BMP2/7 heterodimer is a stronger inducer of bone regeneration in peri-implant bone defects model than BMP2 or BMP7 homodimer

Ping SUN¹, Jingxiao WANG², Yuanna ZHENG³, Yi FAN¹ and Zhiyuan GU¹,³

¹School/Hospital of Stomatology, Zhejiang University, 395 Yan’an St, 310006, Hangzhou, Zhejiang, P.R. China
²Department of Stomatology, the First Affiliated Hospital of Wenzhou Medical College, 2 Fuxue St, 325000, Wenzhou, Zhejiang, P.R. China
³School of Stomatology, Zhejiang Chinese Medical University, Mailbox 97, 548 Binwen Road, Binjiang District, 310053, Hangzhou, Zhejiang, P.R. China
Corresponding author, Zhiyuan GU; E-mail: gzy@zju.edu.cn

This study aimed to compare the effects of bone morphogenetic protein BMP2/7 heterodimer and BMP homodimers on bone regeneration in bone defects model. Identical peri-implant bone defects model were created using proper controls on the frontal skull in 18 minipigs. Collagen sponges with low-dose (30 ng/mL) BMP2/7 heterodimer, BMP2 or BMP7 homodimer were filled in the defects. New bone formation and the expression of type I collagen (Col1), alkaline phosphatase (ALP) and osteocalcin (OCN) were evaluated after 2, 3, and 6 weeks of implantation. BMP2/7 resulted in significantly higher new bone areas percentage in the defect region than BMP2 and BMP7 (p<0.05). Immunohistochemical staining of Col1, ALP and OCN was stronger in BMP2/7 group than BMP2, BMP7 and control group (p<0.05). These results demonstrate that BMP2/7 heterodimer is a stronger inducer of osteoblastogenesis and could be applied at low dose to reduce the cost and side effects of BMP homodimers.

Keywords: BMP2/7 heterodimer, Bone regeneration, Implant, Collagen I, Osteocalcin

INTRODUCTION

Endosseous dental implants are considered the ideal replacements for missing teeth. However, in clinic osseointegration is often compromised by large peri-implant bone defect. Several regional bone regeneration techniques including bone tissue engineering has been reported to solve such peri-implant bone defect¹⁻³.

Bone morphogenetic proteins (BMPs) emerge as promising candidates for bone tissue engineering due to their significant effects on bone and cartilage growth⁴,⁵. The efficacy of BMP homodimers therapy has been proved to be successful in many animal experiments, but the wide application of BMP homodimers in the clinic is limited by the high effective dosage (e.g. up to milligrams) and a series of potential side effects⁶,⁷.

Recent study showed that the bone-inducing efficacy of BMP2/7 heterodimers was 15–20 fold more than that of BMP homodimers and BMP 2/7 heterodimers could be used in relatively low dosage without compromising its osseointegration and bone regeneration efficacy⁸. Similarly, in our in vitro experiment, we found that rhBMP2/7 heterodimer induced cell migration with a significantly lower optimal concentration and higher peak effect and induced cell differentiation with significantly lower threshold concentrations than the respective homodimers⁹. Furthermore, our in vivo experiment using collagen sponges with low-dose (30 ng/mL) BMP2/7 heterodimer to treat freshly created peri-implant bone defects on minipig’s caldaria showed that BMP2/7 heterodimer induced new bone formation in a significantly higher quality and quantity in comparison to BMP2 and BMP7 homodimers by MicroCT and hard tissue slicing¹⁰. These studies suggest that BMP2/7 heterodimer may be an alternative approach for bone tissue engineering to overcome the disadvantages of BMP homodimers.

However, the biofunctional characteristics and mechanisms of BMP heterodimers on osteogenesis remain unclear. Therefore, in the present study we investigated the processes of new bone formation and the expression of Col1, ALP and OCN in a peri-implant bone defect model of minipigs treated with the same low-dose (30 ng/mL) BMP2/7 heterodimers in comparison with BMP2 and BMP7 homodimers.

