Title

Effects of Bone Morphogenetic Protein (rhBMP-2) and Platelet Derived Growth Factor (rhPDGF-BB) on Ectopic Bone Formation In Rats

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Effects of Bone Morphogenetic Protein (rhBMP-2) and Platelet Derived Growth Factor (rhPDGF-BB) on Ectopic Bone Formation In Rats

A thesis submitted in partial satisfaction

of the requirements of the degree Master of Science

in Oral Biology

by

Martin Harootune Mardirosian

2012

ABSTRACT OF THESIS

Effects of Bone Morphogenetic Protein (rhBMP-2) and Platelet Derived Growth Factor (rhPDGF-BB) on Ectopic Bone Formation In Rats

By

Martin Harootune Mardirosian

Master of Science in Oral Biology

University of California, Los Angeles 2012

Professor Tara Aghaloo, Chair

Marshall Urist first described a reproducible method by which to evaluate potentially osteoinductive molecules using a rat ectopic model to induce bone growth *in vivo* in non-bony, intramuscular sites.

Recombinant human bone morphogenetic protein-2 (rhBMP-2) has been shown to induce *de novo* bone formation in ectopic implantations. When applied to an absorbable collagen sponge (ACS) carrier, the protein is retained at the site of implantation and induces bone formation in a non-bony site within two weeks in a subcutaneous rat ectopic model. The combination device of rhBMP-2/ACS has been approved by the FDA for use in anterior lumbar interbody fusions, acute, open tibia1 shaft fractures, and certain dental bone regenerative procedures.

Recombinant human platelet-derived growth factor-BB (rhPDGF-BB) is approved for use in the treatment of certain periodontal defects when used in combination with a β-tricaclium phosphate (TCP) carrier. Thus far, no studies have demonstrated osteoinductive properties of PDGF.

The purpose of this study was to compare the osteoinductivity of rhBMP-2 and rhPDGF-BB separately on both an ACS and carrier TCP when placed subcutaneously in rats.

Thirty-two male immunocompetent 5 month old Sprague-Dawley rats were used in this study. Investigational materials were implanted subcutaneously in the rat thorax, with four randomized implants being placed per rat (e.g., one per quadrant). Animals received implantations of: rhBMP-2/ACS (0.3mg/mL), rhBMP-2/TCP (0.3mg/mL), rhPDGF-BB/TCP (0.3mg/mL), rhPDGF-BB/ACS (0.3mg/mL), ACS alone, or TCP alone. No animal received both rhBMP-2 and rhPDGF-BB. After sacrificing the animals after 2 and 4 weeks, quantitative and qualitative results were assessed using MicroCT and histology.

rhBMP-2 showed considerable induction of bone growth at both 2 week and 4 week samples whether grown on TCP or ACS. MicroCT analysis revealed a significant increase in calcified tissue in rhBMP-2/ACS, rhBMP-2/TCP, and rhPDGF-BB/TCP in comparison to ACS alone control. However, only rhBMP-2/ACS at 2 and 4 weeks, not ACS alone nor rhPDGF-BB, was osteoinductive, as demonstrated by histology. H&E staining demonstrated immature woven bone at 2 weeks, which became more mature at the 4 week time point.rhPDGF-BB lacked the ability to induce bone formation, either with an ACS or TCP carrier .

This study confirmed the osteoinductive properties of rhBMP-2/ACS and rhBMP-2/TCP, but failed to demonstrate that rhPDGF-BB is osteoinductive on either an ACS or TCP carrier. Due to its ability to induce *de novo* bone formation, rhBMP-2 is an important treatment option

for patients with significant alveolar bone defects in the jaws, and will serve as a viable alternative to autogenous bone grafting.

The thesis of Martin Harootune Mardirosian is approved.

Flavia Pirih

Peter K. Moy

Sotirios Tetradis

Tara Aghaloo, Committee Chair

University of California, Los Angeles
2012

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACKNOWLEDGEMENTS

This study was supported in part by a grant from Medtronic Inc.

INTRODUCTION

Bone is a mineralized connective tissue that functions in support, motion, protection, and mineral homeostasis of the vertebrate body. Bone homeostasis is maintained by a tight balance between bone formation and resorption. It's functions are dependent on osteoblasts and osteoclasts to fulfill these duties (1). Changes in the balance of bone homeostasis can lead to systemic or local bone diseases that can lead to fractures and bone defects.

Bone defects themselves are multifactorial with etiologies ranging from trauma and surgical resection to congenital abnormalities. Although various treatment modalities are available depending on the location, size, and reconstructive goals, autogenous bone is still considered as the "gold standard" for bone grafting procedures (2). Autogenous bone grafts are normally harvested from the patient's iliac crest, tibia, mandible, or maxillary tuberosities and have significant associated complications including donor site morbidity and limited supply (3-6). Specifically in the craniofacial region, autografts have unacceptably high failure rates, and allografts are even less predictable (7,8). These issues have lead clinicians and researchers to investigate other sources of graft materials for allografts, alloplasts, and more recently, growth factors.

Regeneration of bone and the maintenance of its homeostasis involves the differentiation of osteoblasts and chondrocytes from progenitor mesenchymal cells and differentiation of osteoclasts from progenitor monocytic cells all directed by a multitude of different growth factors (9). Bone naturally contains a wide variety of these factors such as: transforming growth factor (TGF-β), bone morphogenetic proteins (BMPs), platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and basic fibroblast growth factors (bFGF) all of which have shown positive effects in promoting fracture healing (10-14).

As early as 1965, a bone inducing agent, eventually termed bone morphogenetic protein, in demineralized bone matrix was discovered using a rat ectopic model (15). After purification and sequencing of this protein from organic bone matrix (16), recombinant BMPs were produced and purified, showing that the protein alone was sufficient enough to induce bone formation (17). BMPs are among the most potent regulators of osteoblast differentiation (18), and BMP-2, 4, 6, and 7 all have osteoinductive properties *in vivo* (19,20). BMP-2 can also regenerate bone defects in animal models (21-26), as well as mandibular continuity defects, cleft palate defects, and augment maxillary sinuses in humans (27-31). These results have lead to the FDA approval for rhBMP-2 on an absorbable collagen sponge using a 1.5 mg/cc concentration in orthopedic and maxillofacial surgery.

PDGF is an important growth factor with mitogenic, chemotactic, and angiogenic properties that promotes both soft tissue and bone healing (32-35). PDGF enhances DNA synthesis, increases collagen deposition, and stimulates synthesis of extracellular matrix in wound repair (36). It further promotes wound healing by the promotion of blood vessel formation through the upregulation of VEGF (37) and stabilization of new capillaries by mural cells (38). Local rhPDGF-BB delivery in diabetic animals shows a significantly enhanced rate of fracture healing (39).

