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AAO Foundation Final Report Form
(a/o 5/31/2017)

Please prepare a report that addresses the following:

Type of Award,
Research Aid Award

Name(s) of Principal Investigator(s)
Xuanyu Lu

Title of Project
Characterization of Biomimetically Enhanced Bio-Oss for Bone Regeneration Applications

Period of AAOF Support
07-01-2017 to 06-30-2018

Amount of Funding
5,000

Summary/Abstract of Completed Project Results

Clefts of the lip and palate are the most prevalent congenital craniofacial birth defects. From an orthodontic perspective, the aim of bone grafting at the alveolar cleft is to provide continuity and stabilization of the maxillary arch and to permit tooth eruption and orthodontic tooth movement. Although autologous bone graft is the gold standard, it requires a secondary surgical site and the risks of pain, morbidity, infection and scarring at donor site. Tissue engineering approaches that are aimed at improving the functionality of existing clinical materials may provide clinicians with new alternatives.

For decade, Bio-Oss, the porous bone mineral substitute, has been widely and safely applied to dental bone grafting procedure. It is osteoconductive and functions primarily as a space maintainer. However, the clinical Bio-Oss® does not support cell attachment and is not osteoinductive. Biomimetic strategies that incorporate the native osteogenic extracellular matrix (ECM) within collagen-based materials have been developed to improve the osteoinductive nature of the biomaterials. This application will focus on utilizing this biomimetic strategy to integrate the osteoinductivity to a frequently used bone graft material Bio-Oss.

We hypothesize that: the biomimetically enhanced anorganic bone graft material will impart osteoinductivity by improving stem cell attachment, proliferation and osteogenic differentiation and, ultimately, facilitating new bone formation and remodeling.

The goal of this research project was to establish a stable 3D pro-osteogenic ECM coating

on Bio-Oss®, and investigate the improved osteoinductive capacity of the biomimetically enhanced Bio-Oss (BE Bio-Oss) by improving stem cell attachment, proliferation and osteogenic differentiation by a series of *in vitro* and *in vivo* tests.

Upon the SEM comparison, the particle size and surface morphologies of BE Bio-Oss demonstrated no difference compared to control but exhibited ECM fibers deposition. However, the HMSC proliferation and the expression of osteogenic marker genes, such as *Runx2, Bmp2, colI*, and *OCN* were increased significantly on BE-Bio-Oss. Applied the BE Bio-Oss and control Bio-Oss in the rat critical-sized calvarial bone defects, comparing the bone healing during 4-, 8- and 12-week periods, the μ-CT analysis showed that the bony content (BV/TV), bone structures and bone mineral density were different in BE-Bio-Oss group compare to the control. Under the histological analysis, the BE Bio-Oss demonstrated increased osteogenic cell infiltration and attachment on particle surface as well as enhanced particle remodeling and collagen deposition. Osteogenic markers proteins, DMP1, fibronectin, BMP2, TGFβ and osteocalcin, were strongly expressed in the experimental group compared to Bio-Oss controls. Nano-indentation was not applicable for this experiment due to the cortical bone was not consolidated at 12-weeks.

Together, our data indicate existing anorganic bone graft material, Bio-Oss, possess poor osteoinductive properites. The biomimetically enhanced Bio-Oss could promote better cell attachment, proliferation and osteogenic differentiation *in vitro*, and facilitate stem cell attachments, differentiation and mineralized tissue remodeling *in vivo*. Our results show a methodology to enhance existing anorganic clinical bone graft materials for improved osteoinductive ability.

Response to the following questions:

1. Were the original, specific aims of the proposal realized?
   Yes

2. Were the results published?
   No publish yet, but the manuscript will be submitted to publication in the near future, and AAOF support will be acknowledged.

2. Have the results of this proposal been presented?
   Yes, this proposal has been presented at the Clinic and Research Day at University of Illinois at Chicago, and AAOF support was acknowledged. Also, it's plan to be presented at IADR/AADR 2019.

   Characterization of Biomimetically Enhanced Anorganic Graft Material for Bone Regenerative Application, *Xuanyu Lu, Chun-Chieh Huang, Praveen Gajendrareddy, Sriram Ranindran*, Clinic and Research Day, Chicago, IL, 3-2018

3. To what extent have you used, or how do you intend to use, AAOF funding to further your career?
   This RAA funding contributes significantly to my master thesis research. Without the financial support from AAOF, it's impossible to complete a series of animal experiments about this project.

