Effect of Bifunctional β Defensin 2-Modified Scaffold on Bone Defect Reconstruction

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ABSTRACT: Bone tissue engineering has emerged as an effective alternative treatment to the problem of bone defect. To repair a bone defect, antibiosis and osteogenesis are two essential aspects of the repair process. By searching the literature and performing exploratory experiments, we found that β defensin 2 (BD2), with bifunctional properties of antibiosis and osteogenesis, was a feasible alternative for traditional growth factors. The antimicrobial ability of BD2 against Staphylococcus aureus and Escherichia coli was studied by the spread plate and live/dead staining methods (low effective concentration of 20 ng/mL). BD2 was also demonstrated to enhance osteogenesis, with higher messenger RNA (mRNA) and protein expression of the osteogenic markers collagen I (Col1), runt-related transcription factor 2 (Runx2), osteopontin (Opn), and osteocalcin (Ocn) in vitro (1.5−2.5-fold increase compared with the control group in the most effective concentration group), which was consistent with the alkaline phosphatase (ALP) and alizarin red S (ARS) staining results. We implanted poly(sebacoyl diglyceride) (PSeD) combined with BD2 and rat bone tissue-derived mesenchymal stem cells (rBMSCs) under the back skin of rats and found that the inflammatory response was significantly lower with this combination than with the PSeD/rBMSCs scaffold without BD2 and the pure PSeD group and was similar to the control group. Importantly, when assessed in a critical-sized in vivo rat 8 m diameter calvaria defect model, a scaffold we developed combining bifunctional BD2 with porous organic polymer displayed an osteogenic effect that was 160−200% greater than the control group. The in vivo study results revealed a significant osteogenic response and antimicrobial effect and were consistent with the in vitro results. In summary, BD2 displayed a great potential of simultaneously promoting bone regeneration and preventing infection and could provide a viable alternative to traditional growth factors applied in bone defect repair.

INTRODUCTION

Perioperative infection is an intractable issue of bone defect reconstruction that always challenges doctors and researchers, particularly when the self-healing capacity of the fragile and complex-structured craniofacial bone may be compromised by potential or active bacterial activity. Once an infection is present, bone regeneration is inhibited. The traditional therapy of local or systemic antibiotic use readily triggers drug resistance in clinical practice and always requires long-term rehabilitation. In an orbital bone defect, the connection between the orbit and the paranasal sinus combined with the fistula caused by the bone defect can provide access to the bacteria outside and readily lead to chronic bacterial infections, increasing the difficulty of controlling the infection and causing bone reconstruction delay. Recovery from this situation always requires several months to years using the current clinical treatments, including local and systemic antibiotic administration, because of the long-term external component appliance and related side effects. Furthermore, there are reports revealing that some antibiotics, including gentamycin, can accelerate the degradation and reduce the mechanical strength of the newly formed bone\(^1\) and that the adjunctive appliance of antibiotics displayed no positive impact on implants with an unmodified surface in periimplantitis\(^2\) and the time of impact was limited.\(^3\) Another popular antimicrobial drug is the silver nanoparticle, which is reported to be cytotoxic through interacting with mitochondria and inducing apoptotic pathways. These problems together inspired the development of the concept of autogenous replacement with antimicrobial activity. Recently, in attempting to find a substitute for antibiotics and considering the limited sources of growth factors in bone tissue engineering, antibacterial peptide (ABP) was taken into consideration.

