

ORIGINAL ARTICLE

Expression of bone morphogenetic protein 2, 4, 5, and 7 correlates with histological activity of otosclerosisPÉTER CSOMOR¹, BALÁZS LIKTOR², BÁLINT LIKTOR², ZOLTÁN SZEKANECZ³, ISTVÁN SZIKLAI¹ & TAMÁS KAROSI¹¹Department of Otolaryngology and Head and Neck Surgery, Medical and Health Science Center, University of Debrecen, Debrecen, ²Department of Otolaryngology, Bajcsy-Zsilinszky Hospital, Budapest and ³Department of Rheumatology, Medical and Health Science Center, University of Debrecen, Debrecen, Hungary**Abstract**

Conclusion: This study is the first to establish that bone morphogenetic protein 5 (BMP5) plays a role in the pathogenesis of otosclerosis. These results confirm that elevated expression levels of BMPs, members of the transforming growth factor (TGF)- β superfamily, contribute to the pathologically increased bone turnover in early, active stages of otosclerosis. **Objectives:** Otosclerosis is a complex bone remodeling disorder of the otic capsule, which might be characterized by increased expression of different types of BMPs. TGF- β and BMP are both members of the TGF- β superfamily and play a critical role in bone resorption and new bone formation. It has been suggested that BMP and its receptors may be involved in the pathologically increased bone turnover observed in otosclerosis. **Methods:** Fifty-one otosclerotic and 16 non-otosclerotic ankylosed stapes footplates were histologically analyzed: conventional hematoxylin-eosin staining and BMP2, 4, 5, and 7-specific immunofluorescent assays were performed. Cortical bone fragments ($n = 35$) and incus specimens ($n = 6$) were used as negative controls. **Results:** Active otosclerosis ($n = 39$) was characterized by increased expression of BMP2, 4, 5, and 7. Inactive cases of otosclerosis ($n = 12$) were characterized by negative immunoreaction for BMPs. Non-otosclerotic stapes specimens ($n = 16$) and negative controls ($n = 41$) showed negligible BMP expression. The BMP expression pattern showed a strong correlation with the histological activity of otosclerosis.

Keywords: Bone remodeling disorder, non-otosclerotic stapes fixation, stapes**Introduction**

Otosclerosis is a primary disorder of bone remodeling unique to the human otic capsule and is characterized by disturbed equilibrium of bone resorption and new bone formation [1,2]. Otosclerotic foci usually appear in the stapes footplate and in the pericochlear region and lead to progressive conductive and/or sensorineural hearing loss as a consequence of stapes footplate fixation and pericochlear bone resorption [1]. The fissa ante fenestram often contains fibrous tissue and immature cartilage and it is a preformed anatomic structure with a predilection for both stapedia and cochlear otosclerosis [1]. Otosclerosis is strictly limited

to the human otic capsule: no otosclerosis-like bone lesions have been reported outside of the ear [1,3]. Otosclerosis is a relatively common cause of hearing impairment in the Caucasian white population, with a prevalence of 0.3–0.4% [1]. Histological otosclerosis is much more common and it has been reported to have a prevalence of 8–11% in large unselected autopsy series [3]. According to previous reports, otosclerosis represents approximately 60% of stapes ankylosis cases leading to conductive hearing loss [1,4]. Several hypotheses suggested viral, inflammatory, autoimmune, metabolic, and genetic factors as possible links to otosclerosis; however, the etiopathogenesis has remained unexplained [1,5,6].

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(Received 18 October 2011; accepted 11 December 2011)

ISSN 0001-6489 print/ISSN 1651-2251 online © 2012 Informa Healthcare
DOI: 10.3109/00016489.2011.653669

Several genetic predisposition factors for otosclerosis have been reported over recent decades [1,6]. According to linkage studies, familial aggregation of patients with stapes fixation typically shows an autosomal dominant inheritance with incomplete penetrance and has promoted the identification of 10 otosclerosis-associated loci (OTSC1–10) [1,6]. These loci have been mapped and linked to otosclerosis; however, specific genes and proteins have not been identified yet [1,6]. In most patients, otosclerosis does not follow a regular Mendelian segregation, suggesting a complex disease caused by interconnected environmental and genetic factors [1,6]. It has been discussed that A1 and A2 alleles of type I collagen (COL1A1/A2), different types of bone morphogenetic protein (BMP2, 4, 7), transforming growth factor- β 1 (TGF- β 1), CD46-type measles virus receptor, and RELN genes may also be involved in the development of otosclerosis [7–11]. However, some of these genetic associations cannot explain female dominance, adult onset, and organ-limited pattern of otosclerosis [1,6,7,11].

