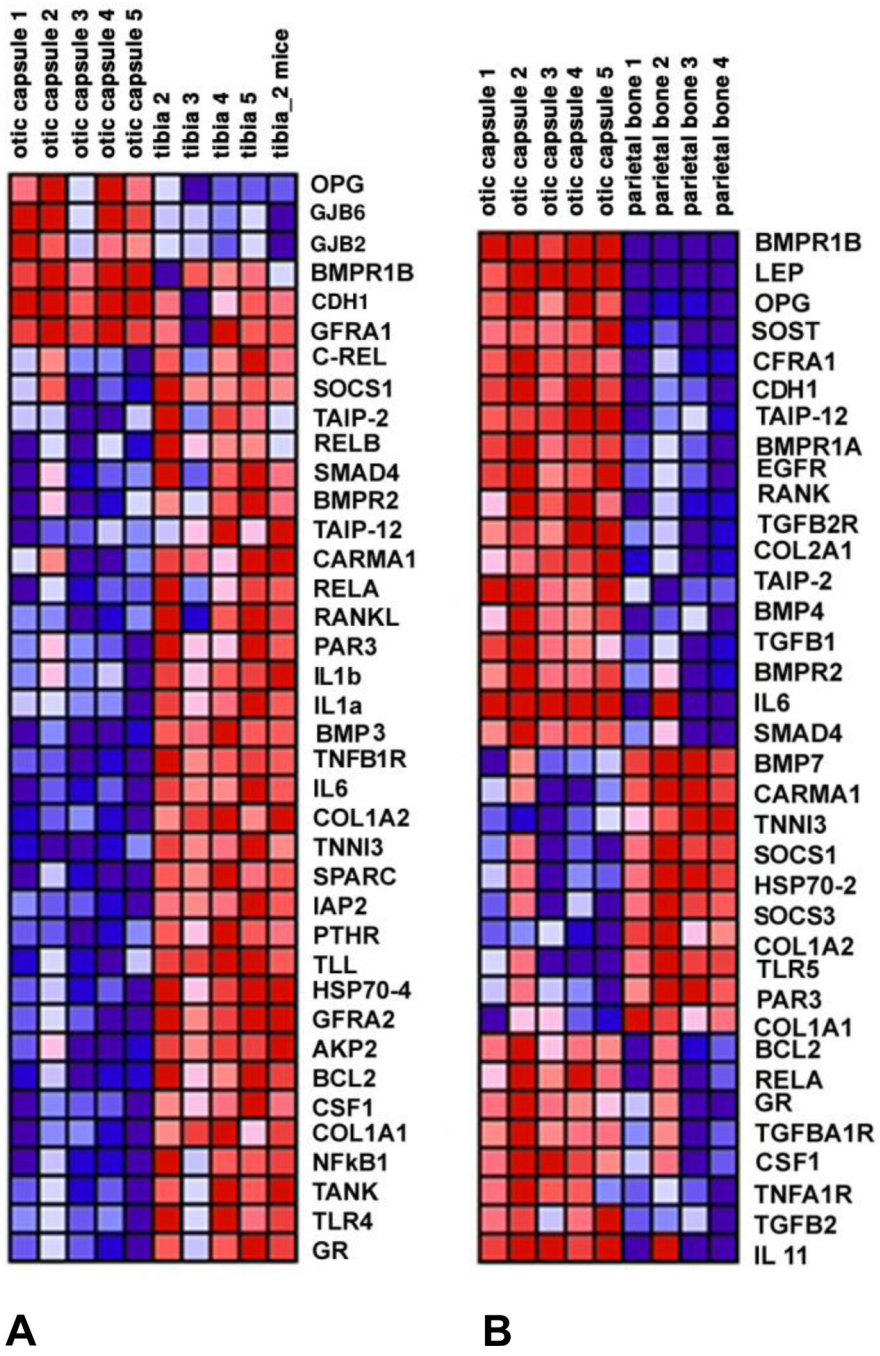

Figure 1.