   My career goal is to be an orthodontic scientist. This founding opportunity not only supported my interest in bone regeneration at basic science level, but also it encourages me to advance my knowledge in Craniofacial Orthodontics and conduct clinical research to evaluate the outcomes of the secondary alveolar graft in craniofacial anomalies patients.

After I finished my orthodontic residency, currently, I am a Craniofacial Orthodontic Fellow at Children's Hospital Los Angeles. Taking care of cleft lip and palate kids as well as seeking the optimized long-term bone graft outcomes will be my specialty in the future. I am very appreciated AAOF support my research and early career development.

Accounting for Project; i.e., any leftover funds, etc.

Please return to AAOF via email attachment to aaofevp@aaortho.org

CHARACTERIZATION OF BIOMIMETICALLY ENHANCED ANORGANIC GRAFT MATERIAL FOR BONE REGENERATIVE APPLICATIONS

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ABSTRACT

In order to integrate the osteoinductive potential to anorganic bone grafts, Bio-Oss ®, we employed our biomimetic strategies to develop an biomimetically enhanced Bio-Oss (**BE Bio-Oss**) to facilitate new bone formation. A series of *in vitro* and *in vivo* test applied to exam cell and tissue responds. Under the SEM comparison, the particle size and surface morphologies of BE Bio-Oss same as control but with ECM fibers deposition. However, the HMSC proliferation and the expression of osteogenic marker genes, such as *Runx2*, *Bmp2*, *Col1* and *Ocn* were increased significantly on BE Bio-Oss. *In vivo*, the μ-CT analysis showed that the bony content and bone structures changed significantly in BE Bio-Oss group. Under the histological analysis, the BE Bio-Oss demonstrated increased osteogenic cell infiltration and attachment as well as enhanced collagen deposition and remodeling. Osteogenic markers DMP1, FN, BMP2, TGFβ and OCN, were strongly expressed in the experimental group compared to Bio-Oss controls. In summary, we integrated the osteoinductive properties to the anorganic bone graft material by biomimetically strategy which evidenced by improved cell attachment, cell proliferation, osteogenic differentiation and enhanced host stromal cell infiltration, collagen depositions, and tissue remodeling.

INTRODUCTION

Clefts of the lip and palate are the most prevalent congenital craniofacial birth defects and several surgeries are usually required to correct these defects and they involve extensive bone grafting procedures [1] . Tissue engineering of the existing bone graft material is an alternative approach to replace the autologous bone graft which is gold standard for grafting of critical size bony defect [2]. For decade, anorganic bone grafts, Bio-Oss®, the porous bone mineral substitute, has been widely and safely applied to dental bone grafting. It is osteoconductive and functions primarily as a space maintainer [3]. However, the clinical Bio-Oss® is not osteoinductive which does not support cell attachment [4], proliferation, not facilitate osteogenic differentiation [5] with very low resorption rate [6]. Recently, biomimetic strategies that incorporate the native osteogenic extracellular matrix (ECM) on various scaffold have been developed to improve the osteoinductive nature of the biomaterials [7-9]. This application will focus on utilizing this biomimetic strategy to enhance the osteoinductivity of a frequently used bone graft material Bio-Oss ®.

HYPOTHESIS/OBJECTIVE

We **hypothesize** that: Osteoinductivity can be biomimetically integrate on to Bio-Oss by generating a 3-D coating of an osteogenic ECM on the surface of the Bio-Oss particles. The biomimetically enhanced Bio-Oss (**BE Bio-Oss**) could promotes greater cell attachment, proliferation and differentiation of mesenchymal stem cells (HMSCs) *in vitro*, as well as enable rapid osteogenic differentiation and new bone formation and remodeling *in vivo.*
The **goal** of this research project is to characterize the BE Bio-Oss for their ability to induce osteogenic differentiation of HMSCs *in vitro* and to evaluate its osteoinductive potential *in vivo*.