In the last 5 years, BMPs and their receptors (BMPRs) have been reported to contribute to the pathogenesis of otosclerosis [8,12]. BMPs are a group of growth factors and belong to the TGF- β superfamily, so they also act as inflammatory cytokines [13,14]. At present, 20 distinct isoforms of BMP have been discovered; however, only 6 (BMPs 2–7) function as bone-specific growth factors [13,14]. BMP1 is not closely related to other known growth factors, since it is a matrix metalloproteinase [13,14]. BMPs 2–7 play an important role in the embryonic phase of endochondral bone development and later in new bone formation and repair [13,14]. BMPs 2–7 are excreted by embryonic chondrocytes, osteoid precursor cells, preosteoclasts, and inflammatory T cells [13,14]. Physiologically, BMPs act as disulfide-linked homodimers and induce bone and cartilage formation through their receptors [12–14]. Signal transduction process mediated by activated BMPRs results in mobilization and recruitment of SA (Smad and Mad related proteins) proteins [13,14]. Finally, SA induces the expression of TGF- β 1 [14]. BMPs and TGF- β 1 are critical regulators of bone turnover within the TGF- β superfamily [13,14]. In addition, an inverse interplay has been confirmed between BMP and TGF- β 1 pathways [13,14]. Disruption of BMP signaling and mutations in BMPs and in their inhibitors (noggin, chordin, sclerotisin) are associated with a variety of bone diseases, including fibrodysplasia ossificans progressiva and congenital stapes fixation with multiple synostoses-synphalangism syndrome [14,15]. Detection of increased BMP expression in otosclerosis might support the ‘embryonic

cartilage reactivation hypothesis’ in the pathogenesis of disease.

This study investigated the expression levels and patterns of different types of BMPs in otosclerotic and non-otosclerotic stapes footplates to elucidate the effective role of BMP association in the pathogenesis of otosclerosis.

Material and methods

Patients and controls

A total of 67 ankylotic stapes footplates ($n = 67$, male = 26, female = 41) removed by stapedectomy were analyzed. The mean age of patients was 42.28 years (range 25–65 years). The diagnosis of ossicular chain fixation was based on clinical, audiometric, and tympanometric findings. Air–bone gap at 1000 Hz was at least 30 dB on pure tone audiometry. Preoperative tympanometry revealed type As tympanograms in 79.1% ($n = 53$) and type A tympanograms in 20.9% ($n = 14$) of stapes fixation cases. Stapes fixation was clinically bilateral in 44 patients (65.7%); however, only one stapes obtained from each patient was analyzed, because over this period, only unilateral stapes surgeries have been performed. Partially removed stapes footplates were not included in the study, because the anterior or posterior poles containing the bone lesions fixing the stapes were retained in the oval window niche. Fragmented and reconstructed footplates were not excluded. Thirty-five cortical bone fragments harvested from the external ear canal during lateral atticotomy ($n = 35$) and six incus specimens removed during canal-wall-down (CWD) mastoidectomy were used as tissue-specific controls of BMP expression of stapes footplates. Cortical bones were obtained from patients with stapes ankylosis, while incus specimens originated from patients with middle ear cholesteatoma. Stapes footplates and control tissue specimens were collected from December 2009 until March 2011 (University of Debrecen, Medical and Health Science Center, Department of Otolaryngology and Head and Neck Surgery, Debrecen, Hungary; and ENT Department of Bajcsy-Zsilinszky Hospital, Budapest, Hungary). All patients gave their informed written consent to our study. The Hungarian Scientific Research Ethical Committee (ETT-TUKEB 84-227/2008-1018EKU) approved this study. The study was carried out according to the Declaration of Helsinki.