PDGF has been used in the past in combination with autogenous bone grafts to further enhance bone regeneration (40). Recent studies have used PDGF in combination with mesenchymal stem cells to achieve 53% bone fill in large bony defects (41). However, the amount of mean generated bone appeared to be lower than that achieved in other alveolar cleft studies using rhBMP-2 or autogenous iliac graft (42). PDGF has also shown improvement in

periodontal defects, which has lead to the FDA approval of rhPDGF-BB on a tricalcium phosphate carrier for periodontal intrabony defects in a product called GEM 21S® (43).

The role of the carrier should be to act as a three-dimensional space maintainer, to keep the protein localized to the site, and to act as a scaffold for new bone deposition and formation. Tricalcium phosphate has been studied for years as a reliable bone substitute. It is considered a bioresorbable ceramic that is capable of allowing osteoconduction. The TCP carrier functions as a space maintainer and a scaffold, but it lacks in its ability to bind growth factors. Absorbable collagen sponge is made of natural fibrillar collagen derived from the Achilles tendon of steers. ACS has been used commercially since 1981 as an absorbable hemostatic agent. It is made up of type I bovine collagen which is similar chemically to the type I collagen found in humans. The ACS undergoes resorption over a 4-12 week period after implantation via cell-mediated degradation by macrophages. The ACS carrier has the unique ability of binding its protein and localizing it to the region, it serves as a scaffold for new bone deposition, but its ability to serve as a three-dimensional space maintainer is rather limited.

With significant clinical applications in bone healing and regeneration and now the commercial availability of rhBMP-2 and rhPDGF-BB on their respective carriers, we sought to directly compare the osteoinductive properties of these two growth factors in a rat ectopic bone formation model.

MATERIALS AND METHODS

Rat Ectopic Bone Formation Model

Thirty-two male immunocompetent 5 month old Sprague-Dawley rats were used in this study. Approval was obtained for all animal experiments through the Chancellor's Committee on Animal Research Oversight at UCLA. Animals were numerically labeled and separated into 2 growth groups: 2 weeks and 4 weeks. Samples were implanted subcutaneously in the rat thorax, with four randomized implants being placed per rat (**Table 1**). Animals received implantations of: rhBMP-2/ACS (0.3mg/mL), rhBMP-2/TCP (0.3mg/mL), rhPDGF-BB/TCP (0.3mg/mL), rhPDGF-BB/ACS (0.3mg/mL), ACS alone, or TCP alone at each quadrant for a total of 4 treatments per rat. rhBMP-2 and ACS were supplied by Medtronic Inc (Nashville, TN) and rhPDGF and TCP were purchased from Osteohealth (Shirley, NY). No animal received treatments of both rhBMP-2 and rhPDGF-BB. After inhalational general anesthetic, each rat received a preoperative plain film radiograph. The back of each rat was shaved and divided into four quadrants (**Table 1**). The implantation site was prepped with povidone-iodine solution, and draped in a sterile fashion. A single trained surgeon performed all surgeries. A one centimeter long incision was made at each quadrant of the back, approximately 1.5-2.0 cm from the midline. The incisions were made subcutaneously and the tissue bluntly dissected creating a pouch at each location. Using a randomized schedule, implants were placed at each location, and sutured with 4-0 vicryl to obtain primary closure. Immediate post operative plain film radiographs were taken. Plain film radiographs were again taken at 2 and 4 weeks.

A large collagen sponge was sectioned into 1.4 cm x 1.4 cm squares and 0.3mg/mL concentration of rhBMP-2, rhPDGF-BB, or saline solution was distributed onto each of the squares. The ACS was hydrated with 200 μl saline, rhBMP-2, or rhPDGF-BB (60 μg each) for a

minimum of 15 minutes and a maximum of 120 minutes before implantation. The samples were then carefully placed into the subcutaneous pockets ensuring that the ACS was not crushed. Tricalcium phosphate samples received a 0.3mg/mL concentration of rhBMP-2, rhPDGF-BB, or saline in a 1:1 ratio to yield 0.2 cc graft material. The TCP also soaked for a minimum of 15 minutes and a maximum of 120 minutes before implantation. The TCP samples were then be placed into the subcutaneous pocket. The surgical procedure was identical for both ACS and TCP groups (**Figure 1**).

Radiographic, Histologic, and Histomorphometric Analysis

Plain film radiographs were taken preoperatively, immediately post operatively and at 2 and 4 weeks to assess bone formation. Sixteen of the rats were sacrificed at 2 weeks and the rest at 4 weeks. Following sacrifice, the tissue masses placed at each quadrant were physically identified and surgically isolated using 5 mm margins around each sample and immediately placed in 10% neutral buffered formalin for 48 hours, after which they were rinsed and stored in a 70% ethanol solution. Samples from each rat were evaluated by an independent investigator who was blinded to the treatment group.

MicroCT imaging was performed at 12 μM isotropic voxel resolution and tissue and bone volume analysis was performed, and visualization and reconstruction was performed using the MetaMorph® Imaging System (Universal Imaging Corporation) to generate 3D and multiplanar reconstructed images. Then, sponges were decalcified using Cal EX decalcifying solution (Fisher Scientific, Pittsburgh, PA), paraffin embedded, sectioned, and stained with hematoxylin and eosin (H&E). Consecutive cross-section cuts were made at various levels to ensure visualization of the implant site and analyzed. Photomicrographs were taken with a Leica DMLB microscope

(Leica Microsystems, Wetzlar, Germany) using BioQuant software (R&M Biometrics, Nashville, TN).

Histologic slides were evaluated and a histomorphometric score was determined based on percentage of bone formation. A grading scale for new bone formation was used by designating a score of 0.0 to mean no bone formation, 0.5 for new bone formation of 1-10%, 1.0 for new bone formation of 11-25%, 2.0 for new bone formation of 26-50%, 3.0 for new bone formation of 51-75%, and 4.0 being an area of new bone formation of 76-100% of the total implant area.

Statistical analysis

The Kruskal–Wallis one-way analysis of variance was used to determine statistical significance of the differences between bone volumes acquired from microCT amongst ACS and TCP samples. Statistics were performed on the ACS group at 2 weeks, ACS group at 4 weeks, TCP group at 2 weeks, and TCP group at 4 weeks, independently on each category. The ACS group at 2 weeks compared microCT bone volume of ACS alone, ACS/PDGF, and ACS/rhBMP-2 and showed a statistical significance between the samples, with a p-value of X. The same statistical test was performed on the ACS group at 4 weeks and showed a statistical significance between the samples, with a p-value of X. Similarly, the TCP group at 2 weeks compared microCT bone volume of TCP alone, TCP/PDGF, and TCP/rhBMP-2 and showed a statistical significance between the samples, with a p-value of X. The same statistical test was performed on the TCP group at 4 weeks and showed no statistical significance. $P<0.05$ was considered as significant.