MATERIALS AND METHODS

Animals

Eighteen 9-month-old minipigs (9 male, 9 female, weighing 16.5–19.8 kg) were used for the in vivo animal study. The animals were housed in Animal Research Centre of Zhejiang University where they were given unrestricted access to food and water during the experimental period. The experimental protocols were approved by the Institutional Animal Care and Use Committee of Zhejiang University (Hangzhou, China).

Experimental protocols

The minipigs were divided into four experimental groups: 1) Collagen with BMP2/7 heterodimer (experimental group); 2) Collagen with BMP2 homodimer; 3) Collagen with BMP7 homodimer; 4) Collagen without BMPs (non-BMP-treated). The samples were subjected to different defects of different animals following a randomization
protocol to balance the influence of the gender and health of animals as well as defect sites.

We set up three time points (2 weeks, 3 weeks and 6 weeks after the implantation). At each time point, total 24 defects were randomly distributed in 6 minipigs (4 defects in each minipig and \( n = 6 \) per group per time point).

Operative procedure

Recombinant human BMP2/7 heterodimer, BMP2 homodimer or BMP7 homodimer (R&D, Minneapolis, USA) was reconstituted to a final concentration of 0.05 \( \mu \text{g/\mu L} \) in a sterile 4 mM HCl solution containing 0.1% bovine serum albumin (BSA). The sterile 4 mM HCl solution containing 0.1% BSA without BMP was used as negative control (non-BMP suspension).

The absorbable collagen sponges (Helistat®, Integra Life Sciences, Plainsboro, USA) were adapted into uniform small pieces (15 mm×4 mm×2.5 mm) under sterile condition. 100 \( \mu \text{L} \) BMP or non-BMP suspension was then adsorbed onto each collagen sponge piece. The final loading of BMP was 5 \( \mu \text{g} \) per collagen sponge piece. The sponge pieces were then dried naturally under sterile condition. Titanium implants (Φ3.1 mm×10 mm, sandblasted and acid etched surface, Guangci Medical Appliance, Cixi, China) were cleaned and sterilized before use.

The minipigs were anaesthetized by intramuscularly injection of 0.3 mL/kg Sumianxing II (Military Veterinary Institute, Changchun, China) with the addition of Penicillium (5×10^4 Unit/kg) and Atropine (0.03 mg/kg) 30 min before the surgery. After applying a local anesthesia (1% Lido- caine with 1:100,000 Adrenaline) to the frontal calvariae of the minipig, a 10 cm long sagittal incision was made on the forehead region under sterile conditions. With a trephine drill (8 mm in diameter, Straumman, Switzerland), four bone defects (8 mm in diameter, 4 mm in depth) on each minipig calvariae were formed according to previous reports \(^{11,12} \) (Fig. 1a). The defects were positioned at least 1 cm apart to avoid biological interactions. Then the titanium implants were inserted in the centre of bone defects with 4 mm long fixtures above the bottom of the defects (Fig. 1b).

The remaining circular bone defects around the implant were filled with prepared collagen sponge with or without BMP. At each time point, the distribution of total 24 defects in 6 minipigs (4 in each) was assigned by random distribution. The total volume of each bone defect after the implantation was 166 mm\(^3\), and 5 \( \mu \text{g} \) BMPs per defect was equivalent to 30 ng/mm\(^3\) (5,000 ng/166 mm\(^3\)). After each of the defect and implant was covered by a piece of Bio-Gide® membrane (40 mm×50 mm, Geistlich PhamaAG, Wolhusen, Switzerland), the upper soft tissues were sutured in two layers. In addition, PENicillin (50,000 U/kg) was given intramuscularly to prevent infection.