BMP 2, 4, and 7 specific immunofluorescent assay

A total of 67 ankylotic stapes footplates, cortical bone fragments ($n = 35$), and incus samples ($n = 6$) were

fixed in 10% (w/v) buffered formaldehyde and decalcified in 0.5 M Na-EDTA (sodium ethylenediaminetetraacetate, 72 h, 4°C) containing 0.02% (w/v) sodium azide. Then specimens were embedded in 15% (w/v) purified gelatin (24 h, 56°C) and refixed in 4% (w/v) paraformaldehyde (24 h, 20°C). Histological blocks were cryoprotected in 20% (w/v) saccharose solution (2 h, 4°C) and sectioned into 10 µm slides at -25°C (MNT-200, Slee, Mainz, Germany). Slides were stored in 0.1 M PBS (phosphate-buffered saline) containing 0.03% (w/v) sodium azide at 4°C.

Five consecutive 10 µm frozen cut sections were examined as follows. (1) Staining with hematoxylin and eosin (H&E); and (2) BMP2, (3) BMP4, (4) BMP5, and (5) BMP7 specific immunofluorescent assays (IFAs). First, sections of each individual specimen were processed by conventional H&E staining. The IFA series of sections were washed and protein blocked in Power Block™ Reagent (BG-BS-1310-25, Biogenex, Fremont, CA, USA). Blocked sections were incubated with 1:20 monoclonal mouse anti-human BMP2 primary antibody solution (20°C, 24 h, continuous shaking) (RD-MAB2260, R&D Systems, Minneapolis, MN, USA), with 1:20 polyclonal goat anti-human BMP2/4 primary antibody solution (20°C, 24 h, continuous shaking) (RD-AF355, R&D Systems), with 1:20 polyclonal goat anti-human BMP5 primary antibody solution (20°C, 24 h, continuous shaking) (RD-AF615, R&D Systems), and finally with 1:20 monoclonal mouse anti-human BMP7 primary antibody solution (20°C, 24 h, continuous shaking) (RD-MAB3542, R&D Systems). Primary antibodies were washed with 0.2 M PBS and sections were incubated with 1:200 Northern Lights™ donkey anti-goat IgG labeled by NL-493 fluorochrome (20°C, 6 h, continuous shaking) (R&D Systems) and with 1:200 Northern Lights™ donkey anti-mouse IgG labeled by NL-637 fluorochrome (20°C, 6 h, continuous shaking) (R&D Systems), respectively. All antibody incubations were performed in a sterile humid chamber. Slides were covered by UV transparent mounting medium (Vectashield™, Vector Laboratories, CA, USA). Imaging was performed under UV light with 510 nm and 470 nm wavelengths and 43–77 ms exposure time and the photographs were archived in jpeg file format (Axioskop2 MOT™, Axiovision 3.0™, Zeiss, Jena, Germany). Individual specimens were analyzed as 10–20 centrally focused, high magnification (× 40) fields according to the BMP2, 4, 5, and 7 specific immunofluorescent reactions. Histological examinations were blinded for two independent researchers: P.C. analyzed the sections stained by H&E, while T.K. interpreted the IFAs. The histopathological activity of otosclerosis was based on the cellularity and the structure of cement

