Comparative Analysis of Osteoinductivity Testing Methods

Rebecca Jaw, MS; Elizabeth Silveira, BS; Joan Yonchek, BS, HT;
James Kirk, PhD; and Chandra Nataraj, BVSc, PhD (RTI Biologics Inc., Alachua, FL)

Introduction:

Most commercially available bone paste products consist of demineralized bone matrix (DBM) with a carrier material to aid in the delivery of DBM. The osteoinductivity (OI) of DBM varies from donor to donor as a result of varying levels of multiple growth factors, matrix integrity and material processing. This quality of DBM (OI) has been assessed using both *in vitro* and *in vivo* assays.

The *in vitro* assays are largely dependent on measuring growth factor levels, usually of bone morphogenetic proteins (BMPs), in the DBM. Levels of growth factors can be assessed directly by antibody-based assays, such as an enzyme linked immunosorbent assay (ELISA), or indirectly by their ability to differentiate and/or support the growth of osteoblast-like cells in culture.

The *in vivo* method relies on more complex chemical and biological properties of DBM, and is usually assessed by means of the ectopic bone formation in rodents, as originally described by Urist. The ectopic assay not only indirectly assesses growth factors, it is also dependent on specific matrix cues, particle size, and processing methods.

With a variety of DBM-based products in the market, it is critical to identify a reliable method to assess the quality of DBM. The desirable features in a quality control assay should be 1) repeatability and 2) sensitivity. The objective of this study was to determine the correlation between DBM-induced ectopic bone formation in the rat and two common *in vitro* assays for assessing DBM osteoinductivity.

Materials and Methods:

DBM

The DBM used for this study was prepared from multiple donors collected at random with no emphasis on age or sex at RTI Biologics, Inc. (Alachua, FL). The demineralization process was performed within processing standards as described by the American Association of Tissue Banks (AATB).

ELISA

Levels of BMP 2/4 and TGF-β1 growth factors were assessed using commercially available ELISA kits (R&D Systems, Minneapolis, MN) on 304 DBM samples. Concentrations of BMPs and TGF-β1 were expressed as pictograms (BMP-2/4) or nanograms (TGF-β1) of growth factor per gram of DBM.

Cell Culture

Mitotic stimulatory activity of 239 DBM samples was assessed using SAOS-2 human osteoblast cells. Here, the ability of DBM to either promote transformation of stem cells / pre-osteoblasts to an osteoblast phenotype[3], or their ability to enhance the growth of osteoblasts/osteoblast-like cells are assessed.[4,5]

Ectopic Bone Formation Model

All 543 DBM samples used in the ELISA and SAOS-2 assays were analyzed for biological activity using the *in vivo* ectopic model. For this assay, samples of each DBM preparation were prepared and implanted into the muscle pouch of athymic nude rats. The rats were euthanized 28 days later, and the explants were reviewed histologically following Hematoxylin-Eosin staining. Biological activity (OI) of DBM was expressed using a semiquantitative histological scoring system (Table 1).

OI Score	% New Bone Formation
0	No New Bone Formation
1	1% - 25% New Bone
2	26% - 50% New Bone
3	51% - 75% New Bone
4	>75% New Bone

Table 1: AATB approved scoring of explant histology

Results:

To reliably predict the inductive potential of DBM preparations, the 304 samples of DBM tested for BMP-2/4 and TGF-β1, and the 239 samples of DBM tested through SAOS-2 cell culture assays were compared to an *in vivo* OI score of 0-4 from the same donor. A representative summary of the data results comparing the growth factor ELISA (Table 2), and the cell culture assay (Table 3) to the *in vivo* ectopic bone formation assay is shown below. The comparisons demonstrate that there are instances when samples exhibit a phenotype *in vitro* which they are unable to recapitulate *in vivo* (see those with *). Please refer to "Demineralized Bone Matrix as an Osteoinductive Biomaterial and *In Vitro* Predictors of Its Biological Potential" for detailed results.

Table 2: Comparing Growth Factor ELISA vs. in vivo Assay

Donor ID	BMP-4/2 (pg/g DBM)	TGF-b1 (pg/g DBM)	Pass Prob. (Algorithm)	Rat OI Score
XX22666	739.6	114667	89.05%	Non-Induct.
XX17798	1876.3	88330	99.01%*	Non-Induct.
XX17250	1354.5	68497	91.24%	Inductive
XX20327	396.9	127399	73.77%*	Inductive

In calculating the algorithm, a DBM with high pass probability is expected to form greater new bone formation when implanted in vivo. For the Rat OI score, explants showing less than 25% new bone formation are considered non-inductive. (Data representative of 304 donors.)

Table 3: Comparing cell culture assay vs. in vivo Assay

Donor ID	In Vitro Score	Rat OI Score
XX22666	84.6 ± 6% (Non-Induct.)	Non-Induct.
XX17798	88.1 ± 6% (Non-Induct.)	Non-Induct.
XX17250	125.9 ± 5% (Induct.)	Inductive
XX20327	150.8 ± 8% (Induct.)	Inductive
XX25859	146.0 ± 9% (Induct.)*	Non-Induct.
XX26382	130.5 ± 9% (Induct.)*	Non-Induct.