METHODS

1. **Anorganic bone grafts**
   Commercial Bio-Oss®
2. **Cell culture**
   Human marrow stromal cells(HMSCs)
3. **Generate BE Bio-Oss**
   As diagram shows [8].
4. **SEM**
   Scanning electron microscope
5. **MTT cell proliferation assay**
6. ***In vitro* osteogenic differentiation**
7. **quantitative RT-PCT**
8. **Rat Calvarial Bone Defect**
   12-week-old male rats, two 5mm defects
9. **Microcomputed Tomography (μ-CT)**
   Sample collected at 4-,8-and 12-weeks after surgery
10. **Histology**
    H&E staining,
    Mallory's Trichrome staining
11. **Immunohistochemistry (IHC)**

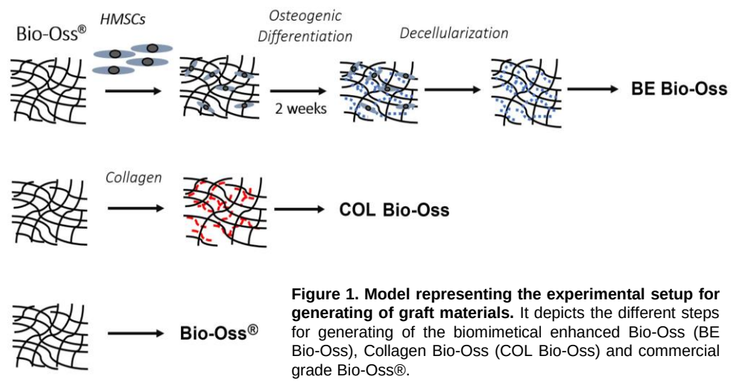

Figure 1. Model representing the experimental setup for generating of graft materials. It depicts the different steps for generating of the biomimetical enhanced Bio-Oss (BE Bio-Oss), Collagen Bio-Oss (COL Bio-Oss) and commercial grade Bio-Oss®.

RESULTS

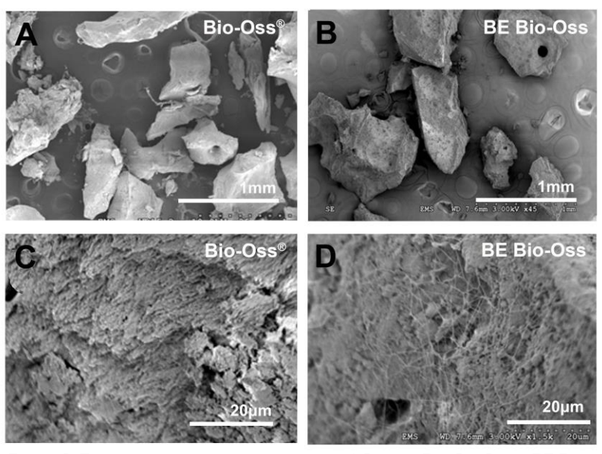

Figure 2. Electron microscopic comparison of Control Bio-Oss® and BE Bio-Oss surface structure. (A,B) are SEM at 45-fold magnification, (C, D) and 1500-fold magnification. (A, C) are from commercial grade Bio-Oss® as control, and (B, D) are from BE Bio-Oss. A scale bar in the right serves as a references for the level of magnification.

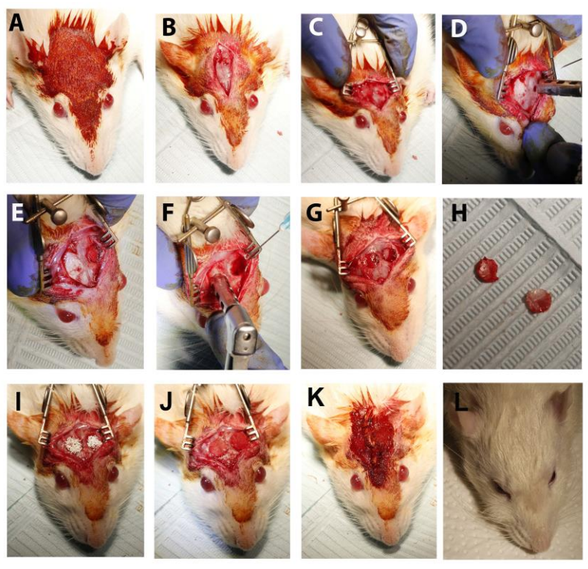

Figure 6. Surgical protocol for the establishing of rat critical-sized calvarial bone defect (5mm) (A - K) on rat calvarial bone and fully healed surgical sits at 4 weeks post-operation (L).