Sample preparation

The animals were sacrificed by intramuscular injection of 0.6 mL/kg Sumianxin II at 2-, 3- and 6-week post-operation (6 minipigs for each time point). The 6 calvariae blocks were harvested and immediately fixed in 10% neutrally buffered formalin. Then each individual bone sample was cut longitudinally in half using a gypsum saw. Totally 24 specimens (6 specimens per group) were achieved at each time point. After being decalcified in
10% ethylenediaminetetraacetic acid at 4°C for 2–4 weeks the specimens were dehydrated with alcohol, and embedded in paraffin for histological and immunohistochemical analysis.

**Histological analysis**
The decalcified tissues were cut into 4 µm thick sections using a Leica microtome (Nussloch, Germany), and mounted on glass slides coated with poly-l-lysine and stained with hematoxylin and eosin. The histomorphology was assessed under a light microscope (BX51, Olympus Japan Inc, Tokyo, Japan) using computer-based image analysis system (Image Pro Plus 6.0, Media Cybernetic, Silver Springs, MD, USA). Four randomly selected sections from the serial sections collected from each sample were analyzed manually. In each section we randomly selected 4 fields of vision in the defect region. The newly formed bone area and the defect region area were recorded according to the instruction of the Image Pro Plus 6.0 Software. And the newly formed bone ratio (i.e. the percentage of newly formed bone area in the defect region) was recorded and compared.

**Immunohistochemistry**
The paraffin-embedded sections were deparaffinized in 100% xylene for 10 min, and then hydrated with 100% ethanol for 5 min, 95% for 3 min, 80% for 3 min, and 70% for 3 min. After being washed with distilled water, the sections were trypsin digested for 8 min for antigen retrieval, then rinsed in phosphate-buffered saline (PBS) for 5 min three times. Afterward, the slides were incubated with 3% hydrogen peroxide for 10 min and rinsed in PBS 5 min three times. Then, the slides were incubated with primary antibodies for collagen I (human polyclonal antibody, Sigma-Aldrich, 1:100 dilution), alkaline phosphatase (rabbit polyclonal antibody, Protein Tech, 1:100 dilution), and osteocalcin (mouse monoclonal antibody, Abcam, 1:400 dilution) for 1 h in a humid chamber at 37°C. After rinsing in PBS, the slides were incubated with goat anti-rabbit or mouse secondary antibodies labeled with horseradish peroxidase (HRP) for 30 min at 37°C. Finally the slides were incubated with DAB chromagen and examined for color change under light microscope (BX51, Olympus Japan Inc). The slides were counterstain with hematoxylin for 1 min. The primary antibody was omitted and substituted with PBS as the negative control.

Image-pro plus 6.0 analysis software was used for Image analysis. The positive staining was recorded and expressed as the value of integrated option density (IOD).

**Statistical analysis**
All data were presented as mean±SD. Differences among the groups were analyzed by repeated measures ANOVA. p values less than 0.05 (two tailed) were considered significant. Statistical analysis was performed using the SPSS/Windows statistical package (version 17, SPSS, Chicago, IL, USA).

**RESULTS**

**Histological findings**
H&E staining of the bone tissues in the four groups at three different time points was shown in Fig 2.

At 2 weeks post-operation, in the control group there was a little osseous repair at the bottom and the lateral margin of the peri-implant defect. In the centre of the defect, a little newly formed woven bones and the undegraded collagen sponge with a few numbers of cells could be observed.

In the defect region of BMP2 or BMP7 treated group, newly formed woven bones was observed from the defect border to the center together with the undegraded collagen sponge.

In the BMP2/7 treated group, significantly more new bones formed were observed in the border and center of the defect, compared to the other three groups. We also found that some newly formed trabecular bones had osteoblasts adhering on its surface. In the new bone formation areas seams of cuboidal and columnar osteoblasts were found. Solitary immature woven bone was present in the defect region.

At 3 weeks post-operation, new bone formation was obvious in peri-implant defect in each group compared to 2 weeks post-operation. Osteoblasts seams were observed lining the trabeculae in most portions typical. The control group showed the least bone formation among all the four groups.