Heat map of real-time quantitative RT-PCR data comparing the otic capsule with tibia (A) and parietal bone (B). Red indicates high and blue low levels of gene expression.

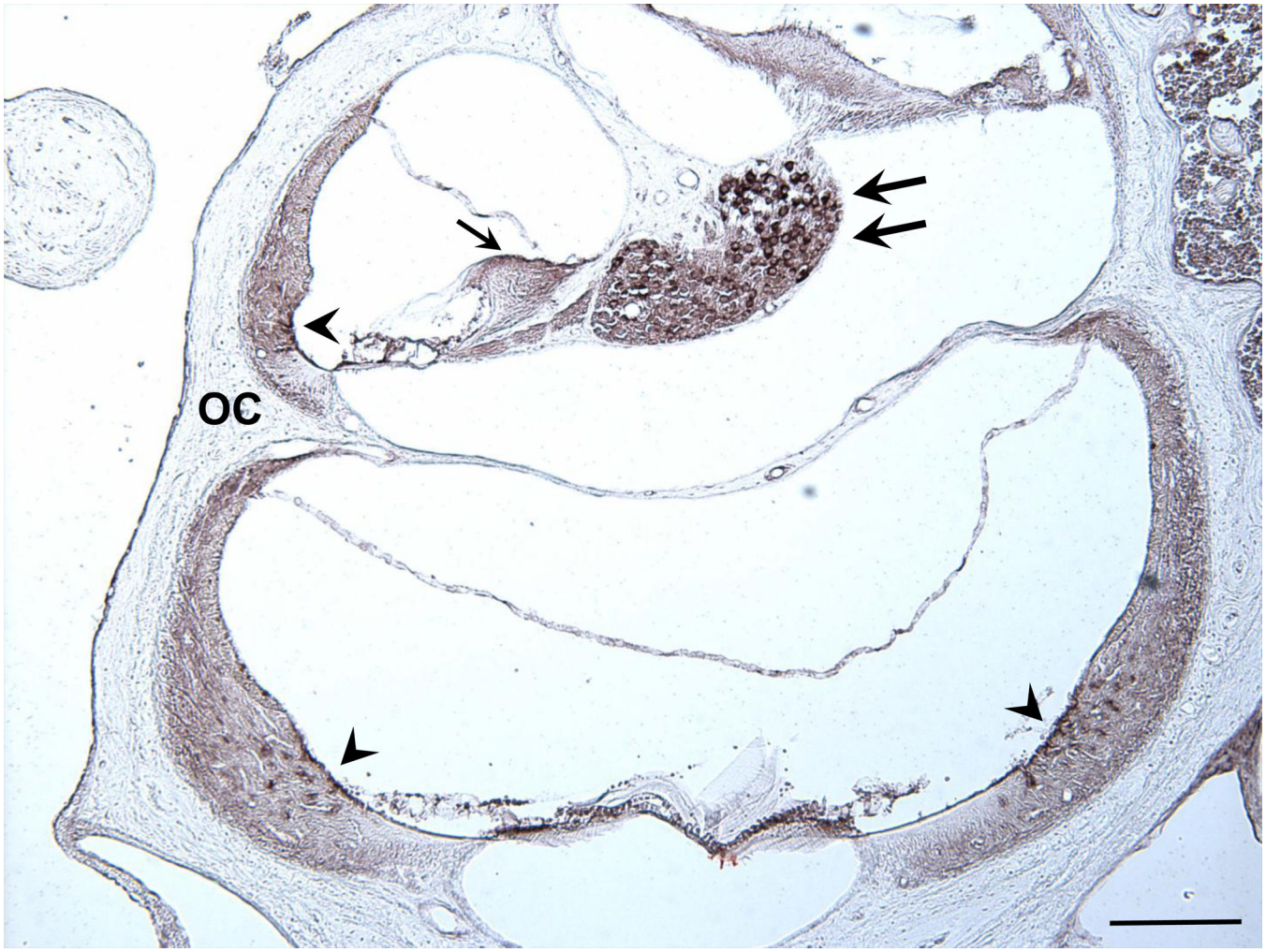

Figure 2.
A cross section through two cochlear turns hybridized with *opg* antisense probe. A strong signal (shown in dark brown) is present in root cells of the spiral ligament (arrowhead), interdental cells of the spiral limbus (single arrow) and spiral ganglion neurons (double arrows). A weaker signal is present in the otic capsule (OC). Hybridization with a sense probe produced no signal. Scale bar = 200 μm.