RESULTS

The animals underwent uneventful clinical healing. Sutures were removed at the 2 week time point. One rat died in the 4 week PDGF category, and the experiment was repeated on this individual animal as specified in our protocol. 16 rats were scarified at 2 weeks and the other 16 at 4 weeks. Each growth category included 8 rhBMP-2 containing rats and 8 rhPDGF-BB containing rats, with no rats receiving simultaneous treatment of both proteins. Each sample isolated underwent plain radiographic, microCT, histological, and histomorphometric evaluation. Four week samples from ACS with both saline and PDGF were difficult to identify since the sponge had resorbed and no bone had formed. However, tissue from the surgical site was harvested and subjected to analyses as described below.

Radiographic Evaluation

Preoperative radiographs did not reveal any abnormal radiologic pathology. Immediate post operative radiographs showed distinct nodules of TCP morphologically identical implanted samples. At 2 and 4 week time points, TCP containing nodules were still radiographically visualized. Differences were not detected between TCP samples with saline, PDGF, or BMP-2 at 2 or 4 weeks. In the ACS samples, radioopacities are seen in the BMP-2 groups with plain radiographs at the 2 and 4 week time points. However, in the ACS with saline or PDGF, no radiopaque nodules are seen (data not shown).

MicroCT Analysis

3D reconstructions of all explanted samples were performed. The MicroCT is able to visualize mineralized tissue, but is not able to differentiate between mineralized alloplastic graft material and new bone formation. No microCT image was produced at 2 or 4 weeks after

grafting with ACS/saline or ACS/PDGF, indicating lack of mineralization or new bone formation. In comparison, ACS/BMP-2 at both 2 and 4 week time periods demonstrate a mineralized nodule on microCT. At 2 weeks, the mineralized surface is mainly concentrated at the surface, which became more dense at the 4 week time point (**Figure 2**). The difference in mineralization between ACS/saline vs. ACS/BMP-2 and ACS/PDGF vs. ACS/BMP-2 at both 2 and 4 weeks was statistically significant ($p<0.05$; **Figure 3**).

MicroCT analysis of TCP samples are more difficult to evaluate for comparisons between groups because the difference between new bone and mineralized TCP particles cannot be identified. In all groups including TCP/saline, TCP/PDGF, and TCP/BMP-2, microCT images of mineralized tissue were seen, without any obvious difference in quality of mineralization at either 2 or 4 weeks (**Figure 4**). When the mineralization was quantified on microCT, a significant increase was seen in the TCP/BMP-2 group as compared to the TCP/saline and TCP/PDGF groups at 2 weeks ($p<0.05$). However, no significant differences were seen between the groups at 4 weeks (**Figure 5**).

Histology

Histology was performed to confirm the presence of new bone formation. It allowed us to overcome the difficulties in identifying new bone formation of our TCP samples using Micro CT analysis alone. Conventional H&E staining was used to identify the tissue formed in the rat ectopic model. Similar to the microCT data, no bone tissue was identified in the ACS/saline or ACS/PDGF groups at 2 or 4 weeks. After 2 weeks, the ACS/saline group demonstrated collagen sponge within a fibrous tissue capsule with the presence of inflammatory cells and fibrous tissue interspersed throughout the remaining sponge. By 4 weeks in the ACS/saline group, a smaller amount of collagen sponge was seen, again with the presence of fibrous tissue around and

interspersed throughout the remaining sponge. The ACS/PDGF group also demonstrates the presence of the collagen sponge, but with a noticeable increase in vascularity and inflammatory cells as compared to the ACS/saline group at both 2 and 4 weeks. However, similar to the ACS/saline group, no bone or cartilaginous tissue is seen at either time point. For the ACS/BMP-2 group, the collagen sponge is also present at 2 weeks, with an outer border of immature bone formation. The outer perimeter of the sponge is almost entirely encased by woven bone formation. However, minimal to no bone formation is seen in the inner part of the collagen sponge at 2 weeks. At 4 weeks in the ACS/BMP-2 group, the collagen sponge is mostly resorbed and replaced by more mature lamellar bone formation. Here, some bone formation is moving to the center of the sponge location, with the presence of immature appearing bone marrow structures centrally located. The bone formed is vital and densely stained with the presence of osteocytes in the lacunae and osteoblasts laying down osteoid material at the edges of the more mature bone (**Figure 6, 7, & 10**).

In both TCP/Saline and TCP/PDGF groups, well formed nodules of TCP particles surrounded by a fibrous capsule are seen with the presence of fibrous tissue throughout the sample. However, no bone formation is seen at 2 or 4 weeks. Similar to the ACS samples, TCP/PDGF samples demonstrate an increase in vascularity as compared to the TCP/Saline samples. In contrast, TCP/BMP-2 samples show TCP particles, with new bone formation at the edges of sample. TCP particles can be seen in close proximity to the new trabecular bone, which contains osteocytes in the lacunae. At 4 weeks, an increase in new bone formation can be seen in the TCP/BMP-2 group. Numerous islands of trabecular bone are present surrounding remaining TCP particles. The trabecular islands are thicker, again with osteocytes present in lacunae of the

newly formed bone and osteoblasts lining the bone surface. Bone marrow structures are also present, interspersed throughout the trabeculae and TCP particles (**Figure 8, 9, & 10**).

Histomorphometry

All samples containing ACS with saline, ACS with rhPDGF-BB, TCP with saline, and TCP with rhPDGF-BB received a bone score of 0.0 due to the fact that they exhibited less than 1% of new bone formation throughout the implant area. ACS/BMP-2 at 2 weeks samples contained 5 samples with 1-10% bone formation and 7 samples with 11-25% bone formation. TCP/BMP-2 at 2 weeks contained 4 samples with 1-10% bone formation, 6 samples with 11-25%, and 2 samples with 26-50% bone formation. ACS/BMP-2 at 4 week samples contained 7 samples with 11-25% bone formation and 5 samples with 25-50% bone formation. TCP/BMP-2 at 4 weeks samples consist of 8 samples with 26-50% bone formation and 4 samples with 51-75% bone formation (**Table 3**).

DISCUSSION

An ideal graft material is one that creates no donor site morbidity, contains presence osteogenic or osteoinductive signals, the ability to stabilize the construct while maintaining vascularity, a low risk of infection or antigenicity, ease of availability and manipulation, and high reliability (44). Although allografts and autografts fill some of these requirements, the ideal graft material has not yet been identified. Autogenous bone is still the graft material to which all others are compared since it possesses osteogenic, osteoinductive, and osteoconductive properties (45). Most available alloplast and allograft materials promote bone formation via osteoconduction, with the exception of some osteoinductive activity in specific allograft preparations (46). However, the osteoinductive properties of allograft materials are consistent between preparation and age of the donor. A recent study even showed osteoinductive variability between different lots of the same allograft product (47). In evaluating the complication rate, especially in craniofacial reconstruction, with autografts, allografts, and alloplasts, it is not surprising that clinicians are embracing the recent clinical availability of recombinant growth factors for patients with bone deficiencies.