Received: December 11, 2019
Accepted: February 7, 2020
Published: February 18, 2020
ABP is a small molecular peptide consisting of 20–60 amino acid residues. Most ABPs are characterized by strong alkalinity, thermal stability, and broad-spectrum antimicrobial activity, which is the basis for the stable and long-term antibacterial effects. Researchers have already applied ABPs to bone tissue engineering for implant-associated bone infections. Among the ABPs, β-defensins (BDs) are reported to have wide availability and favorable prices. BDs are secreted by epithelial cells and white blood cells when the body encounters physical or chemical stimulation and bacterial infection.5,6 Regarding their antimicrobial ability, they have been demonstrated to be effective against virus infection,7 in the activation of the innate immune of periodontal tissues,8 and in the protection of the reproductive system from pathogens.9 There is also evidence that BDs contribute to cell proliferation and differentiation, including stimulation of T cell proliferation,9,10 regulation of macrophage activation and orientation,11 and promotion of dendritic cell maturation with Toll-like receptors.12,13 Scientists have applied defensins in tissue reconstruction, including epithelium wound healing.14 Among the BDs, β-defensin 2 (BD2) can act like traditional ABPs, supporting resistance to bacterial infections, including Actinobacillus pleuropneumonia infection.15 It was shown that bone marrow mesenchymal stem cells (BMSCs) transfected with recombinant adenovirus expressing human BD2 could promote wound healing.16 BD2 has been corroborated to be able to directly promote the proliferation and differentiation of osteoblast-like MG63 cells, which belong to the osteogenic cell line but cannot be implanted in the body because of the risk of carcinogenesis.17 However, the osteogenesis potential of BD2 on strains of the primary cells without the risks of overproliferation and carcinogenesis remains to be discussed. Based on earlier studies, we speculated that BD2 could promote mesenchymal stem cells, for example, by stimulating BMSC osteogenic differentiation and thereby accelerate bone regeneration in vivo via the bifunction of osteogenesis and antimicrobial activity against microorganisms.

In recent years, we have proven that poly(sebacoyl diglyceride) (PSeD) with exposed hydroxyl groups can be linked to bifunctional active molecules, including growth factors, peptide, and chemical groups, making it a potential alternative treatment for bone regeneration.18–21 These results consolidated the experimental basis for BD2-related bone tissue engineering.

In the present study, we demonstrated the antibacterial ability and the optimum osteogenic concentration of BD2 on rat BMSCs (rBMSCs) in vitro. Subsequently, we combined PSeD with rBMSCs and BD2 (PSeD/rBMSCs/BD2) to repair a critical craniofacial defect in rats and detected the osteogenic effect of BD2 in vivo. We found that BD2 was an effective antibacterial starting from 20 ng/mL. At the same concentration, the relative osteogenic messenger RNA (mRNA) and protein levels were increased such that this could promote the osteogenic differentiation of rBMSCs and play an important role in the prevention and treatment of infection during the perioperative period. Furthermore, within a certain concentration range, BD2 displayed no biotoxicity on rBMSCs, such that BD2 application in the clinical setting will likely be safe. The combination of PSeD/rBMSCs/BD2 also showed excellent effectiveness in rat critical calvaria defect reconstruction in vivo.

The combination of BD2, rBMSCs, and PSeD is a bifunctional scaffold that could concurrently treat perioperative infections and promote osteogenesis, providing an innovative and efficient solution for bone defect reconstruction.

■ RESULTS AND DISCUSSION

Bone defect repair appears to be the main target of bone tissue engineering. Scientists have paid great attention to traditional growth factors in previous years. Bone morphogenetic protein (BMP) can induce the differentiation of BMSCs to osteoblasts and promote the proliferation of osteoblasts and chondroblasts.22 Experiments indicated that the calcium phosphate cement scaffold carrying BMP-2 effectively induced osteogenesis, osteoinduction, and osteoconduction and could successfully repair a leporid orbital defect.23 In addition, a coral scaffolding carrying recombinant human BMP-2 (rhBMP-2) was able to repair orbital defects in a canine24 and a rabbit model.25 Researchers found that rhBMP-2 application was accompanied by side effects, including tissue swelling and seroma, and once the implant with rhBMP-2 was removed, the swelling was soon reduced.26 Because of a strong and positive correlation between frequencies of side effects and the rhBMP-2 doses in the repair of a rat critical craniofacial defect, determining the effective dose of rhBMP-2 to balance bone regeneration and maturation appears to be essential.27 Vascular endothelial growth factor (VEGF) itself can promote angiogenesis, increase vessel permeability, and accelerate the recruitment of MSCs and osteoprogenitor cells. On the association of VEGF with BMP-2, this ensures cell survival, stimulates bone mineralization, and promotes the shape and absorption of cartilage, while at the same time interfering with the signaling pathway between BMPs and VEGFs.28

Infection is another complex problem during the perioperative period. In the case of open fractures, infection is always associated with nonunion, such that we need to consider antibiosis as well as bone defect reconstruction. However, none of the traditional growth factors are able to fulfill both the osteogenic and antibacterial activities. The traditional method of treating infection is antibiotics, which are broadly and effectively used in the treatment of infected bone defects.29 However, antibiotics may fail when the bacteria has already caused an infectious nonunion. Additionally, prophylactic antibiotic use may lead to antibiotic overprescription.