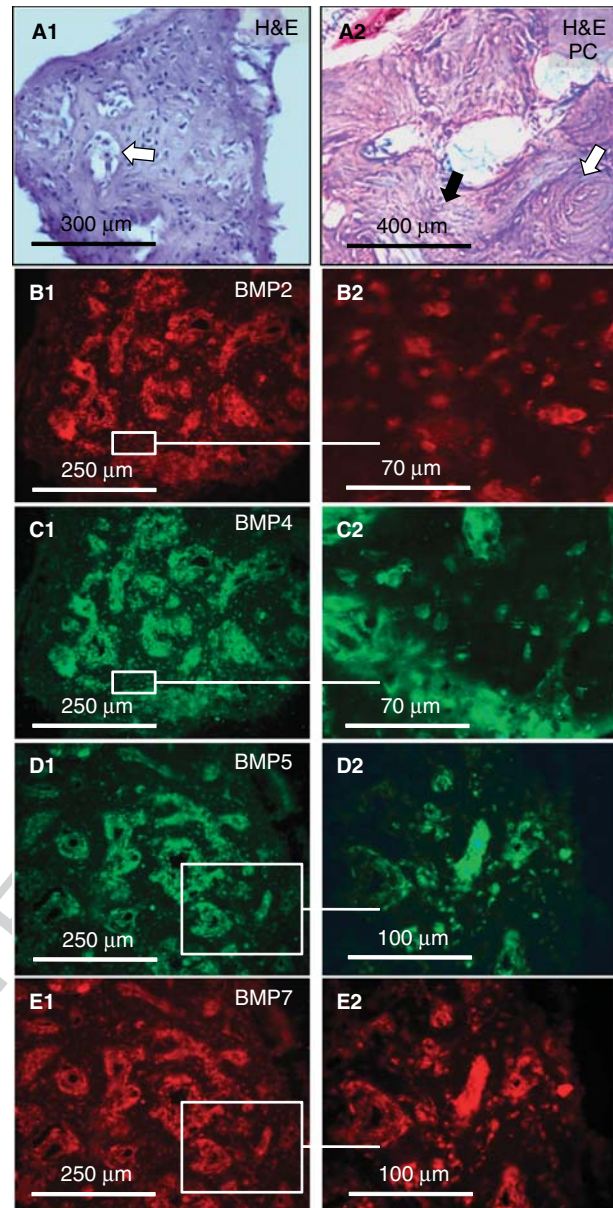


Figure 1. Immunofluorescent demonstration of BMP expression in active otosclerosis. (A1) Active focus of otosclerosis with hypercellular osteoid substance and several pseudo-vascular spaces (white arrow) (H&E, right ear). (A2) Phase-contrast (PC) microscopic view of the previous section. The cement lines show an irregular concentric (white arrow) and woven (black arrow) pattern. Immunofluorescent micrographs with different magnification views for BMP2 (B1, B2), BMP4 (C1, C2), BMP5 (D1, D2), and BMP7 (E1, E2) display intense granular cytoplasmic immunoreactions in the lesion-forming cells.

lines by conventional H&E staining. Since otosclerosis is a multifocal disease, the histopathological activity of a stapedia osteolytic focus is not in full correspondence with the clinical activity of otosclerosis [1,4]. We used the following histopathological criteria for the diagnosis of otosclerosis. (1) Active otosclerosis is displayed by wide pseudo-vascular