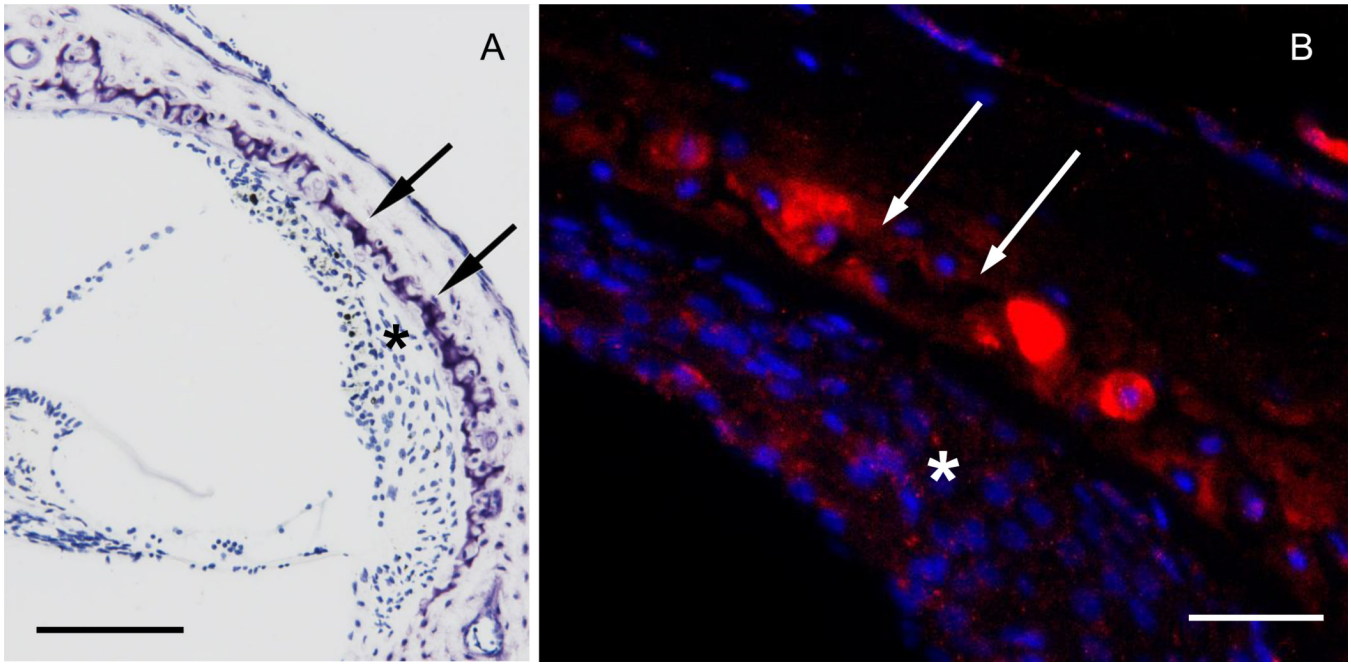

Figure 3.

Bmpr1b localizes to cartilaginous cell rests of the otic capsule. Cochlear cross section stained with azure (A), and with anti sense probe for *bmpr1b* (B). The red fluorescent signal (B) is most intense in cartilaginous rests (double arrows) and weaker in the spiral ligament (asterisk). Cell nuclei are in blue. Scale bar = 100 μm (A), 50 μm (B).