Due to its ability to regenerate critical sized defects in both animals and humans, the osteoinductive properties of BMP-2 are well known (21-26,28-31). Since 2002, rhBMP-2/ACS marketed as INFUSE® Bone Graft has been FDA-approved as an autograft replacement for certain interbody spinal fusion procedures. Then in 2004, INFUSE® Bone Graft was approved for open tibial fractures with an intermedullary (IM) nail fixation. Most recently in March 2007, INFUSE® Bone Graft was approved as an alternative to autogenous bone grafts for sinus augmentations, and for localized alveolar ridge augmentations for defects associated with extraction sockets (48).

The current study is consistent with others that demonstrate ectopic bone formation in an animal model after treatment with rhBMP-2 (25,26,49-51). Similar to these reports, 2 weeks was adequate time to induce significant bone formation on microCT, and histology demonstrated vital trabecular islands produced containing active osteoblastic bone formation and the presence of hematopoietic marrow (25). Our results clearly demonstrate an increase in bone formation and maturity after 4 weeks on histology. Interestingly, studies evaluating longer time points such as 8-26 weeks demonstrate less bone formation than that observed at these earlier time points (25,26). These studies differ from ours by either the source of BMP-2 production in *E. coli* (25) vs. Chinese hamster ovary (CHO) cells in the current study or the carrier, polylactic acid (PLA) (26) vs. an absorbable collagen sponge in the current study. Therefore, conducting these longer time point experiments would be interesting in this model system. Results with ACS/BMP-2 were expected since many studies on the osteoinductive effects of BMP-2 on an ACS have been performed (25,26,49,50,52,53). However, our increased bone formation in the BMP-2/TCP group was quite interesting because only few studies have evaluated the osteoinductive effects of BMP-2 on a TCP carrier (52,54).

In contrast to BMP-2, the potential osteoinductive properties of PDGF are less clear. It is well known that PDGF has potent mitogenic, chemotactic, and angiogenic properties that allows it to induce soft tissue wound healing (33,55-57). For these potent soft tissue effects, PDGF was FDA approved to treat lower extremity diabetic ulcers (58). In animal studies, PDGF induced bone regeneration in calvarial defects when implanted on a poly(L-lactide) scaffold (59). However, it has been shown to have a role in matrix mineralization and promoting osteoblastic differentiation of precursor cells (35,60). Although PDGF has positive effects on periodontal tissue repair, our study failed to demonstrate a clear osteoinductive effect either on the ACS or

TCP carrier (43,61). PDGF has been shown to increase the rate of fracture repair in diabetic animals due to possible enhancement in the release of additional signaling factors from mesenchymal stem cells that are involved in chondrogenesis and bone formation (36). Histology did show an increase in vascularity in the PDGF samples, which does have an important role in bone regeneration. In fact, previous studies suggest enhanced angiogenesis and osteogenesis with the combination of PDGF with other growth factors like IGF-1 and VEGF (61-63).

Recent studies show the existence of enhancers of BMP-2 as well. One article indicates that TGF-β1 enhances rhBMP-2 induced bone formation up to five times more than bone formed with rhBMP-2 alone. Moreover, TGF-β1 increased filling of bone defects and caused an increase in osteoblasts and a decrease in osteoclasts during later stages of matrix calcification (64). This is also true of rhBMP-2 in combination with other factors such as Nell-1, which showed a synergistic increase in bone formation when compared to rhBMP-2 alone (65). It would also be interesting to evaluate a potential additive or synergistic effect with the combination of rhBMP-2 with rhPDGF-BB both on ACS and TCP carriers in possible later studies.

With current breakthroughs in growth factors available today future research should be directed towards finding the ultimate carrier. An ideal carrier needs to provide mechanical strength for bridging of bone defects and resistance against soft tissue pressure while still maintain porosity for ingrowth of bone cells and turnover into bone. The ideal carrier would be loadable with biologically active molecules and allow for retarded delivery and controlled presentation (66). Studies have shown that long-term delivery of BMP-2 induces more ectopic bone formation than short-term delivery of an equivalent dose (67,68). With a better carrier we should be able to provide a controlled release of growth factor over a longer period of time rather than a burst of release of biologic activity. A comparative study of long and short term carrier

used Heparin-conjugated poly(lactic-co-glycolic acid) nanospheres suspended in fibrin gel as a long-term delivery system, and fibrin gel a short-term delivery system (69). The study showed a maximal dose of BMP-2 capable of forming substantially more bone when delivered over a slow released longer period of time. Advances in long term carriers coupled with the osteoinductive effects of rhBMP-2 could ultimately create the future gold standard for bone grafting.

CONCLUSION

The importance of identifying potent osteoinductive molecules cannot be disputed, and the most commonly used method is the ectopic rodent model (25,26,49,50,52,53). Therefore to clarify the potential osteoinductive properties of PDGF, it was directly compared to BMP-2. Plain radiograph, microCT, histology, and histomorphometry confirmed the ability of BMP-2 on an ACS and TCP carrier to induce bone formation. In contrast, saline or PDGF on both ACS and TCP carriers was unable to form bone at either time point.

FIGURES AND TABLES.