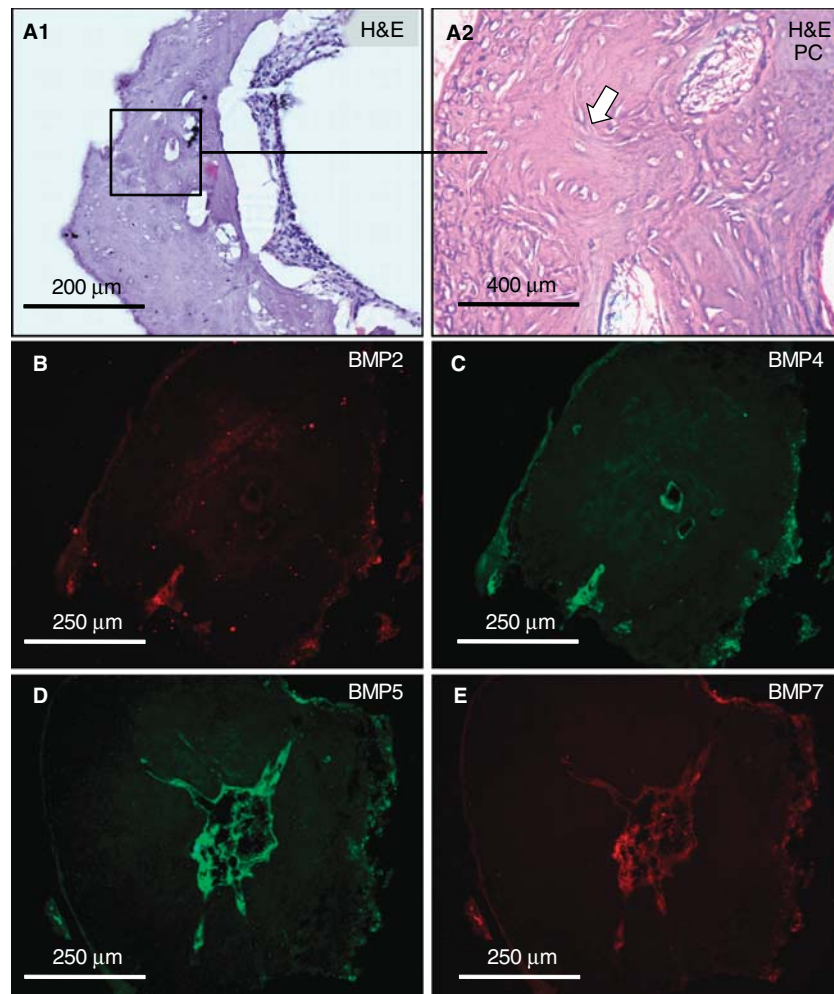


Figure 2. Immunofluorescent demonstration of BMP expression in inactive otosclerosis. (A1) Inactive otosclerotic focus at the posterior pole of the stapes footplate (H&E, right ear). The lesion is characterized by hypocellularity and empty cellular halos. (A2) Phase-contrast (PC) microscopic view of the previous section. Cement lines show lamellar pattern (white arrow). The remnants of osteoblasts and osteoclasts (empty halo cells) show negative immunofluorescent staining for BMP2 (B), BMP4 (C), BMP5 (D), and BMP7 (E).

spaces filled with increased numbers of large, misshapen, and multinucleate osteoclasts. Cement lines show a woven pattern. (2) Inactive otosclerosis is marked by obliterated vascular spaces and resorption lacunae with decreased numbers of osteoclasts (empty halo cells). Cement lines show a lamellar pattern.

Results

Ankylotic stapes footplates ($n = 67$), cortical bone fragments ($n = 35$), and incus specimens ($n = 6$) were analyzed by BMP2, 4, 5 and 7 specific IFAs and by conventional H&E staining, respectively. Histological diagnosis of otosclerosis was established in 51 ankylotic stapes footplates (Figures 1 and 2, Table I). Stapes footplates were involved by single and dominant otosclerotic foci. Among these specimens, foci of

otosclerosis were considered to be active in 39 stapes footplates and inactive in 12 cases (Figures 1 and 2, Table I). The localization of otosclerotic foci differed among the 51 patients with manifest involvement of the stapedia footplate by otosclerosis at the anterior pole ($n = 31$), posterior pole ($n = 7$), bipolar ($n = 6$) or obliteration ($n = 7$). In the 16 non-otosclerotic stapes footplates, histologic examinations revealed annular, stapediostibular calcification with marked thickening of the stapes footplate [4] (Figure 3, Table I). According to the clinical history and otoscopic findings of non-otosclerotic patients, tympanosclerosis due to chronic otitis media or familiar stapes fixation could be excluded.

Active otosclerosis cases were characterized by simultaneously increased expression of BMP2, 4, 5, and 7 that resulted in intense granular immunoreaction (Figure 1). Both osteoclasts and osteoblasts showed considerable positivity for different types of BMPs,

Table I. Assessment of expression of bone morphogenetic proteins (BMPs) 2, 4, 5, and 7 in ankylosed stapes footplates with different histopathologies and in controls.

Histopathology (H&E) (<i>n</i> = 108)	BMP2	BMP4	BMP5	BMP7
Otosclerosis (<i>n</i> = 51)				
Active focus (<i>n</i> = 39)	+++	++++	+++	+++
Inactive focus (<i>n</i> = 12)	-	+	-	-
Non-otosclerotic stapes fixation (<i>n</i> = 16)				
Annular calcification (<i>n</i> = 16)	-	-	-	-
Controls (<i>n</i> = 41)				
Cortical bone (<i>n</i> = 35)	-	-	-	-
Incus (<i>n</i> = 6)	-	-	-	-