For the cell culture assays, DBM preparations having an in vivo score in excess of 100% (including standard deviation) are considered capable to support osteoblast growth, i.e. inductive. (Data representative of 220 donors.)

Conclusions:

The results from this study indicate the importance of an appropriate assay to qualify DBM preparations with regard to their biological activity. While *in vitro* assays are reasonable predictors of inductive potential of DBM preparations, the data shown above indicates there are limitations.

The *in vivo* model used in this study (ectopic bone formation with DBM) follows the endochondral ossification pathway, and is supported by a variety of animal models, including primemates.[8] This pathway employs multiple steps in bone formation namely, vascularization, chemotaxis, transformation of stem cells into chondrocytes and osteoblasts, deposition of new collagen, osteoclast activity, and generation of bone marrow.[6,8,9]

Thus, analyses predicate on the presence of a few growth factors may not always correlate with evidence of new bone formation *in vivo*. Published studies also indicate that multiple growth factors are involved in new bone formation in addition to the BMPs and TGF, such as VGEF, IGF, and FGF.[10,11] Even within the BMP family, members appear to have a differential effect on bone forming cells depending on the stage of stem cell differentiation.[12] In as much, employing *in vitro* methods to qualify an unknown preparation of DBM may result in improper categorization of their osteoinductive ability. The *in vivo* assay, on the other hand, appears more sensitive, as it encompasses not only properties inherent to the DBM, but also on interaction of the DBM with the host that results in bone formation.

In conclusion, the results of this study amplify the notion that a multitude of factors and their relative interplay, rather than a single factor, are likely to determine the potency of any particular lot of DBM. As a result, the complexity of bone formation may demand different or multiple *in vitro* measures to predict DBM osteoinductivity with high confidence.

References

1. Katz, Jordan M; Nataraj, Chandra; Jaw, Rebecca; Deigi, Elizabeth; Bursac, Predrag. Demineralized bone matrix as an osteoinductive biomaterial and *in vitro* predictors of its biological potential. Published online 8 September 2008 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jbm.b.31195.
2. Jaw, Rebecca YY; Sied, Chris; Wironen, John F. Quantitative Assay for BMP-2/4 and TGF-b1 Extracted for Human Bone. Society for Biomaterials 27th Annual Meeting. St. Paul, Minnesota 2001
3. Zhang, M., Powers, R.M., Wolfinbarger, L. A quantitative assessment of osteoinductivity of human demineralized bone matrix. J. Periodontol. 68(11):1076-84. 1997.
4. Adkisson, H.D., Shoenberger, J.S., Gillis, M., Wilkins, R., Jackson, M., Hruska, K.A. Rapid quantitative bioassay of osteoinduction. J. Orthoped. Res. 18:503-511. 2000.
5. Huang, D.S., Kao, R.T., Shteyer, A., Kaban, L. Mitogenic response of cells in culture to demineralized bone matrix. J. Oral Maxillofac. Surg. 46(6):460-464. 1988.
6. Urist, M.R. Bone formation by autoinduction. Science. 150:893-899. 1965.
7. Douglas, J., and Clarke, A. Response to demineralized bone matrix implantation in foals and adult horses. Am. J. Vet. Res. 56(5):649-55. 1995.
8. Ripamonti, U., Magan, A., Ma, S.S., van den Heever, B., Moehl, T., Reddi, A.H. Xenogeneic osteogenin, a bone morphogenetic protein, and demineralized bone matrices, including human, induce bone differentiation in athymic rats and baboons. Matrix. 11(6):404-411. 1991.
9. Ripamonti, U., Ma, S.S., Cunningham, N.S., Yeates, L., Reddi, A.H. Reconstruction of the bone-bone marrow organ by ostegenin, a bone morphogenetic protein, and demineralized bone matrix in calvarial defects of adult primates. Plast. Reconstr. Surg. 91(7):27-36. 1993.
10. Wildeman, B., Schmidmaier, G., Lubberstedt, M., Haas, N.P., Raschke, M. Differentiation but not proliferation of osteoblasts is stimulated by IGF-1 and TGF-b1 in vitro. Orthopedic Research Society, 49th Annual Meeting, 2003.
11. Cui, Y.X., Allen, W., Li, G. rhBMP-2, rhVGEF and rhOSF-1 in human osteoblasts and microvascular endothelial cell chemotaxis. Orthopedic Research Society, 49th Annual Meeting, 2003.
12. Cheng, H., Jiang, W., Haydon R., Breyer, B., Zhou, L., Feng, T., Vanichakarn, P., Szatkowski, J., Park, J.Y., Phillips, F., He, T.C. Bone morphogenetic proteins 2, 6 and 9 exhibit potent osteogenic activity in osteoblastic progenitor cells. Orthopedic Research Society, 49th Annual Meeting, 2003.