The ABPs are safe and effective substitutes for antibiotics. These polypeptides have relatively shorter peptide chains compared with proteins like BMP-2, such that it is easier for scaffolds to carry them than a whole protein. In addition to the wide array of antimicrobial ability, some ABPs have been demonstrated to inhibit implantation rejection.30 BDs, produced by epithelium and immune cells, have the traditional function of maintaining the dynamic equilibrium between the host and the microorganism and preventing the potential infection of the enteric, productive, oral, and respiratory systems.15 BDs have also been reported to be effective in an Staphylococcus aureus-infected rat calvaria defect31 but displayed no extra osteogenic effects compared with the uninfected wild-type BMSC group in this study. Indeed, few researchers have focused on the osteogenic ability of BDs. Among the BDs, BD2 has been reported to contribute to the antibacterial as well as the wound-healing process. This encouraged us to investigate the differentiation potential of BD2. In the present study, we assessed the antimicrobial ability and the osteogenic effects of BD2 in vitro. Furthermore, we combined it with the porous scaffold PSeD and then evaluated the anti-inflammatory ability and osteogenic response in vivo.
It was previously proven that BD2 has an antimicrobial activity. However, the reported minimum inhibitory concentration (MIC) of human BD2 against wild-type strains of *S. aureus* of 4–10 μg/mL is much greater than the
biological activity concentration (0.1−100.0 ng/mL) of rat BD2 we used in the present study. Therefore, we decided to test the antibiosis of BD2 against both Gram-negative bacteria (Escherichia coli) and Gram-positive bacteria (S. aureus) at different concentrations within the biological activity concentration range. We examined the antimicrobial activity of different BD2 concentrations (0, 10, 20, 40, 80 ng/mL). From the preliminary study, we found a MIC for BD2 of 20 ng/mL (Figure 1). We inoculated the bacteria into a broth containing 20 ng/mL BD2. After incubation for 6 h, we found that E. coli

![Figure 3. Effects of BD2 on cellular proliferation and cell inflammation of rat BMSCs. The rat BMSCs were cultured separately in a control group of simple minimum Eagle’s medium α (MEMα) and with different BD2 concentrations (10, 20, 40, 80 ng/mL). (A) Cellular viability was analyzed by the OD 450 nm value. (B) Cellular viability was calculated based on equipercentile equating and normalized to the control group. (C) TNFα, caspase 3, and IL-6 mRNA expression levels of cells cultured in pure MEMα or MEMα with 20 ng/mL BD2 for 3 days were analyzed by quantitative polymerase chain reaction (qPCR). Data are presented as the mean ± SD, n = 3, and P-values are calculated using one-way ANOVA. All statistical significance is shown in comparison with the control group, *P < 0.05, **P < 0.01, ***P < 0.001.

![Figure 4. Photomicrographs of H&E staining sections of different implants with surrounding tissues. (A) H&E staining at week 2, (1, S) control group with no implant; (2, 6): PSeD; (3, 7): PSeD + BMSCs; (4, 8): PSeD + BMSCs + BD2 with PSeD marked with # and the simulated implantation site of the control group marked with * under a 40-fold microscope (1−4) and under a 400-fold microscope (5−8). (B, C) Immunofluorescent staining of macrophage-specific markers, CD86 for M2 and CD68 for M1. There was no significant difference among the four groups. Scale bar for the first row in (A)−(C) is 250 μm; scale bar for the second row in (A) is 25 μm.](https://dx.doi.org/10.1021/acsomega.9b04249)
(Figure 2A) and *S. aureus* (Figure 2B) were inhibited in the BD2 group by gross observation of the spread plate method. The live/dead dyeing method showed similar results (Figure 2C,D). Furthermore, we diluted the treated *E. coli* (Figure 2E) and *S. aureus* (Figure 2F) with broth to 1:100 and found that the optical density (OD) 600 nm value, which indicated the surviving bacteria activity, remained steady for 6 h compared with the significant increase with time of the control group. All of the tests demonstrated that 20 ng/mL BD2 could effectively inhibit both Gram-negative and Gram-positive bacteria activity.

