Osteoblast Cultures from the Stapes of Patients with Otosclerosis Demonstrate Hypermineralization, which is Corrected by Alendronate

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INTRODUCTION
Otosclerosis, a focal disease that exclusively affects the otic capsule of the temporal bone, is among the most common causes of acquired deafness in the US. It affects between 0.1 to 1% of the general population. A relationship between otosclerosis and the potential for developing osteoporosis is apparent in many of these patients. The histopathology of otosclerosis involves three stages: spongiosis, fibrosis, and sclerosis with the early stage having excessive bone remodeling and the later stage resulting in hypermineralization. To better understand the pathophysiology of otosclerosis, we developed and characterized for the first time osteoblast-like cultures grown from otosclerotic stapes and compared them to osteoblast and normal stapes cultures. Cultures were treated with bisphosphonates since there is some evidence that bisphosphonates, currently used for the treatment of osteoporosis, may be helpful in preventing hearing loss associated with otosclerosis.

METHODS
Osteoblast-like cultures were grown from otosclerotic stapes (OSO; 9 patients) and compared to human osteoblasts (HOB: 10 patients) cultured from peripheral bone fragments discarded during orthopaedic procedures of patients matched by age and sex, and normal stapes (NSO; 4 patients). Specimens were de-identified, morselized and cultured in DMEM/F-12, 10%FBS and antibiotics to obtain cell outgrowth. Cells were passaged once before plating at 10,000 cells/cm². Each patients’ cells were used for separate experiments. To measure cell adhesion, cells were cultured for 4h, rinsed, trypsinized, and Coulter counted. To determine proliferation rates, [3H]-thymidine incorporation was determined in the last 4h of 72h of culture. For mineralization, cells were switched after 1 week to mineralization medium (α-MEM, 10% FBS, antibiotics, 2 mM β-glycerol phosphate, 100 µg/ml ascorbic acid). Mineralization was quantified using Eagle Diagnostics Kit (DeSoto, TX). RNA was extracted from cell pellets using the RNeasy kit from Qiagen (Valencia, CA). Relative transcript levels were measured by quantitative PCR in triplicate using ABI PRISM 7300 sequence detection system (Applied Biosystems, Foster City, CA) following protocol for SYBR-Green, and normalized to GAPDH. The primers for amplifications were for Runx2, alkaline phosphatase, osteocalcin, type I collagen, osteopontin, RANKL & OPG

RESULTS
Cells were assayed after 4h of culture and demonstrated significantly (p<0.001) higher OSO attachment compared to NSO and HOB. By 72h all cultures had no significant differences in DNA content due to OSO having decreased proliferation rates. At 72h, OSO proliferation was significantly (p<0.01) lower than for HOB and NSO. OSO cultures displayed significantly higher calcium content (fig 1, OSO C vs. HOB C **=p<0.05) and mineralized nodules detected by xylene orange staining than NSO and HOBs at 14d and 21d. At 7, 14 and 21d, Runx2 expression was found in all cultures. Type I collagen, alkaline phosphatase, bone sialoprotein and osteocalcin were expressed at varying levels but the most significant difference was found in osteopontin, which was expressed more than 10-fold in NSO compared to OSO at 7d. RANKL and OPG levels were higher in OSO compared to NSO and HOB. All cultures were also treated with the bisphosphonate, alendronate (ALN, Sigma) 10⁻¹⁰M-10⁻⁸M. ALN did not significantly affect HOBs. However, OSO adhesion and proliferation rates were no longer significantly different from HOB after 1 week pretreatment with ALN. A dose-dependent decrease in calcium content was found in ALN-treated OSO cultures with a significant decrease (fig 1, *=p<0.05, 14d) at all concentrations so that they were not significantly different from HOB. ALN had no significant effect on HOB calcium content.

DISCUSSION
Otosclerotic osteoblasts, OSO, demonstrate significantly increased cell adhesion, mineralization, and decreased proliferation compared to osteoblasts from normal stapes and peripheral bone. OSO also had decreased osteopontin levels suggesting that osteopontin deficiency may play a role in hypermineralization Alendronate treatment altered the properties of OSO so that they were no longer significantly different from osteoblast cultures of HOBs.

SIGNIFICANCE
Human osteoblast cultures can be used to study otosclerosis and demonstrate hypermineralization and significantly altered adhesion and proliferation rates in cultures from otosclerotic patients compared to cultures from normal stapes and peripheral bone. In addition, alendronate may have therapeutic potential in normalizing the properties of otosclerotic osteoblasts.

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Figure1

Calcium Content (µg/ml)

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<th>Concentration</th>
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<th>HOB</th>
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