Subjective intensity assessment of immunofluorescence assays (IFAs) by the analysis of 10 centrally focused magnification fields ($\times 40$): +, weak annular immunoreactivity; ++, weak homogeneous immunoreactivity; +++, strong homogeneous or weak granular immunoreactivity; +++++, strong confluent and robust granular reaction.

indicating active bone remodeling and new bone formation within the lesion (Figure 1). The BMP immunoreactivity varied between different parts of the stapes samples. The superstructures were negative for BMPs, while the footplates containing the lesion and the hyaline cartilage layer of the vestibular surface displayed very similar expression patterns. The optimal exposure time was 43 ms for BMP-expressing otosclerotic specimens that were strongly positive by IFA. In BMP-negative samples (non-otosclerotic stapes footplates, control bone specimens), exposure time was extended until 77 ms to avoid false-negative results. In contrast, in inactive otosclerotic foci, no significant BMP expression could be demonstrated on the surface of cellular remnants (Figure 2). In these cases, expression levels and patterns of BMPs were strongly associated with the histopathological activity of otosclerosis (Table I). Non-otosclerotic stapes fixations displayed negative immunoreactions for BMPs (Figure 3, Table I). Cortical bones and incus specimens applied as negative controls for stapes fixation displayed negative BMP-specific immunoreactions (Figure 4, Table I). Finally, expression of different types of BMP was strongly associated with the histologic diagnosis of otosclerosis (Table I).

Discussion

Although several disease-associated monogenic loci have been mapped and linked to otosclerosis,

experiments to identify causative genes and their proteins have been unsuccessful [1,6]. In addition, candidate gene association studies on COL1A1, COL1A2, and RELN have generated conflicting results [7,11]. A notable difference with our study is that diagnosis of otosclerosis was based on the medical history and audiometric findings of patients with stapes fixation without histopathological analysis of the surgically removed stapes footplates [6,9,11]. It might be explained by the limitations of stapedotomy, which is not a suitable method to obtain stapes footplate fragments containing the fixing lesions.

This study aimed to carry out precise immunohistological assessment of BMP expression in otosclerotic and non-otosclerotic stapes footplates compared to bone-specific controls. The results shown in this report are of value, since only three studies have reported a potential association between increased BMP expression and otosclerosis [8,12,16]. Furthermore, we confirmed the observations reported by Lehnerdt et al. that suspected BMP2, 4, and 7 as real causative agents in the pathogenesis of otosclerosis [8]. According to our current knowledge, this is the first study in the literature to evaluate the role of BMP5 in the pathogenesis of otosclerosis. The present study indirectly supports the proposition that increased expression of BMP2, BMP4, BMP5, and BMP7, four members of the TGF- β superfamily, also plays a role in the otosclerotic bone remodeling disorder.

It has been suggested that different types of BMP and BMPRs may be involved in the pathologically increased bone turnover observed in otosclerosis [8,12,16]. As pioneers, Lehnerdt et al. have performed immunohistochemical analysis of BMPs and BMPRs in otosclerotic stapes footplates [8]. Significantly increased BMP2, BMP4, and BMP7 expression was demonstrated within the otosclerotic foci compared with normal and non-otosclerotic bone specimens [8]. Among BMPRs, BMPR-1B and BMPR-2 showed increased immunoreactivity, whereas BMPR-1A always exerted negative reaction [12]. Thus, the effects of BMPs may be mediated through type 1B and type 2 BMPRs in otosclerosis [12]. Later, the same group carried out immunofluorescent typing of different BMP isoforms in active otosclerotic tissues. Again, consequent expressions of BMP2, BMP4, and BMP7 were demonstrated [12]. Schrauwen et al. have reported the potential role of various BMP isoforms in the risk of otosclerosis [16]. There was a significant association between clinical otosclerosis and the increased expression of BMP2 and BMP4 [16]. Authors have reported two otosclerosis-associated single nucleotide polymorphisms (SNPs) in the genes of BMP2 and BMP4 [16].