Regarding the antibacterial activity of BD2, the results of Soman et al. in *Anas platyrhynchos* using avian BD2 suggested that BD2 has the ability to kill *E. coli*, but they did not consider the antibacterial effect against *S. aureus*, which together with *E. coli* are the most common bacteria in bone defect wound and the main bacterial pathogens of human osteomyelitis. In our study, we found that the MIC of BD2 against *S. aureus* was less than for *E. coli*, as similarly reported earlier. Furthermore, the MIC of BD2 was much lower at 20 ng/mL in the present study by 3 orders of magnitude compared with the earlier reported MIC of 32–128 μg/mL. One reason that we achieved similar antimicrobial effects with a lower MIC may be the ABP’s dynamical behavior to the phospholipid membrane at a low concentration as reported earlier. Another probable reason is the different origin of the BD2 and bacteria. Application of the low effective BD2 concentration would bring economic savings and greater efficiency of the BD2 application.

Biological safety is a precondition of clinical application. Warnke et al. reported that the proliferation of human MSCs and osteoblasts was similarly unaffected when incubated with recombinant human BD2. In our study, we confirmed that BD2 had no cytotoxicity for rBMSCs according to the cell counting kit-8 (CCK-8) results.

We investigated the viability of rBMSCs treated with different BD2 concentrations using the CCK-8 assay. The cell cultures diluted with 10–80 ng/mL BD2 performed the same as the control group (Figure 3A,B), indicating that BD2 did not inhibit cell proliferation of rBMSCs, such that we could apply this concentration range safely to animals. Furthermore, we investigated the mRNA levels of inflammation-associated cytokines in the BD2 (20 ng/mL BD2) and control groups. We found that the cells cultured in 20 ng/mL BD2 expressed 35–50% lower levels of tumor necrosis factor-α (TNFα), caspase 3, and interleukin 6 (IL-6) compared with the control group, which indicated that BD2 can effectively inhibit the expression of inflammatory cytokines in rBMSCs.

Then, we detected a peripheral inflammatory response in rats after implantation. We found that all of the treatment groups (PSeD/rBMSCs/BD2, PSeD/rBMSCs, PSeD) and the control group showed similar behavior and no specific inflammation reaction. Haemotoxylin and eosin (H&E) staining (Figure 4A) and immunofluorescent staining (Figure 4B,C) related to the expression of the M1/M2 macrophage (CD68 for M1 and CD86 for M2) results suggested that the surrounding soft tissue of all of the groups appeared to display a normal histological structure and host response to the implants.

Our study further confirmed an osteogenic effect of BD2 in vitro. The data we reported here indicated that BD2 could promote bone regeneration as well as infection defense. It not only had bacteriostatic and bactericidal effects in vitro but also promoted osteogenesis of rBMSCs via the upregulated expression of related genes and proteins (collagen I (Col1), osteocalcin (Ocn), osteopontin (Opn), and runt-related transcription factor 2 (Runx2)). The osteogenic regulation by BD2 was correlated to concentration within a certain range, reaching a peak effect at 20 ng/mL. Most of the ABP-related studies indicated that with an increase of antimicrobial peptide concentration, bacteria elimination and cell adhesion and proliferation could be more effectively promoted. Similarly...
Figure 6. Micro-CT and morphological results of reconstructed skulls. To investigate the in vivo osteogenic effect, we continuously traced the growth tendency of rat critical calvaria defects for 8 weeks. (A) Micro-CT images showing the difference in bone regeneration among the control, PSeD, PSeD/BMSCs, and PSeD/BMSCs/BD2 groups at weeks 1 and 8. The red circles indicate the original defect area (8-mm-diameter circle). Subsequently, we cut the skull into coronal sections and applied the resin as the carrier to embed the skulls. Following embedding, the skulls were stained with van Gieson dye. (B) Van Gieson staining results indicated that the PSeD/BMSCs/BD2 group performed the best while the remaining three groups were ranked according to the order PSeD/BMSCs group, PSeD group, and control group. In these images, the new bone with collagen was marked in red. Following decalcification, the skulls were stained with Masson’s trichrome. (C) Masson’s trichrome staining results for the four groups; red marks collagen I, which indicates mature bone tissue, whereas the blue area shows the immature area. (D–F) Morphometric analysis of the bone volume/total volume (BV/TV), bone surface (BS), and bone mineral density (BMD). *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 7. Fluorescence analysis of newly formed bones. (A) Confocal microscope images for each group. Row 1 (yellow) represents new bone formation marked by calcein injected at week 6, row 2 (red) represents alizarin red at week 9, row 3 (green) represents tetracycline at week 3, and row 4 represents merged images of the three fluorochromes for the same group. (B) The graph shows the percentages of the fluorochrome area of new bone formation out of the whole bone area. With respect to the different administration times, the new bone tissues were marked in a different color at different stages of osteogenesis. The resulting osteogenic area increased in the following order: control group, PSeD group, PSeD/rBMSCs group, and PSeD/rBMSCs/BD2 group. Scale bar is 100 μm. All statistical significance is shown in comparison with the control group, *P < 0.05, **P < 0.01, ***P < 0.001.
in our study, an increase of BD2 concentration was associated with more effective antimicrobial activity, as was its osteogenic effect. However, when BD2 was >80 ng/mL, the osteogenic effect appeared to be reduced. We suggest that because of the high BD2 concentration, a signal was sent to an upstream receptor, which in turn suppressed the expression and transmission of downstream signals. Nevertheless, the specific mechanism needs to be discussed and explored in our future research.