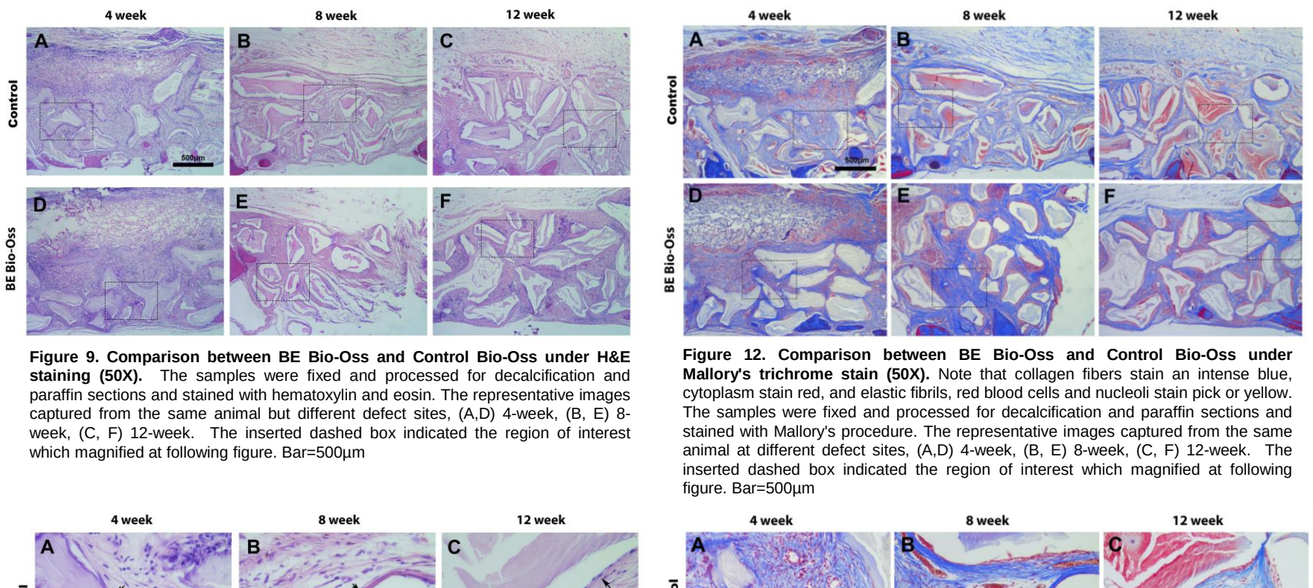

Figure 9. Comparison between BE Bio-Oss and Control Bio-Oss under H&E staining (50X). The samples were fixed and processed for decalcification and paraffin sections and stained with hematoxylin and eosin. The representative images captured from the same animal but different defect sites, (A,D) 4-week, (B, E) 8-week, (C, F) 12-week. The inserted dashed box indicated the region of interest which magnified at following figure. Bar=500μm

Figure 12. Comparison between BE Bio-Oss and Control Bio-Oss under Mallory's trichrome stain (50X). Note that collagen fibers stain an intense blue, cytoplasm stain red, and elastic fibrils, red blood cells and nucleoli stain pick or yellow. The samples were fixed and processed for decalcification and paraffin sections and stained with Mallory's procedure. The representative images captured from the same animal at different defect sites, (A,D) 4-week, (B, E) 8-week, (C, F) 12-week. The inserted dashed box indicated the region of interest which magnified at following figure. Bar=500μm