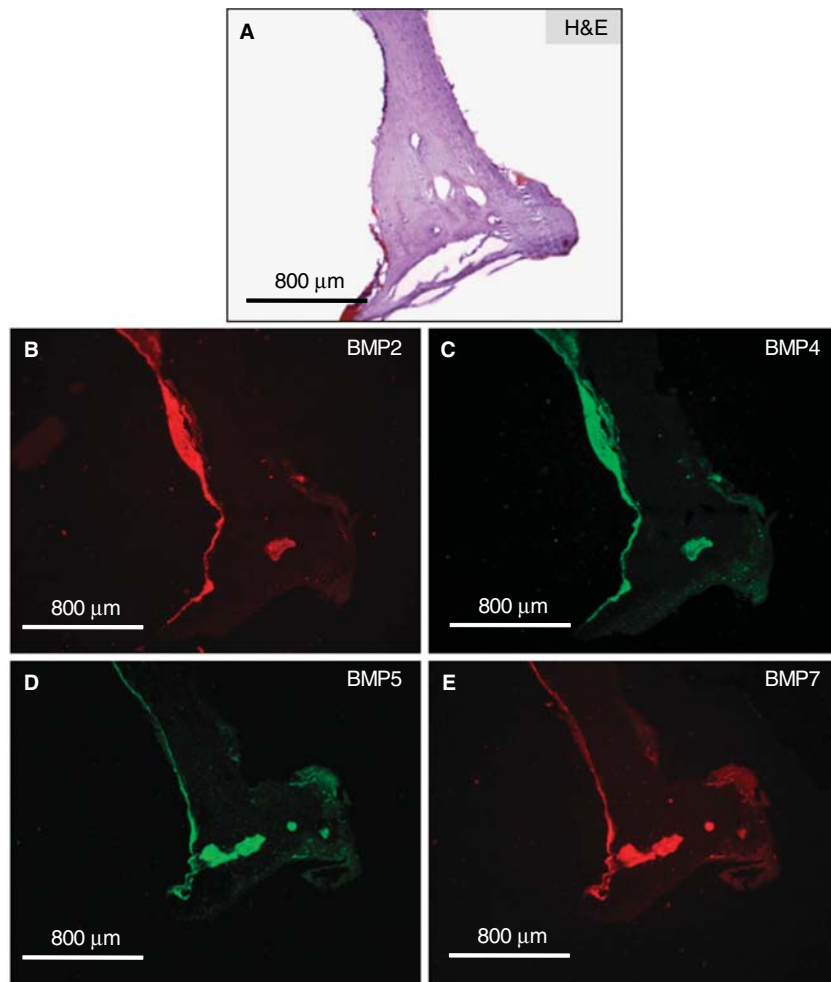


Figure 3. Immunofluorescent demonstration of BMP expression in non-otosclerotic stapes fixation. (A) Non-otosclerotic, annular calcification of the stapes footplate with hypocellular and angled anterior pole (H&E, right ear). The osteocytes show negative immunoreactions for BMP2 (B), BMP4 (C), BMP5 (D), and BMP7 (E).

The first non-coding SNP, rs3178250 (BMP2), was located in a 3' untranslated region, while the second, rs17563 (BMP4), led to amino acid exchange in BMP4 that was strongly associated with otosclerosis [16]. These observations are reasonable to assume, since both BMPs and TGF- β are key regulators of new bone formation. In addition, there are several interconnected events in the BMP and TGF- β pathways that result in inverse interactions between the two protein groups [17,18].

In the embryonic phase and in the early postnatal period, a regular enchondral ossification develops in the otic capsule and in the stapes footplate, which is completed after 1 year [1]. Osteoblastic and osteoclastic activity, normally associated with bone turnover, is rarely, if ever, seen in the adult otic capsule [1,2]. There is a potential explanation for this phenomenon: low-grade bone turnover protects the membranous labyrinth and the organ of Corti from

serious biomechanical changes during life [1,2]. The otic capsule is formed by three bone-like layers, i.e. the endosteal layer next to the perilymphatic space, the interosseal globules (*globuli inerossei*), which are embryonic cartilage remnants, and the periosteal layer [1]. The enchondral layer of the otic capsule contains peculiar, embryonic chondrocytes, which persist throughout life [1,19]. Similar cells can be found in the vestibular layer of the stapes footplate. It has been reported that these remnants may be the sites of the earliest otosclerotic foci [1,19]. However, these cells disappear from otosclerotic foci and are replaced by pre-osteoclasts and active osteoclasts [19]. In contrast to normal adult otic capsule, osteoclasts and osteoblasts of otosclerotic foci express a special embryonic glycoprotein (CD51/61 – osteoclast functional antigen) [19]. In addition, increased expression of BMPs, TGF- β , tumor necrosis factor (TNF)- α , and apoptosis inhibitors (hCIAP1/2) also supports