The newly formed bone trabeculae of BMP2 or BMP7 treated group built up a structure of network. In BMP2/7 heterodimer group a lattice of newly formed trabecular bone was in the endosteal surface and bone was found to be surrounded by an osteoblastic rim in some areas.

At 6 weeks post-operation, the control group still showed limited bone regeneration. The collagen sponges were almost completely degraded in BMP treated groups, and were replaced by newly formed bone trabeculae. The bone trabeculae induced by BMP2/7 were significantly thicker and denser than those induced by BMP2 or BMP7. Extensive bone formation was observed on the periosteal and endosteal surfaces with remoulding of the cortical bone. In addition, the structure of newly formed bone tissue was almost the same as the normal ones around the defect.

**Histomorphometric analysis of new bone areas percentage**
Next we analyzed the newly formed bone area ratio (i.e. the percentage of newly formed bone area in the defect region) and the results were represented in Fig. 3.

1. At 2-week post-operation
Two weeks after implantation, the bone areas percentage in the defect region was 6.04±1.22% in the control group, 19.70±2.96% in BMP2 group, 14.50±2.47% in BMP7 group, and 30.41±5.36% in BMP2/7 group, dramatically higher than the former three groups. It is obvious that all the three BMP treated groups achieved more bone regeneration compared with control group (p<0.05).

2. At 3-week post-operation
Three weeks after implantation, the bone areas
Fig. 2  H&E staining of the bone tissues in the four groups at 2 (a–d), 3 (e–h) and 6 (i–l) weeks post-operation. a, e, i: control group; b, f, j: BMP7 group; c, g, k: BMP2 group; d, h, l: BMP2/7 group. The arrows indicated the position of the dental implant. Magnification: 40×

Fig. 3  Analysis of the newly formed bone area ratio in the four groups at different time post-operation. The percentage of newly formed bone area in the defect region was calculated based on H&E staining and expressed as $\bar{x} \pm s$. *$p<0.05$ vs. control group; $^a_p<0.05$ vs. rhBMP7, rhBMP2; $^d_p<0.05$ vs. 2 weeks, 3 weeks.
percentage in the defect region increased to 20.55±2.73%, 31.61±3.31%, 25.72±2.82% or 40.72±5.39% in the control group, BMP2, BMP7 or BMP2/7 group, respectively. Statistical analysis revealed that while significant difference was observed between BMP-treated groups and control group, the difference between BMP2/7 group and BMP2 or BMP7 group was also significant (p<0.05).

3. At 6-week post-operation

Six weeks after surgery, the bone areas percentage in the defect region increased a lot in all four groups. Especially for BMP2/7 group, the bone areas percentage was 59.44±6.42%, significantly higher than that of BMP2 group (43.07±2.74%) or BMP7 group (36.42±3.39%) as well as the control group (27.88±2.07%) (p<0.05).

Immunohistochemistry

Type I collagen (COL1) is the major organic component of the mineralized bone matrix. By immunohistochemical staining we could detect its expression in bone matrix at 2-, 3- and 6-week post-operation (Fig. 4). Notably, COL1 expression peaked at 3 weeks (p<0.05). Comparison of COL1 expression in the four groups showed that COL1 expression was significantly higher in BMP2/7 group than other groups at 2-, 3- and 6-week (p<0.05). COL1 expression was higher in the BMP2 and BMP7 groups than the control groups, but without any significant difference between the two groups at 2-, 3- and 6-weeks (p>0.05).

ALP is expressed by osteoblasts and participates in the mineralization of the bone matrix. We detected ALP protein in osteocytes, osteoblasts and bone matrix (Fig. 5). ALP expression was much higher in the BMP 2/7 treated group than in the other groups at 2-, 3- and 6-week post-operation (p<0.05). When we performed intra-group comparison, ALP expression was significantly higher at 2 weeks in relation to 3 weeks and 6 weeks post-operation in all four groups (p<0.05).

