

博士學位論文

塩基性線維芽細胞増殖因子（bFGF）徐放化システムを
併用した OCP/コラーゲン複合体による骨再生の有用性；
ラット頭蓋骨欠損モデルを使用した評価

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Doctoral Dissertation

Bone regeneration using an OCP/collagen composite
supplemented with a bFGF drug delivery system:
Evaluation in a rat calvarial bone defect model

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ABSTRACT

Bone regeneration methods using biomaterials, such as a bioactive ceramic (hydroxyapatite) and bioabsorbable ceramic (β -tricalcium phosphate), have been clinically applied as reconstruction materials for various bone defects. However, bone formation by these ceramics is limited in tissue engineering approaches. An octacalcium phosphate (OCP)/collagen (Col)-conjugated composite is biodegradable and highly osteoconductive, with OCP particles within Col enhancing the migration of osteoblasts, and, thus, it has potential as a new bone regeneration material. However, the application of exogenous osteogenic cytokines with this material has not yet been examined. In the present study, basic fibroblast growth factor (bFGF) delivered by biodegradable gelatin hydrogels for sustained release was incorporated into the OCP/Col composite, and the bone regeneration ability of this system was investigated in a rat calvarial defect model. Histological, immunohistochemical, and CT examinations revealed that new bone formation was markedly greater by the OCP/Col composite with the bFGF drug delivery system than by the OCP/Col composite alone. The present results demonstrate the potential of OCP/Col with the bFGF drug delivery system for the treatment of difficult bone defects.

Introduction

Reconstruction of bone defects is performed primarily by autologous bone grafting. The mandible and ilium are often selected as graft materials, and these nonantigenic, autologous grafts unite with the surrounding bone tissues. However, various disadvantages of autologous grafts including pain and deformity of the donor sites have been reported.¹

To overcome these issues, artificial biomaterials, such as a bioactive ceramic (hydroxyapatite, HA) and bioabsorbable ceramic (β -tricalcium phosphate, β -TCP), have been clinically applied as reconstruction materials for bone defects. However, osteogenesis by these ceramics is limited and associated with many complications, such as infection, foreign body reactions, and post-graft displacement.²

Recently, an octacalcium phosphate (OCP)/collagen (Col)-conjugated composite has been developed. OCP/Col composite is biodegradable and highly osteoconductive, with OCP particles within Col enhancing the migration of osteoblasts. This synthetically engineered bone graft biomaterial may provide a promising alternative approach for the clinical treatment of bone defects caused by trauma, resection of the tumors, and congenital deformities.^{3,4}

Another approach that has attracted attention is the use of cytokines with bone-inducing activities, such as bone morphogenetic protein (BMP), basic fibroblast growth factor (bFGF), and insulin-like growth factor (IGF). bFGF has been widely used in clinics to induce new bone formation and was recently shown to play an essential role in the promotion of angiogenesis and osteogenesis in areas in which repair occurs by intramembranous ossification.^{5,6,7,8} Due to the relatively short half-life of bFGF, its single administration alone may be insufficient for an *in vivo* response.⁹ To overcome this limitation, a drug delivery system (DDS) has been developed using gelatin particles as bFGF carriers, which enables the stable and long-term supply of bFGF.¹⁰

While the combination of OCP and bFGF-DDS may become newly established bone regeneration approach, the potential of the combination therapy in osteogenesis has not been studied. In the present study, we examined the effects of the OCP/Col composite on osteogenesis and investigated whether an additional treatment with bFGF-DDS effectively enhanced angiogenesis and osteogenesis in a rat calvarial defect model.

Materials and Methods

Human cells: Human bone marrow-derived mesenchymal stem cells (hBMSC, PT-2501, Lonza Japan Co., Tokyo) and human osteoblasts (hOB, C-12720, Takara Bio, Inc., Shiga) were used.

Experimental animals: Four-week-old male Wistar rats (mean body weight 280–324 g, SLC Corp., Shizuoka, Japan) were used (n=72). They were housed in a clean rack with a uniform temperature (22°C) and humidity (50%) and a 12-hour light-dark cycle. Radiation-sterilized (3 mG) solid food and water was provided *ad libitum*. All animal procedures were conducted in accordance with the regulatory standards of the Kindai University Animal Experiment Committee (No. K AME-2020-047).