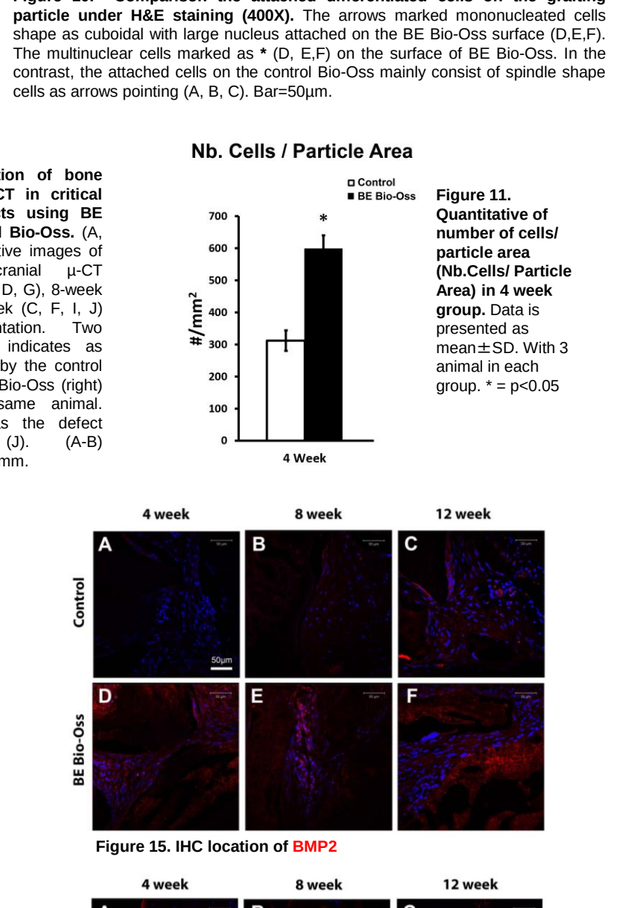

Figure 10. Comparison the attached differentiated cells on the grafting particle under H&E staining (400X). The arrows marked mononucleated cells shape as cuboidal with large nucleus attached on the BE Bio-Oss surface (D,E,F). The multinuclear cells marked as * (D, E,F) on the surface of BE Bio-Oss. In the contrast, the attached cells on the control Bio-Oss mainly consist of spindle shape cells as arrows pointing (A, B, C). Bar=50μm.

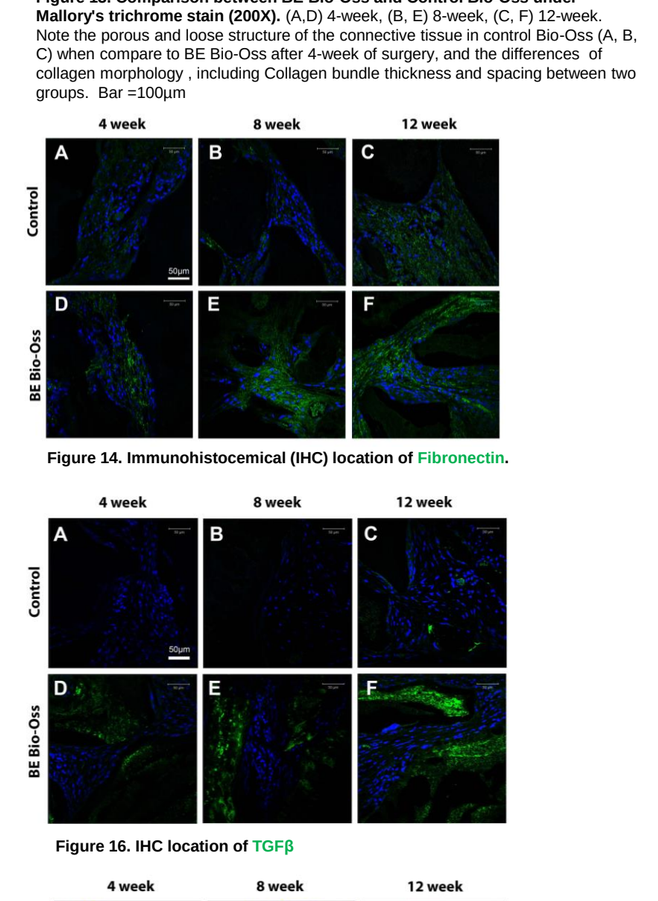

Figure 13. Comparison between BE Bio-Oss and Control Bio-Oss under Mallory's trichrome stain (200X). (A,D) 4-week, (B, E) 8-week, (C, F) 12-week. Note the porous and loose structure of the connective tissue in control Bio-Oss (A, B, C) when compare to BE Bio-Oss after 4-week of surgery, and the differences of collagen morphology , including Collagen bundle thickness and spacing between two groups. Bar =100μm

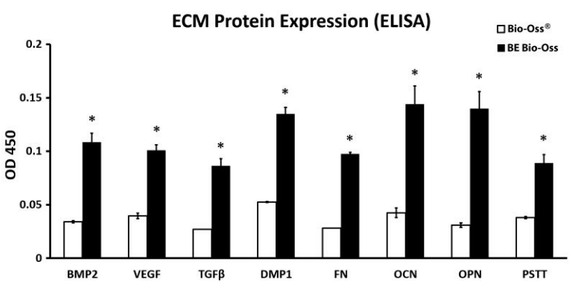

Figure 3. The ECM proteins were detected on BE Bio-Oss by ELISA assay. The commercial grade Bio-Oss® used as control. The OD values were measured under wavelength 450nm. Data are presented as mean ± SD for quadruplicates samples, * = p<0.05, BE Bio-Oss verse control.