The osteocalcin protein, an indicator of the mineralization of the bone matrix. We detected osteocalcin staining we could detect its expression in bone matrix at 2-, 3- and 6-week post-operation (Fig. 6). At 2-, 3- and 6-week, the expression of osteocalcin was significantly higher in the BMP 2/7 group than in the other groups (p<0.05).

DISCUSSION

BMPs induce endochondral bone formation by stimulating the differentiation of mesenchymal progenitor cells10. BMP2 and BMP7 have been proven to be efficacious in stimulating bone formation in animal models6-18. BMP2 is known to induce osteogenesis and new bone formation19-20. BMP7 is a multifunctional member of the BMP family with multiple effects on bone formation and regeneration21-23. BMP2 and BMP7 homodimers were reported to accelerate bone formation efficiently. However, the use of the BMP homodimers is associated with a high cost5-26 and potential side-effects27. BMP 2/7 heterodimers have been proposed to overcome the disadvantages of BMP homodimers.

In this study, we adopted commercially available purified recombinant human BMP heterodimer and homodimers with the same low-dose (30 ng/mL) to compare the bone regeneration process of these materials histologically and immunohistochemically in a minipig bone defects model.

The minipig was chosen as the test model, because its calvarial bone regenerate rate is similar to that in humans and previous studies have indicated that calvaria bone defects are acceptable model for peri-implant bone defects11,26. The critical size defect (CSD) was defined by Hollinger and Schmitz27,28 as the bone defect of a large size where no spontaneous complete osseous regeneration of the created defects occurs during the lifetime of the animals, and CSD was adopted to test bone repair materials in a hierarchy of animal models. Studies have demonstrated that osseous regeneration depends on the localization of the defect, the size of the defect, the species, and the age of the animal27-29. Schlegel et al12 established monocortical defects in calvarias of adult pigs and examined the spontaneous physiologic healing process microradiographically and histologically. No complete osseous defect regeneration was recognizable after 52 weeks, indicating that the monocortical CSD in the calvaria of adult pigs fulfills the requirements for a CSD. Therefore, this peri-implant CSD model was adopted in the present study. Moreover, Bio-Guide® membrane was used to cover the implants and collagen sponges after implantation in order to prevent the soft tissue from growing into the defect region.

The minimal dose of BMP homodimers to induce bone regeneration of bone defects in minipig’s calvaria was not reported before. The recommended dose of BMP2 in the manufacturer’s instruction is about 400 ng/mm3. It was reported that BMP2 could induce bone regeneration in CSD of rat calvaria at the dose of 30 to 240 ng/mm330. In our previous study, we found that the effective concentration of BMP2/7 to induce osteoblastogenesis was significantly lower than those of BMP2 and BMP7 homodimers31. Accordingly, 5.0 µg (equivalent to 30 ng/mm3 in bone defects) BMP was used in this study.

Our histological analysis showed that no sufficient bone regeneration was found in the control group in each time point. In contrast, significant bone regeneration was observed in BMP2/7 group at 2, 3 and 6 week post-operation. As expected, the new bone area ratio in the defect region of BMP treated groups was significantly higher than that of control group at each time point. Moreover, the new bone area ratio in the defect region was significantly higher in BMP2/7 group than BMP2 and BMP7 groups, indicating that BMP2/7 is stronger to induce bone regeneration than BMP2 and BMP7. These results are in agreement with previous studies which showed that BMP2/7 heterodimer exhibited greater efficacy to enhance bone regeneration or stable spine fusion than BMP2 or BMP7 homodimer21,22.