We evaluated the level of osteogenesis stimulated by 20 ng/mL BD2 compared with the control group of the MEMa culture medium alone. The results suggested that 20 ng/mL BD2 could effectively promote osteogenesis of rBMSCs at the mRNA (Figure 5A) and protein levels (Figure 5B). Similar results were obtained for the osteogenic differentiation for days 7 and 14 as reflected by alkaline phosphatase (ALP) and alizarin red S (ARS) staining, respectively, both through microscope observation (Figure 5C,E) and with the naked eye (Figure 5D,F).

In the subsequent study, we chose 20 ng/mL when considering the biological effect and economic factors. Furthermore, for the in vivo experiments to correspond with the in vitro experiments, we calculated the optimum amount of BD2 required per unit cell, which showed that 200 ng of BD2 was needed for 5 x 10^6 cells used in each dorsum subcutaneous pouch as well as each rat critical calvaria bone defect reconstruction.

For continual observation of bone reconstruction, 1 and 8 weeks after generating the calvaria bone defect and reconstructive surgery, the morphology of randomly selected rats was reconstructed by living microcomputed tomography (micro-CT). During the experimental observation, we found that two of six rats of the control groups without BD2 adjunction had bacterial infection with a purulent discharge from the wound and were dead 2 days postsurgery. By contrast, among the groups with added BD2, there was no obvious wound infection. The results of the tracing observation revealed that the combination of PSeD/rBMSCs/BD2 was superior to the others, with the mean reconstruction area rates of the rest of the groups in the order from PSeD/rBMSCs to PSeD alone, being the least in the control group (Figure 6A) in the transverse section. This was also proved by the following morphometric analysis of the bone volume/total volume (BV/TV), bone surface (BS), and bone mineral density (BMD) (Figure 6D-F). At week 12, all of the skulls were dissected and a three-dimensional isosurface generated. Moreover, the results of van Gieson (Figure 6B) and Masson’s trichrome staining (Figure 6C) also corroborated the superiority of the PSeD/rBMSCs/BD2 combination. Furthermore, the luminograms and fluorochrome analysis of tetracycline, calcein, and Alizarin red S (ARS) staining, respectively, both through microscope observation (Figure 5C,E) and with the naked eye (Figure 5D,F).

In conclusion, our study suggested that BD2 could make antibacterial effects in a concentration-dependent manner. The rBMSCs cultured with BD2 not only expressed higher levels of osteogenic genes and proteins but also produced more osteogenesis-related ALP and calcium precipitates in vitro. Moreover, BD2 combined with rBMSCs and PSeD could effectively repair a rat critical calvaria defect, suggesting this to be an innovative biodegradable composite with the bifunction of antibiosis and osteogenesis. In conclusion, with its antibacterial and osteogenic bifunctional effects, BD2 provides new ideas for bone defect repair and appears promising for clinical application.