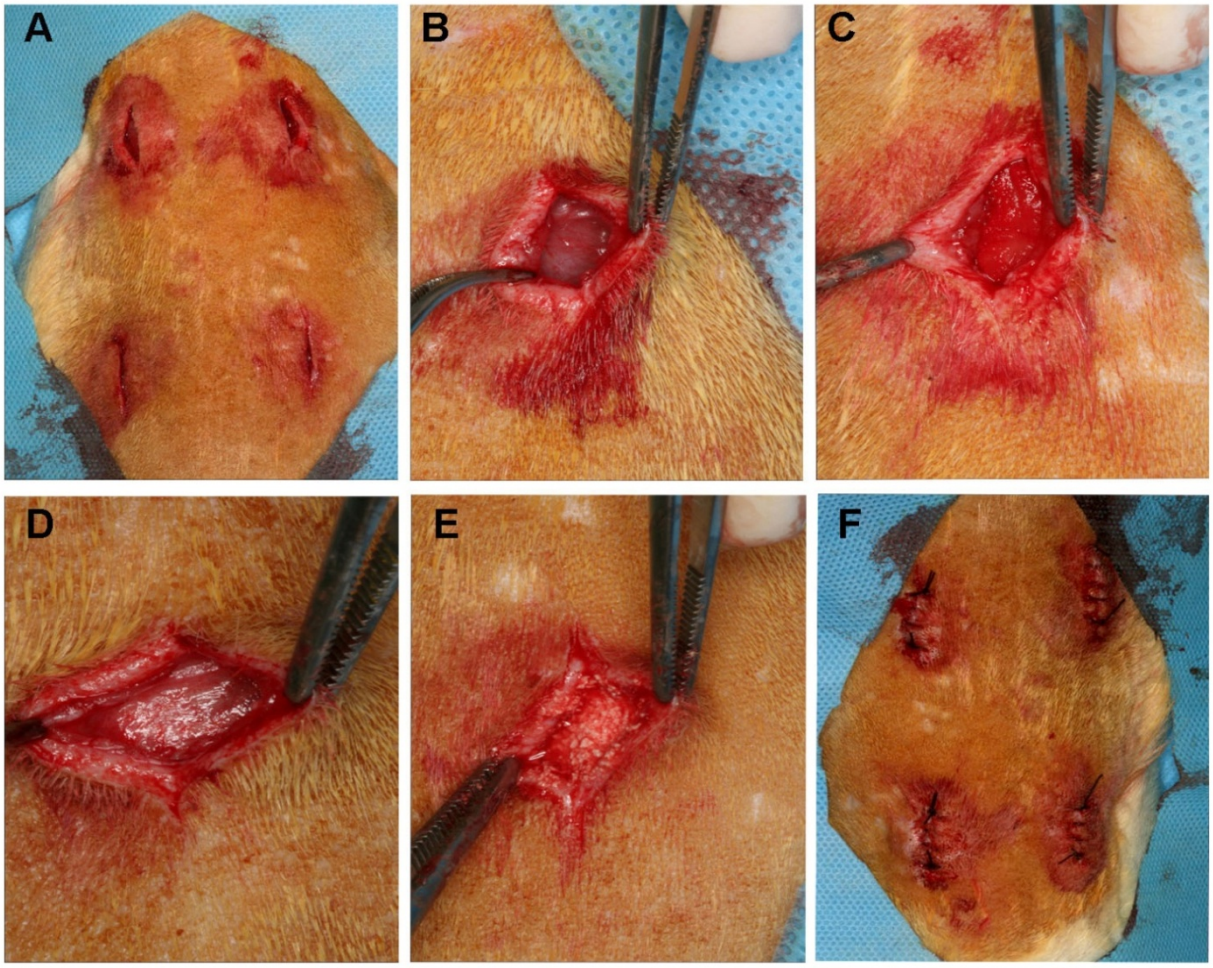

Figure 1: Surgical procedure of ACS and TCP implantation
Description: (A) Four defects created on the rat; (B) Subcutaneous pouch opened; (C) Implantation of ACS; (D) Subcutaneous pouch; (E) TCP implantation; and (F) Wounds closed with vicryl sutures.

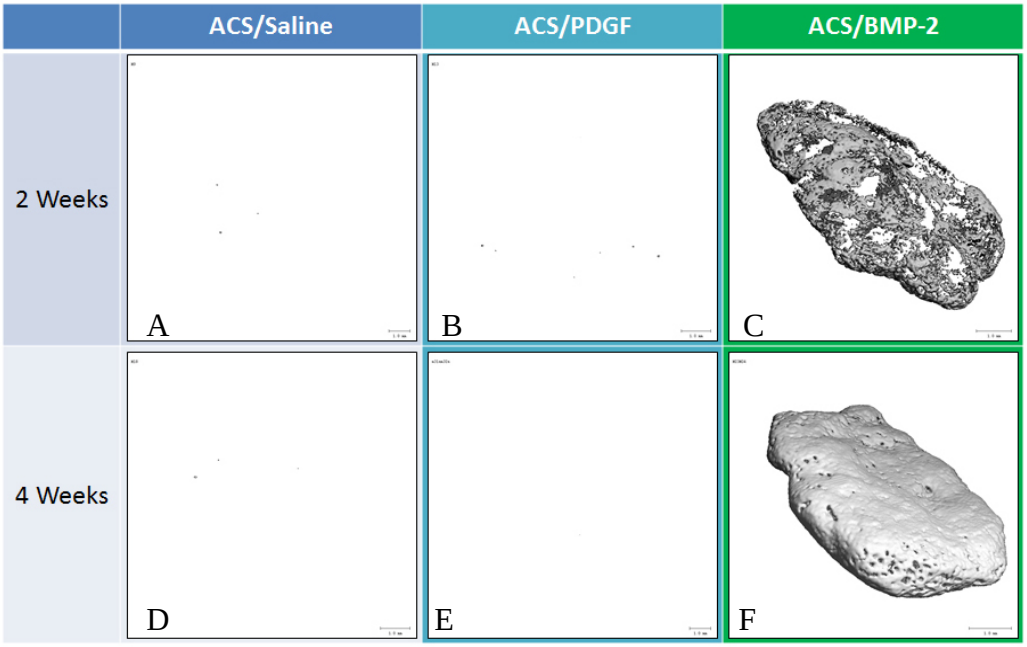

Figure 2: MicroCT 3D Reconstruction for ACS Samples at 2 & 4 weeks with grafting using Saline (A,D), PDGF (B,E), or BMP-2 (C,F)

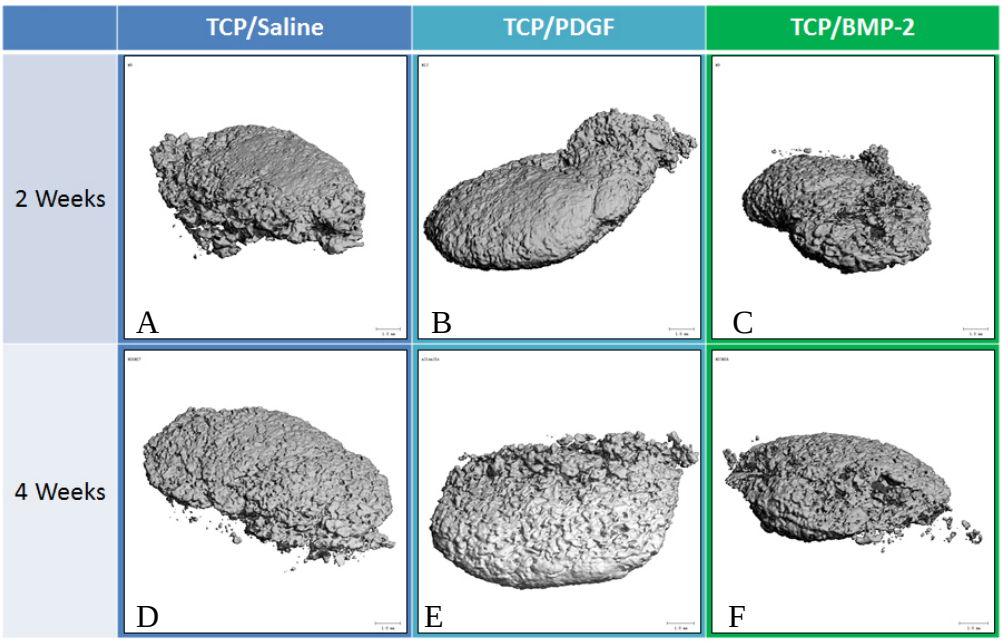

Figure 3: MicroCT 3D Reconstruction for TCP Samples at 2 & 4 weeks with grafting using Saline (A,D), PDGF (B,E), or BMP-2 (C,F)