Furthermore, we examined the expression of three osteoblastogenesis related proteins collagen type I, alkaline phosphatase and osteocalcin by
Fig. 4  Immunohistochemistry staining of COL1 expression in the defect area in the four groups at different time post-operation. a–d: representative images showing the staining of COL1 in the defect area at 3 w post-operation. a: control group; b: BMP7 group; c: BMP2 group; d: BMP2/7 group. e: IOD values of COL1 in the four groups at 2 w, 3 w or 6 w post-operation were calculated and expressed as $\bar{x} \pm s$. *$p<0.05$ vs. control group; **$p<0.05$ vs. rhBMP7, rhBMP2; ***$p<0.05$ vs. 2 weeks, 3 weeks. The arrows indicated positively stained matrix. Magnification: 200×
Fig. 5  Imunohistochemistry staining of ALP expression in the defect area in the four groups at different time post-operation. a–d: representative images showing the staining of ALP in the defect area at 2 w post-operation. a: control group; b: BMP7 group; c: BMP2 group; d: BMP2/7 group. e: IOD values of ALP in the four groups at 2 w, 3 w or 6 w post-operation were calculated and expressed as $\bar{x}\pm s$. *$p<0.05$ vs. control group; **$p<0.05$ vs. rhBMP7, rhBMP2; ***$p<0.05$ vs. 2 weeks, 3 weeks. The arrows indicated positively stained osteoblasts. Magnification: 200×
Fig. 6  Immunohistochemistry staining of osteocalcin expression in the defect area in the four groups at different time post-operation. a–d: representative images showing the staining of osteocalcin in the defect area at 3 w post-operation. a: control group; b: BMP7 group; c: BMP2 group; d: BMP2/7 group. e: IOD values of osteocalcin in the four groups at 2 w, 3 w or 6 w post-operation were calculated and expressed as $\bar{x}\pm s$. *$p<0.05$ vs. control group; a,b $p<0.05$ vs. rhBMP7, rhBMP2; c,d $p<0.05$ vs. 2 weeks, 3 weeks. The arrows indicated positively stained osteoblasts. Magnification: 200x
immunohistochemical staining. Bone homeostasis is maintained by a delicate integrated action of osteoblasts, osteocytes, and osteoclasts. Bone formation can be driven by factors such as bone morphogenetic proteins, cytokines, and growth factors, which induce the differentiation of precursor cells into the osteoblast phenotype. Once differentiated, osteoblasts produce several proteins, such as type I collagen (COLI), osteocalcin (OCN), and alkaline phosphatase (ALP), which will generate newly formed bone, and then undergo differentiation under an osteocyte phenotype. ALP and OCN are markers of osteoblast differentiation and activity (early and late markers, respectively). Therefore, Coll, ALP and OCN are bone markers associated with the differentiation of osteocyte. Type I collagen is an early marker of de novo bone formation which comprises approximately 90% of the organic bone matrix. Alkaline phosphatase is the early-stage marker of osteoblast and plays an important role in the mineralization of the bone matrix. Osteocalcin is a terminal marker of de novo bone formation which is produced by osteoblasts, odontoblasts, and hypertrophic chondrocytes. Osteocalcin is the most abundant noncollagenous protein in the bone. Our results showed that the expression of these three proteins in the defect region was significantly higher in BMP2/7 group than BMP2 and BMP7 groups at 2-, 3- and 6-weeks post-operation. These immunohistochemical findings were in accordance with our previous in vitro study showing that rhBMP2/7 was more powerful to induce the expression of collagen type I, alkaline phosphatase and osteocalcin in MC3T3-E1 preosteoblast than rhBMP2 and rhBMP7.

Taken together, these data demonstrate that rhBMP2/7 heterodimer was more efficient to induce bone formation than BMP2 and BMP7 homodimer in peri-implant bone defects with the same low-dose (30 ng/mL), and this is accompanied by the higher expression of Coll, ALP and OCN in the defect region.

CONCLUSIONS

Compared to rhBMP2 and rhBMP7 homodimers, BMP2/7 heterodimer is a stronger inducer of osteoblastogenesis. BMP2/7 heterodimer could be applied in the bone tissue engineering at low dose to reduce the cost and side effects associated with BMP homodimers.

ACKNOWLEDGMENTS

This work was supported by Fund of Health Bureau of Zhejiang Province (No. 2010QNA014) and Fund of Department of Education of Zhejiang Province (No. Y200908995).

REFERENCES


