All surgical procedures were approved by the Boston University IACUC. Medtronics Inc. provided the human DBM.

Demineralized bone matrix (DBM) is a commercially available biomaterial rendered from cadaveric human bones and is comprised of collagen, non-collagenous proteins, growth factors, residual calcium phosphate mineral and cellular debris. DBM is currently FDA-approved for spinal fusion surgery [1,2]. DBM has both osteoconductive and osteoinductive properties. It is generally believed that the inductive properties are derived from various bone morphogenetic proteins (BMPs) within DBM. The activity of DBM is primarily determined in vivo by implanting it into a muscle and evaluating its ability to induce ectopic bone formation [1]. DBM however, is used clinically by placing it in proximity to the periosteal surface at a surgery site. We hypothesized that the amount of DBM-induced ectopic bone would be dependent on the tissue microenvironment and/or cell responsiveness to DBM’s osteoinductive and osteoconductive properties at a specific implant site. To test this hypothesis, we compared ectopic bone formation of DBM implanted on the periosteal surface to that of DBM implanted in a muscle pouch, either alone or supplemented with varying concentrations of BMP2. Collagen sponges, with and without BMP2 supplementation, were used as controls.

Methods: All surgical procedures were approved by the Boston University IACUC. Medtronics Inc. provided the human DBM (Grafton® Putty) via an MTA. Fifty milligrams of DBM or saline-moistened collagen sponge was implanted adjacent to the periosteal surface of the mid-diaphyseal region of the femur or placed within a muscle pouch of the upper hind limb of the immune-deficient mice, B6,129S7-Rag1tm1/MOM/J. For all collagen sponges and for DBM implanted into the muscle pouch, implanted material was wetted with a BMP2 solution at varying amounts (0.1-5.0μg). A subset of animals received periosteal implanted DBM wetted with the BMP antagonist Noggin (5.0-10.0μg). Ectopic bone formation at the periosteal surface was followed for 31 days to temporally define the developmental stages of the bone formation. RNA was isolated from the implants, femur and muscle contiguous to the ectopic bone from 2-31 days. RT-qPCR quantified mRNA expression of BMP2 and of genes associated with stem cell recruitment (Nanog, Oct4, Sox2), chondrocytes (Aggrecan and Collagen10a), and osteoblast/osteocyte (Osteocalcin and SOST). Analysis of mRNA expression for the DBM implants in the muscle was extended to day 40 due to lesser extent of bone formation. Plain-film radiographs, micro-CT analysis and histology were used to assess ectopic bone formation at day 16.

Results: DBM implanted on the periosteal surface induced extensive de novo bone compared to the muscle implant. Analysis of the implant at the periosteal surface across time showed an increase in bone volume fraction (BV/TV) through day 24 followed by a slight decrease until day 31. The ectopic bone adjacent to the periosteal surface showed elevated stem cell markers at day 2 followed by a steady decrease through day 31. The early peak in stem cell markers was followed by peaks in expression of chondrocyte markers at day 8 and osteoblast/osteocyte markers at days 12 and 16 (Figure 1). Assessment of BMP2 expression showed a strong peak at day 8 in the implant tissues, after which the levels decreased. In contrast, BMP2 expression in the surrounding muscle of the ectopic bone adjacent to the periosteal surface was strongly induced and showed elevated expression through a 12-day period after which it tapered off. Addition of Noggin to the DBM blocked ectopic bone formation in a dose-dependent manner (Figure 2). DBM implanted in the muscle pouch did not induce ectopic bone even after 40 days, although the tissue at the site of implantation showed weak expression of Sox9 and Col2a1. However, addition of exogenous BMP2 to muscle-implanted DBM induced ectopic bone formation in a dose-dependent manner and in amounts three-fold greater than that seen in BMP2-supplemented collagen sponges implanted in the muscle pouch (Figure 3). Finally, BMP2-supplemented collagen sponges in the muscle pouch adjacent to the periosteal surface exhibited an identical dose response to BMP2 as was seen for the BMP2-supplemented DBM in the muscle pouch, which itself was comparable to DBM implanted alone on the periosteal surface.

Discussion: The results of this study demonstrated that ectopic bone formation in response to either DBM or BMP2 is tissue specific, with the placement of DBM on the periosteal surface showing a much more robust ectopic bone formation than seen when DBM is placed in the muscle. These results also demonstrate that independent of site, ectopic bone formation is dependent on BMP signaling, in that Noggin-supplemented DBM was not able to initiate bone formation. Since DBM contains such low levels of BMP2 (61.3-29.0 ng/g) and BMP7 (84.7-78.6 ng/g) with high intra-and inter-preparation variability [2], it is unlikely that additive BMP signaling is solely responsible for the synergism of BMP2 and DBM in the induction of bone formation. Rather, these findings are suggestive that DBM contains non-BMP, osteo-inductive and/or -conductive factors that synergize with BMP, perhaps in the recruitment of osteogenic stem cells. The comparative differences of the inductive nature DBM on the periosteal surface versus in the muscle, and the dose-dependent response to BMP2-supplemented DBM in the muscle, are further suggestive of some limiting factors within the muscle microenvironment that prevent ectopic bone formation. These limiting factors may include an inability to initiate endogenous BMP2 expression to augment exogenous BMP activity, the
presence of BMP antagonists within the muscle, and an insufficiency in local stem cell numbers and/or responsiveness to BMP2.

**Significance:** Demineralized bone matrix is a commonly used biomaterial in orthopaedic surgery, yet the composition that is required for maximum results in bone regeneration is largely unknown. Testing of DBM bioactivity is optimal on the periosteal surface. Very low levels of BMP supplementation greatly augment the bioactivity of DBM. There is a need to further characterize the non-BMP bioactivities in DBM that synergize with BMPs.

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