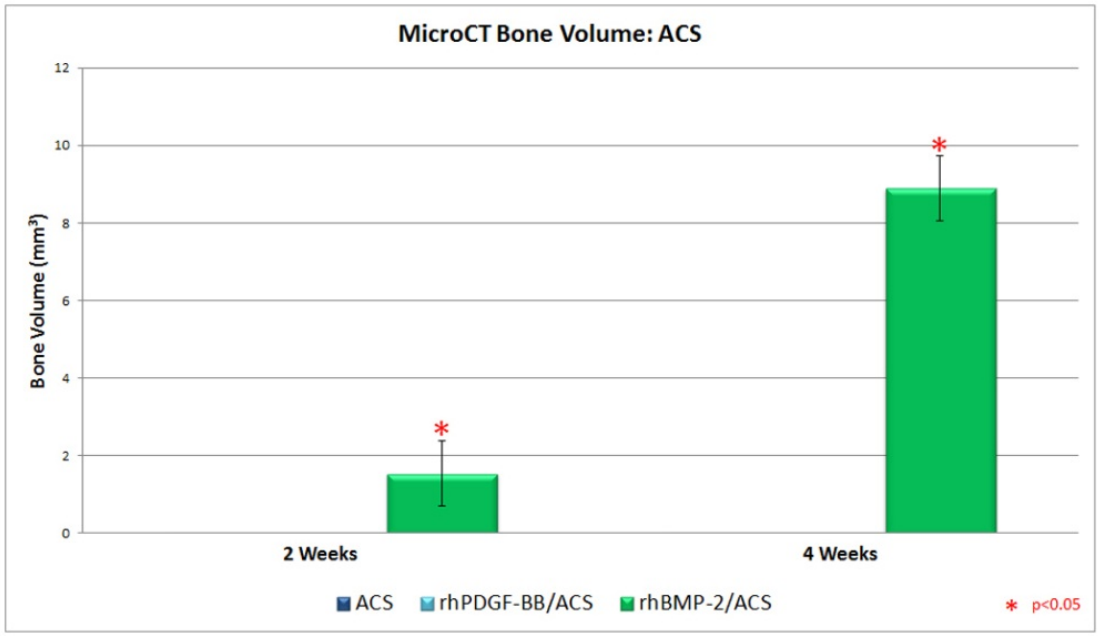

Figure 4: MicroCT Bone Volume for ACS Samples 2 & 4 Weeks. Only rhBMP-2/ACS shows an increase in bone volume with a greater value at 4 weeks vs 2week.

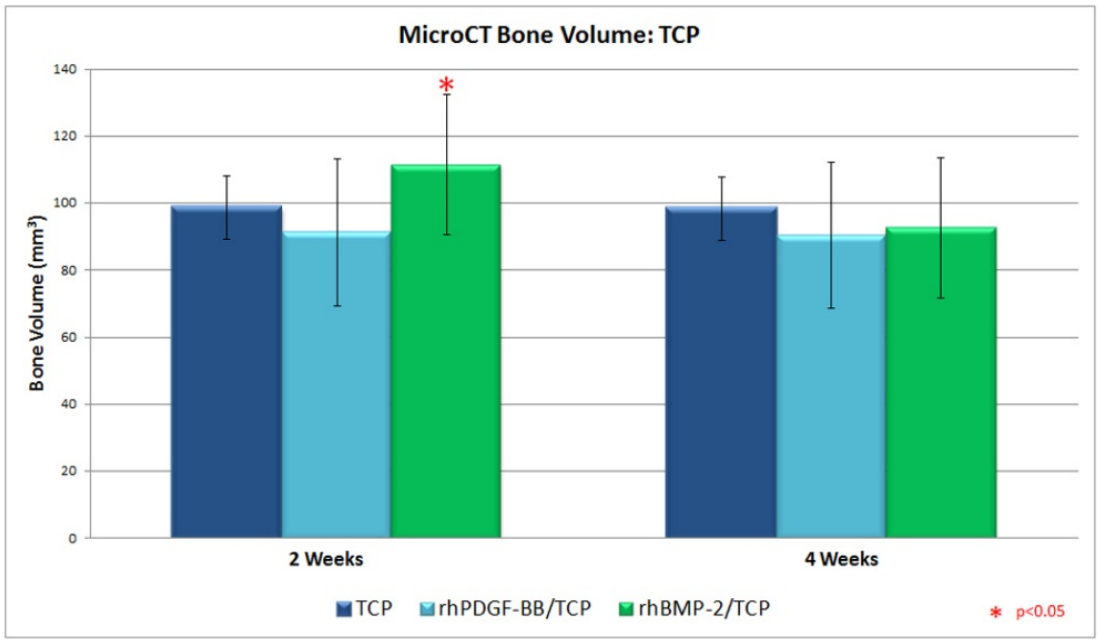

Figure 5: MicroCT Bone Volume for TCP Samples 2 & 4 Weeks. Generalized increase in bone volume for all samples with a statistically greater volume at 2 weeks in rhBMP-2/TCP samples.

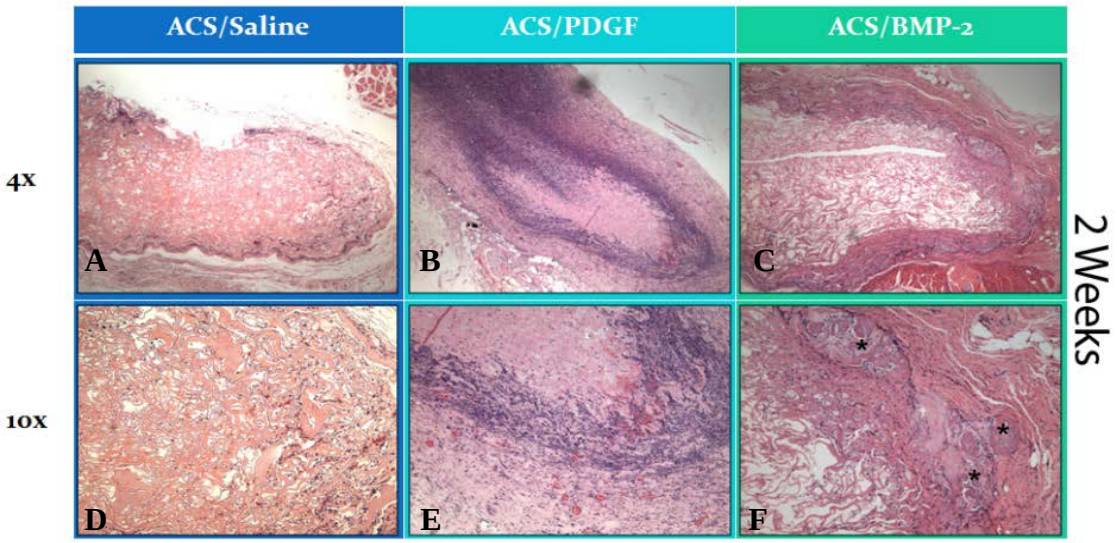

Figure 6: Histology 4X/10X Magnification for ACS Samples 2 & 4 Weeks

Description: (A) ACS/saline at 2: collagen sponge within a fibrous tissue capsule with the presence of inflammatory cells and fibrous tissue. (B) ACS/PDGF at 2 weeks: collagen sponge, with a noticeable increase in vascularity and inflammatory cells. (C) ACS/BMP-2 at 2 weeks: collagen sponge with an outer border of immature bone formation and an outer perimeter of encased by woven bone. * New Bone.