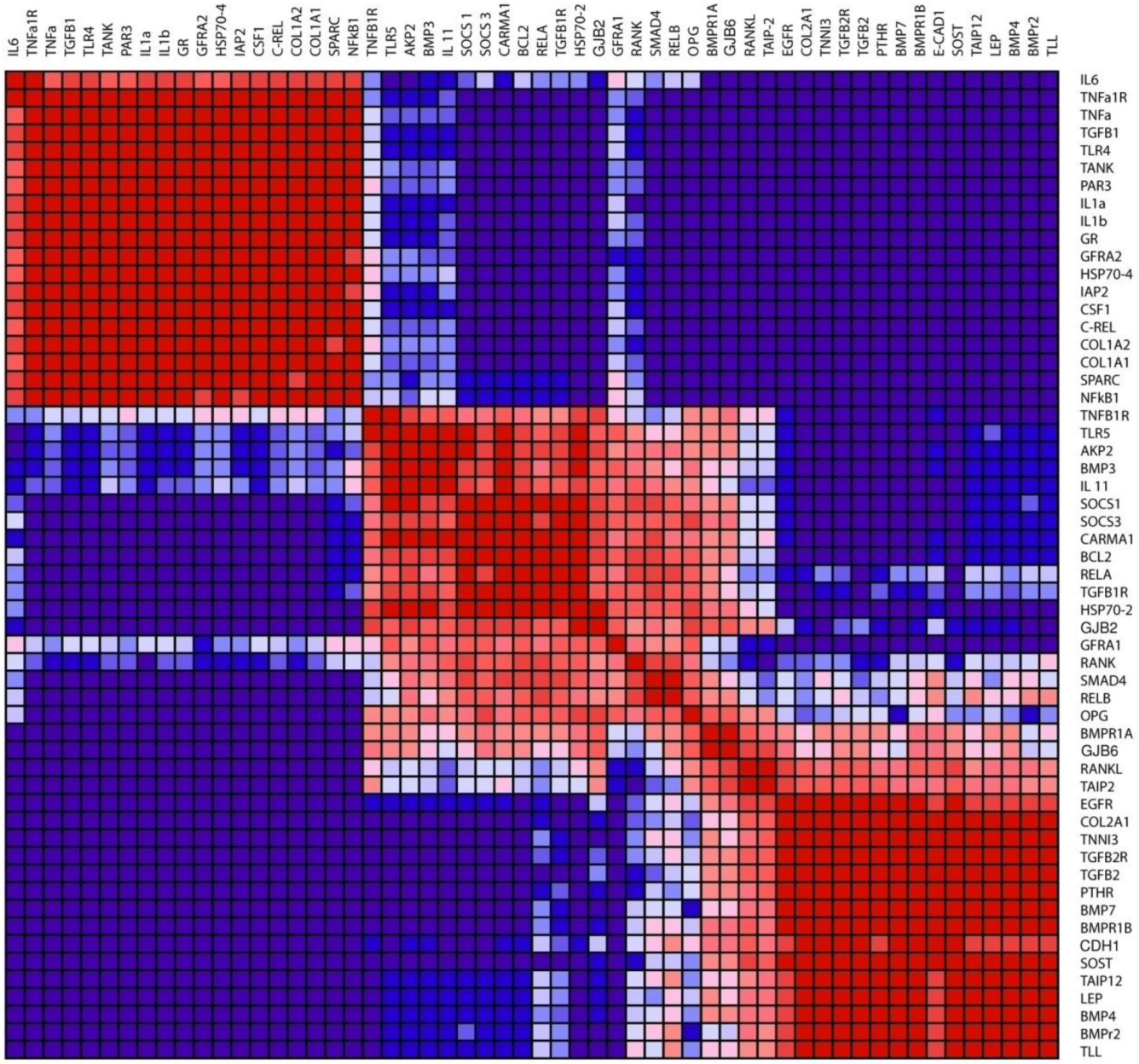

Figure 4.

Heat map of genes that share similar expression patterns within the otic capsule. Consensus SOM clustering was performed assuming three clusters.