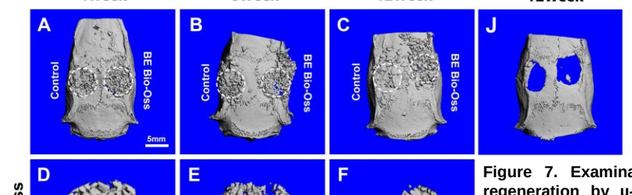

Figure 7. Examination of bone regeneration by μ-CT in critical size calvarial defects using BE Bio-Oss and Control Bio-Oss. (A, B, C) are representative images of three-dimensional cranial μ-CT images of 4-week (A, D, G), 8-week (B, E, H) and 12-week (C, F, I, J) after surgical implantation. Two 5mm bony defects indicates as inserted circles filled by the control Bio-Oss(left) and BE Bio-Oss (right) separately in the same animal. Negative control was the defect received no graft (J). (A-B) Bar=5mm, (D-I) bar=1mm.

Figure 11. Quantitative of number of cells/ particle area (Nb.Cells/ Particle Area) in 4 week group. Data is presented as mean±SD. With 3 animal in each group. * = p<0.05

Figure 4. Growth rate of HMSCs on the BE Bio-Oss, Control Bio-Oss and commercial Bio-Oss® assessed by MTT assay on day 1, 3, 6, 10 of cell culture. Data were obtained as 5 duplicated cultures, and values are showed as mean ± SD (* =p<0.05 verse control Bio-Oss).

Figure 8. Quantitative μ-CT analysis of bone regeneration in bony defects. It illustrates the results from 3D morphometric analysis of the regenerated tissue covering the calvarial defect comparison between BE Bio-Oss and control. Data are displayed as mean ± SD. with n=3 in each group. *= p<0.05.

Figure 15. IHC location of BMP2

Figure 14. Immunohistocemical (IHC) location of Fibronectin.

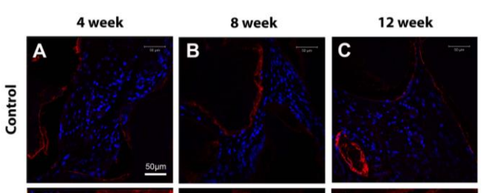

Figure 16. IHC location of TGFβ

Figure 5. Real-time PCR analysis of osteoblastic markers on 1-week of HMSCs cell culture on different graft materials *in vitro*. Expression of each target gene, *Ocn, Col1, Bmp2, Vegf, Osx, Runx2, Alpl and Fgf2*, was standardized to housekeeping gene and rationed as relative fold changes over Bio-Oss® expression level. The values are expressed as mean ± SD. Data were attained from 3 different cell culture. * =p<0.05 BE Bio-Oss verse Control Bio-Oss.

Figure 17. IHC location of DMP1.

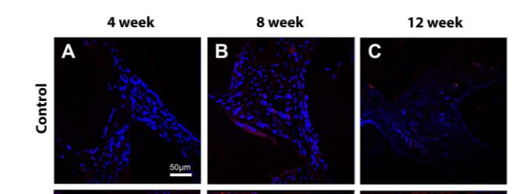

Figure 18. IHC location of Osteocalcin.

CONCLUSIONS

The improved **Osteoinductive** properties of the Biomimetically Enhanced Anorganic Bone Graft material was evidenced by:

- Improved cell attachment, through *in vitro and in vivo*
- Enhanced cell proliferation, *in vitro*
- Enhanced host Stromal cell infiltration, *in vivo*
- Increased collagen depositions, *in vivo*
- Increased expression of osteoinductive proteins, *in vivo*

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- **Animal Protocol : 14-085**