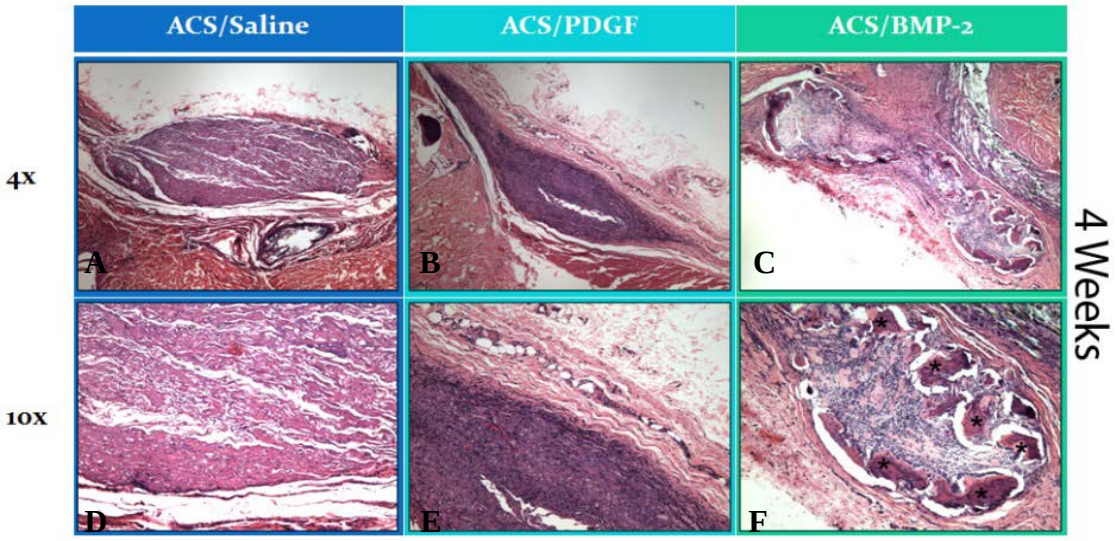

Figure 7: Histology 4X/10X Magnification for ACS Samples 2 & 4 Weeks

Description: (A) ACS/saline at 4 weeks: resorbing collagen sponge within a fibrous tissue capsule with the presence of inflammatory cells and fibrous tissue. (B) ACS/PDGF at 4 weeks: collagen sponge, with a noticeable increase in vascularity and inflammatory cells. (C) ACS/BMP-2 at 4 weeks: resorbed collagen sponge and mature vital lamellar bone formation with osteocytes in the lacunae and osteoblasts laying down osteoid. *New Bone

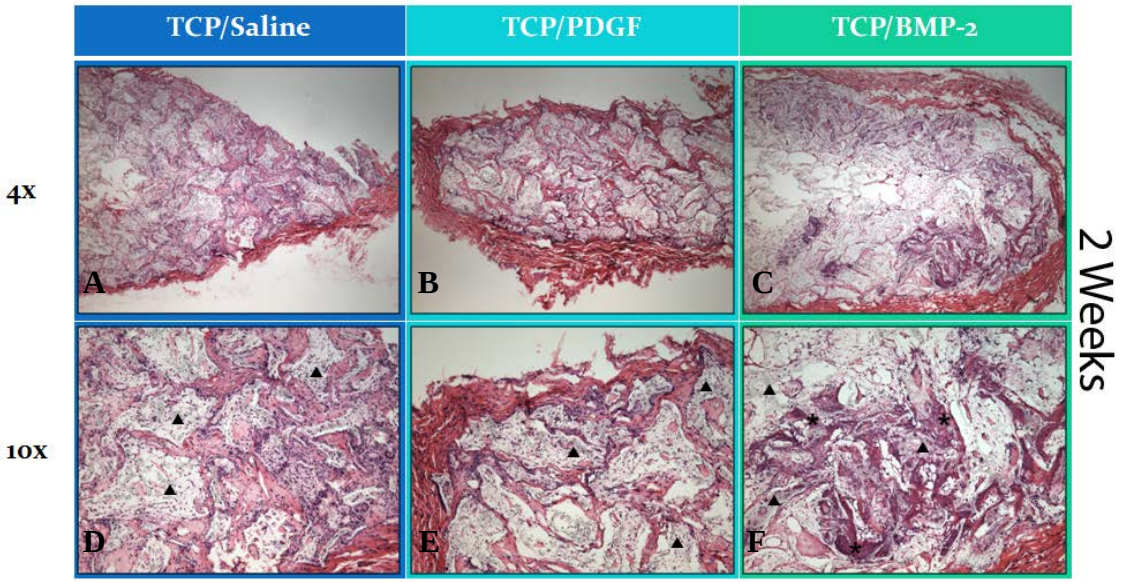

Figure 8: Histology 4X/10X Magnification for TCP Samples 2 & 4 Weeks

Description: (A) TCP/Saline at 2 weeks: well formed nodules of TCP particles surrounded by a fibrous capsule. No bone formation (B) TCP/PDGF at 2 weeks: well formed nodules of TCP particles surrounded by a fibrous capsule with an increase in vascular channels. No bone formation. (C) TCP/BMP-2 at 2 weeks: TCP particles in close proximity to the new trabecular bone containing osteocytes in the lacunae. ✳ New bone ▲ TCP particles