### EXPERIMENTAL SECTION

**Synthesis of PSeD.** Initially, we mixed epoxy propyl alcohol (7.8 mL, 116.9 mmol) and triethylamine (31.5 mL, 224.1 mmol) in 180 mL of anhydrous toluene and precooled in an ethanol–water mixture bath (−15 °C) for 30 min. Sebacoyl chloride (11.37 g, 47.6 mmol) was added to the precooled mixed solution under a nitrogen gas atmosphere and was stirred and mixed well to stimulate an esterification reaction and produce monomer diglycidyl sebacate. Following stirring for 6 h, the mixture was filtered and concentrated. Silica gel was prepared by mixing n-hexane and ethyl acetate in a ratio of 3:1. Using the silica gel as the substrate, the mixed solution was subjected to flash chromatography to obtain a white solid, namely, diglycidyl sebacate.

The chief ingredients of PSeD were sebacate glycidyl ester and sebamic acid, which performed the epoxy ring-opening polymerization, that is, using tetrahydrofuran as a solvent, ethylene glycol ether and sebamic acid dissolved in dimethyl formamide and then were diluted with ethyl acetate, precipitation performed using ethyl ether, and vacuum-drying at room temperature overnight, to ultimately generate PSeD.

**Cross-Linking of PSeD.** PSeD was mixed and melted with sebamic acid (1.1 wt %). This was heated under high temperature at 120 °C for 20 h to remove air bubbles and then placed in a vacuum-drying oven (1.1 Torr) at 120 °C for 21 h for cross-linking. In addition, the in vitro material studies also required two-dimensional cross-linking between PSeD and cell slides to facilitate cell adhesion, proliferation, and observation. We used a vacuum-drying oven to cross-link the PSeD materials under a high temperature of 150 °C with 24-mm-diameter round coverslips. The cross-linked materials were stored at room temperature and were autoclaved before cell experiments. The porous three-dimensional scaffold was constructed by salt fractionation with NaCl salt particles with a uniform diameter of 75–150 μm.

**Antibacterial Tests.** Antibacterial tests were performed against Gram-negative *E. coli* (atcc29522) and Gram-positive *S. aureus* (atcc6538). First, 10^7 colony-forming units (CFUs)
bacteria were cultured in broth separately with different BD2 concentrations (0, 10, 20, 40, 80 ng/mL). We selected the minimum initial concentration of BD2 against both E. coli and S. aureus in the spread plate and live/dead staining methods, and then, we compared the BD2 group with the phosphate-buffered saline (control) group in three different methods. Following coculture with $10^7$ CFU bacteria for 6 h, (1) $60 \mu L$ of fluid was inoculated in agar plates and incubated at $37 \degree C$ overnight according to the method reported earlier, (2) the bacteria after experimental treatment were stained by life/death dyeing for 15 min and the results were recorded by microscopic observation, (3) to monitor the long-term inactivation efficiency of BD2 on bacteria, we diluted the liquid culture with broth to 1:100, added it into 96-well plates, and determined the OD 600 nm value every 30 min for 6 h; this procedure was based on the protocol reported earlier.

Cell Culture. Bone marrow was extracted from the femur and tibia of 5-week-old male Sprague Dawley rats, which were obtained from the Experimental Animal Room of Shanghai Ninth People’s Hospital according to the protocol of Reis and Borges, and all experimental procedures were approved by the Animal Research Committee of Shanghai Ninth People’s Hospital, Shanghai JiaoTong University School of Medicine. The rBMSCs before the third generation were cultured in minimum Eagle’s medium $\alpha$ (MEM$\alpha$) (Gibco, Grand Island, NY) containing 10% fetal bovine serum (Gibco) and 100 units/mL penicillin at $37 \degree C$ under an atmosphere of 5% CO$_2$. Recombinant rat BD2 (Abcam, Cambridge, MA) was diluted to different concentrations (10, 20, 40, 80 ng/mL) according to its biological activity concentration range (0.1–100.0 ng/mL) in the MEM$\alpha$ medium. After 24 h of cell attachment, rBMSCs were cultured in the treated culture medium. In the treatment group, experimental cells were cultured in MEM$\alpha$ with 40 ng/mL BD2, while in the control group, the cells were in MEM$\alpha$ alone.