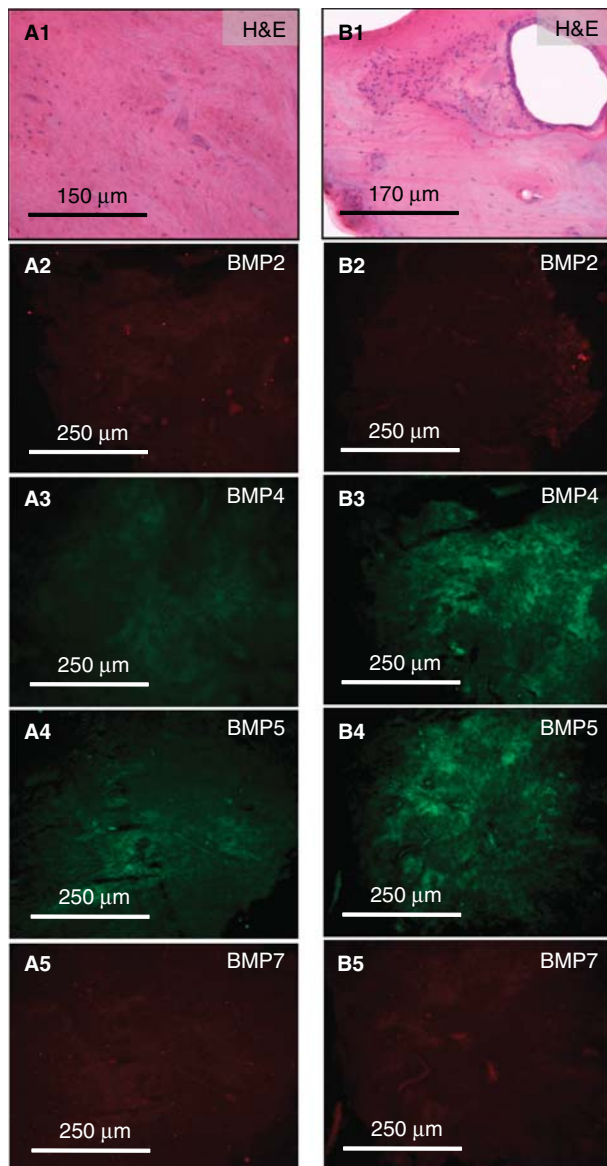


Figure 4. Immunofluorescent demonstration of BMP expression in bone-specific controls. (A1) Body of the incus that represents a normal structure of cement lines and blood vessels (H&E, left ear). The osteocytes represent negative immunoreactions for BMP2 (A2), BMP4 (A3), BMP5 (A4), and BMP7 (A5). (B1) Cortical bone fragment harvested from the posterior wall of the external auditory canal (H&E, left ear). The osteocytes do not display detectable expression of BMP2 (B2), BMP4 (B3), BMP5 (B4), or BMP7 (B5).

the embryonic chondrocyte reactivation hypothesis in otosclerosis [1,8,9,20]. According to previous reports, chondroblasts and osteoblasts might be reactivated and transformed into osteoclasts due to the inflammatory response induced by measles virus infection [5,19,20].

In conclusion, otosclerosis can be considered as a complex bone remodeling disorder that is influenced by genetic, environmental, and metabolic factors.

According to current and previous results, it can be suggested that different types of BMPs may play a significant role in the early molecular events of active otosclerosis. In the light of this association, further studies are necessary to determine the role of BMP in the pathophysiology of otosclerosis to find the most promising medical (biological) treatment for early active stages.

Acknowledgments

This work was supported by grants from the Hungarian Scientific Research Fund (OTKA PD75371, K81480) and Mecénatúra Fund of University of Debrecen (DEOEC Mec 17/2008).

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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