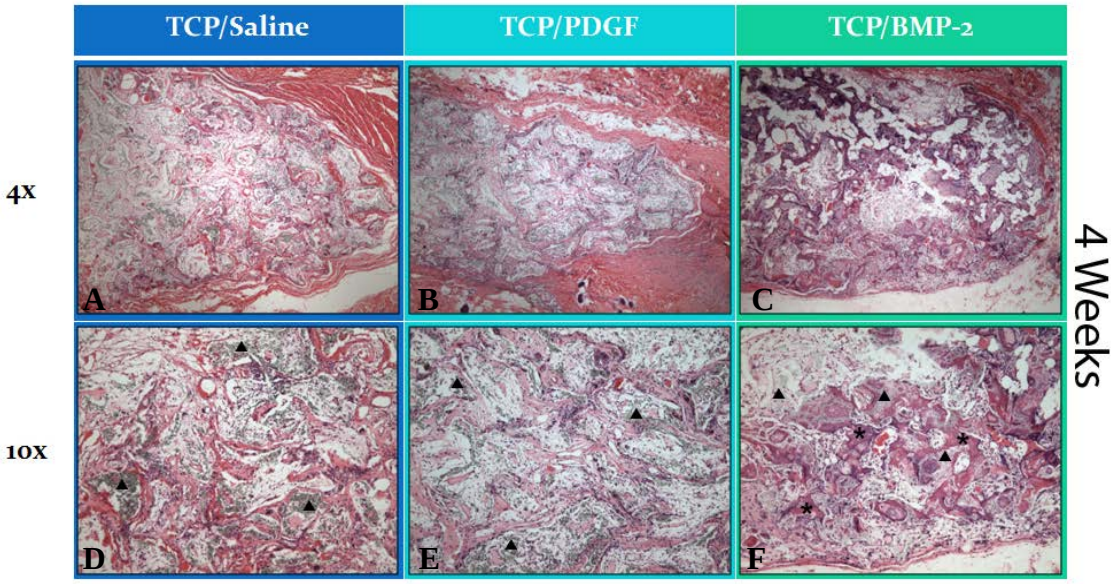

Figure 9: Histology 4X/10X Magnification for TCP Samples 2 & 4 Weeks

Description: (A) TCP/Saline at 4 weeks: well formed nodules of TCP particles surrounded by a fibrous capsule. No bone formation. (B) TCP/PDGF at 4 weeks: well formed nodules of TCP particles surrounded by a fibrous capsule with an increase in vascular channels. No bone formation. (C) TCP/BMP-2 at 4 weeks: Numerous thicker islands of trabecular bone surrounding remaining TCP particles. TCP/BMP-2 at 4 weeks: Numerous thicker islands of trabecular bone surrounding remaining TCP particles. ✳ New bone ▲ TCP particles

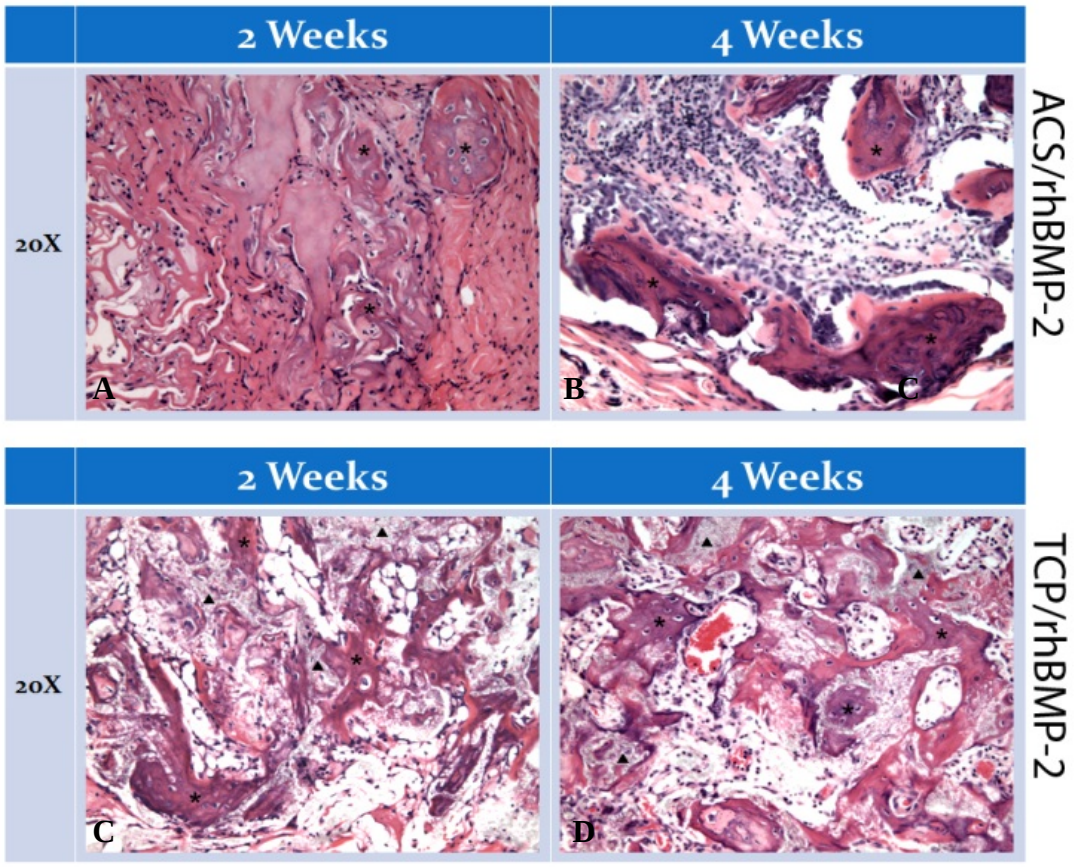

Figure 10: Histology 20X for rhBMP-2 Samples 2 & 4 Weeks

Description: (A) ACS/BMP-2 at 2 weeks: immature woven bone formation. (B) ACS/BMP-2 at 4 weeks: mature vital lamellar bone formation with osteocytes in the lacunae and osteoblasts laying down osteoid. (C) TCP/BMP-2 at 2 weeks: TCP particles in close proximity to the new trabecular bone containing osteocytes in the lacunae. (D) TCP/BMP-2 at 4 weeks: Numerous thicker islands of trabecular bone surrounding remaining TCP particles. TCP/BMP-2 at 4 weeks: Numerous thicker islands of trabecular bone surrounding remaining TCP particles. ⁎ New bone ▲ TCP particles

2 (Rostral Left of Animal)	1 (Rostral Right of Animal)
3(Caudal Left of Animal)	4(Caudal Right of Animal)

Table 1: Identification numbers for the relevant quadrants on the rat thorax.

Histomorphometric Score		**0**	**0.5**	**1**	**2**	**3**	**4**
Bone Formation %		**0%**	**1-10%**	**11-25%**	**26-50%**	**51-75%**	**76-100%**
2 Weeks	**ACS/BMP-2**	0	5	7	0	0	0
	TCP/BMP-2	0	4	6	2	0	0
2 Weeks	**ACS/PDGF**	0	0	0	0	0	0
	TCP/PDGF	0	0	0	0	0	0
2 Weeks	**ACS/Saline**	0	0	0	0	0	0
	TCP/Saline	0	0	0	0	0	0
4 Weeks	**ACS/BMP-2**	0	0	7	5	0	0
	TCP/BMP-2	0	0	0	8	4	0
4 Weeks	**ACS/PDGF**	0	0	0	0	0	0
	TCP/PDGF	0	0	0	0	0	0
4 Weeks	**ACS/Saline**	0	0	0	0	0	0
	TCP/Saline	0	0	0	0	0	0

Table 2: Histomorphometry demonstrating percentage of bone formation.

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