In Vitro Biotoxicity Detection. In total, $5 \times 10^4$ cells were seeded in 96-well plates in MEM$\alpha$ with different BD2 concentrations (0, 10, 20, 40, 80 ng/mL). The CCK-8 reagent was added to the BD2-containing MEM$\alpha$ and then incubated at $37 \degree C$ for 4 h. Cell proliferation was quantified using a cell counting kit (CCK-8) (Dojindo Chemical Laboratory, Kumamoto, Japan). The formazan dye generated by the reaction between the reagent and live cells was measured by spectrophotometry at 450 nm, and the cellular viability was determined based on the control group set at 100%.

Cells cultured in MEM$\alpha$ with or without 20 ng/mL BD2 were digested, and the extracted mRNA was used to detect inflammation-related factors (tumor necrosis factor $\alpha$ (TNF$\alpha$), caspase 3, interleukin-6 (IL-6)) by quantitative real-time RT-PCR (qPCR), the method of which is described in detail below.

In Vitro Osteogenic Detection. qPCR Analysis. Total RNA was harvested from rBMSCs cultured in different BD2 concentrations for 3 days using the EZ-press RNA purification kit (EZBioScience, Shanghai, China), and complementary DNA (cDNA) reverse transcription was performed using the PrimeScript RT reagent kit (Takara, Dalian, China). The qPCR analysis was performed using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and a 7500 Real-Time PCR detection system (Thermo Fisher Scientific, Waltham). The primer sequences used, including for collagen I (Col1), osteocalcin (Ocn), osteopontin (Opn), runt-related transcription factor 2 (Runx2), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were reported earlier. The relative mRNA levels measured were normalized to the GAPDH expression level.

Western Blot Analysis. After culturing rBMSCs in different BD2 concentrations for 7 days, the protein was extracted from the cells using radioimmunoprecipitation assay (RIPA) buffer (Cell Signaling Technology, Boston) and loading buffer (Takara, Ostu, Shiga, Japan). The protein was separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred to 0.22 $\mu m$ poly(vinylidene fluoride) membranes (Millipore, Billerica, MA). The membranes were blocked in 5% bovine serum albumin and tagged with primary antibodies diluted to 1:100 for Col1, Ocn, Ocn, Runx2 (Santa Cruz Biotechnology, Santa Cruz), or $\beta$-actin (Abcam) for 8 h at 4 $\degree C$. Subsequently, the membranes were probed with horseradish peroxidase-conjugated secondary antibodies (Sigma, Darmstadt, Germany) and scanned with an Odyssey V3.0 image scanner (LI-COR, Lincoln, NE). The gray level of the immuno-labeled bands was normalized to $\beta$-actin.

Alkaline Phosphatase (ALP) and Alizarin Red S (ARS) Staining. Following the culture of rBMSCs in different BD2 concentrations for 7 days, the cells were fixed in 95% ethanol and stained with ALP staining solution for 2 h according to the protocol reported earlier. Staining with ARS solution was performed as previously reported overnight on day 14.

Preparation for in Vivo Experiments. We prepared 8-mm-diameter PSeD materials and sterilized them using an autoclave sterilizer and then placed them in a 24-well plate using sterilized tweezers. From the in vitro experiments, an adequate BD2 concentration for antibacterial and osteogenic effects was defined as 20 ng/mL; this was used to culture rBMSCs in six-well plates ($2.5 \times 10^6$ rBMSCs/2 mL/well). For each 8-mm-diameter bone defect as well as subcutaneous biocompatibility detection unit, the cell load of the scaffold was approximately $12.5 \times 10^6$, and the required amount of antimicrobial peptides for each 8-mm-diameter scaffold was 20 ng; because the diluted concentration of the original antimicrobial peptides was 625 ng/mL, 30 $\mu L$ of this was added to each scaffold.

In Vivo Biocompatibility Detection. Six 8-week-old female Sprague Dawley rats were used for this study. Four different dorsum subcutaneous pouches were created through a single incision, and each rat was implanted with three different disk-shaped materials, including PSeD combined with BD2 (200 ng) and rBMSCs ($5 \times 10^6$ cells), PSeD combined with rBMSCs ($5 \times 10^6$ cells), and PSeD alone, and one blank group as the control. The animals were euthanized by general anesthesia, and the implanted materials were harvested with the surrounding soft tissue via subcutaneous dissection and fixed in 4% paraformaldehyde. The samples were cut into 8 $\mu m$ sections, and hematoxylin and eosin (H&E) staining and macrophage-related immunofluorescent staining (CD68 for M1, CD86 for M2) were performed according to a standard protocol to detect the peripheral inflammatory response after implantation of the materials.

In Vivo Osteogenic Detection. Animal Experiments. All experimental procedures were performed following animal protocols approved by Animal Research Committee of Shanghai Ninth People’s Hospital, Shanghai JiaoTong University School of Medicine. Animals were raised in the Animal Center of Shanghai JiaoTong University School of Medicine Affiliated Ninth People’s Hospital.
The calvarium of twelve 8-week-old female Sprague Dawley rats was shaved, cleansed with 75% ethanol, and exposed under general anesthesia according to the method reported earlier. A critical-size round bone defect (8 mm diameter) was created using a dental trephine (8 mm external diameter) (Nouvag AG, Goldach, Switzerland). The implants were divided into four groups: (1) PSeD scaffold seeded with rBMSCs (5 × 10^6 cells) and BD2 (200 ng) (PSeD/rBMSCs/BD2), (2) PSeD scaffold seeded with rBMSCs (5 × 10^6 cells) (PSeD/rBMSCs), (3) PSeD scaffold (PSeD), and (4) blank group (control). The 12 rats were randomly assigned to the four groups (n = 3). Following implantation, the surgical incision was sutured layer by layer and finally disinfected in 75% ethanol. At weeks 3, 6, and 9, tetracycline (Sigma, 25 mg/kg), calcein (Sigma, 20 mg/kg), and alizarin red (Sigma, 30 mg/kg) were injected via the abdomen, respectively, for fluorescent labeling according to the protocol reported previously.

Microcomputed Tomography (Micro-CT) Analysis. At week 1 and week 8 after surgery, the rats were administered general anesthesia and a circular region of interest of 8 mm diameter defined as the original bone defect was examined by micro-CT (Bruker SkyScan1076, Bruker, Karlsruhe, Germany) referring to the parameters used previously, including an X-ray tube potential of 40 kV, tube current of 250 μA, and voxel resolution of 35 mm. The skull was a three-dimensional isosurface rendered by software (MicroView, GE Healthcare, Waukesha, WI).

Fluorescence and Morphological Analyses. After 12 weeks of treatment, the rats were euthanized under general anesthesia and decapitated. The skulls were dissected and fixed in 4% paraformaldehyde, embedded in poly(methyl methacrylate), and dehydrated in an increasing concentration of ethyl alcohol (70–100%). Finally, the embedded skulls were cut into 300 μm sagittal sections using a microtome (EXAKT310, EXAKT Technologies, Norderstedt, Germany). We took pictures using a confocal microscope (Nikon A2, Nikon, Tokyo, Japan) of the sections to detect the fluorescence intensity of tetracycline, calcein, and alizarin red. Following van Gieson staining, we took pictures of the gross image of the sections and measured the area of the fluochrome-stained bone using Image-Pro 5.0 (Media Cybernetic, Silver Springs). The remaining skulls not already cut were decalcified for 1 month and stained with Masson’s trichrome, taking pictures of the sections.

Statistical Analysis. All of the data are presented as the mean ± standard deviation (SD). The analysis was performed using one-way analysis of variance (ANOVA) and paired t-test through SPSS, Version 22.0 (SPSS, Chicago, IL). A P-value < 0.05 was considered statistically significant.

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Notes

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ACKNOWLEDGMENTS

This research was supported by the National Natural Science Foundation of China (81600766, 81800695), the Municipal Education Commission Foundation of China (81600766, 81800695), Shanghai Municipal Education Commission—Gaofeng Clinical Medicine Grant Support (20161419), Shanghai Young Doctor Training Programme, Thyroid Research Program of Young Doctors (2017-N-14), the National Key R&D Program of China (2018YFC1106100, 2018YFC1106101), The Science and Technology Commission of Shanghai (17DZ2260100), and the Natural Science Foundation of Shanghai (18ZR1401900).

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