Scratch assay: Osteogenic cells migrate to the cranial defect of the implanted scaffold (Col and OCP/Col) from the surrounding tissue or bone marrow. In order to investigate whether the osteogenic cell migration could be promoted by bFGF, a scratch assay was performed using hBMSC and hOB. hBMSC and hOB were cultured in 24-well plates at a density of 50,000 cells/well. Growth media for hBMSC (PT-3001 MSCGM™ Mesenchymal Stem Cell Growth Medium BulletKit™, Lonza) and hOB (C-

27001 Osteoblast Growth Medium, Takara Bio, Inc.) were obtained from each company. When cell confluency reached 80-90%, medium was replaced with DMEM containing 10% FBS, 1% PS, 0.1% AB, and bFGF (10 ng/ μ L). A group not treated with bFGF served as a control. A sterile 200- μ L pipette tip was used to gently create a cell-free gap. Images were taken 24 hours later of the scratch with an inverted microscope (CKX53, Olympus Co., Tokyo). Scratch closure was quantified by measuring the cell-free area using ImageJ (National Institutes of Health, Bethesda, MA).

Preparation of the OCP/Col composite: OCP was prepared by direct precipitation¹¹ and sieved granules (particle size: 300–500 μ m) were sterilized by heating at 120°C for 2 hours. Col was prepared from porcine skin (Nippon Meat Packers, Tsukuba, Japan) and adjusted to a final concentration of 3% at pH 7.4. OCP granules were mixed with concentrated Col, and then lyophilized and molded into disks (diameter: 5 mm, thickness: 1 mm). OCP and Col (OCP/Col) composites were subjected to a dehydrothermal treatment (150°C, 24 hours) in a vacuum drying oven and subsequently sterilized by irradiation.¹²

Preparation of bFGF-DDS and evaluation of bFGF release: To prepare bFGF-DDS, gelatin hydrogels were manufactured as bFGF carriers.¹³ Two hundred microliters of 10% gelatin solution (PI=5, bovine bone gelatin, Nitta Gelatin, Osaka) was added to 5 mL of olive oil (150-00276, FUJI FILM Wako Pure Chemical Corporation, Osaka, Japan). The mixture was left to stand at 40°C for 1 hour, stirred, and cooled to 4°C to make particles. Excess olive oil was removed by adding 1.5 mL acetone and centrifuging at 4°C and 5,000 rpm for 5 minutes. This step was repeated three times and the mixture was then dried in a refrigerator at 4°C for 1 hour to collect precipitating gelatin particles. Gelatin particles were cross-linked by adding 1 mL of 0.1% polyoxyethylene sorbitan monooleate and 5 μ L of 25% glutaraldehyde to 1 mg of gelatin particles, followed by stirring at 4°C for 24 hours. The gelatin particle suspension was centrifuged at 5,000 rpm for 5 minutes, and glycine solution (10 mM, pH=7) was added to pelleted gelatin particles. The solution was stirred at room temperature for 1 hour and washed three times with distilled water by centrifugation. Ultrapure water was added to gelatin particles, and the solution was filtered using strainers with pore sizes of 70 and 30 μ m to collect selected particles with diameters 30-70 μ m that were then snap-frozen in liquid nitrogen, freeze-dried, and sterilized using ethylene oxide gas. To incorporate bFGF into gelatin hydrogels, 100 μ g of bFGF (Trafermin, Kaken Pharmaceutical, Tokyo) was dissolved in 60 μ L of Ca²⁺ and Mg²⁺-

free PBS and added to 10 mg of gelatin hydrogels, which were left to stand at 37°C for 1 hour.

bFGF was labeled by adding 20 μ L of a 0.2-mg/mL Chloramine T solution to a mixture of 1 μ L of a water solution of 125 I and 40 μ L of a 3-mg/mL bFGF solution and stirring.^{12,13} The solution (20 μ L) was left to stand for 3 hours and was then impregnated with gelatin hydrogels (5 μ g) for 1 hour to examine the binding capacity of bFGF with gelatin hydrogels at 37°C. Two experimental groups were used to examine the release behavior of bFGF: (1) 125 I-labeled bFGF-saturated gelatin hydrogels (n=6) and (2) 125 I-labeled solution of bFGF in PBS (n=6). Samples were harvested at various times from 15 minutes to 1 hour, and radioactivity was measured using a gamma counter (ARC-310B, Aloka, Tokyo).

Establishment of the rat calvarial defect model and the *in vivo* experimental

design: Tolbutamide (3 mg/kg) and atropine sulfate (0.04 mg/kg) were subcutaneously injected into rats as pre-anesthesia, followed by an injection of isoflurane (Pittmann and Moores, Mundelein, IL, USA) to induce and maintain anesthesia. The skin over the calvarium was shaved and disinfected with povidone iodine (Isodine®, Meiji Seika, Tokyo) and local anesthesia was administered along the incision line with lidocaine hydrochloride containing epinephrine at 100,000:1 (xylocaine containing epirenamin® 1% E, AstraZeneca, Osaka). An incision was made in the periosteum with electrocautery and the calvarium was exposed. The periosteum was detached and removed, and full-thickness bone defects (5 \times 5 mm) were created on the parietal bone on both sides of the sagittal suture of each rat to assess the capacity of bFGF-DDS to promote bone formation.

Defects were subjected to the following treatments: (1) no treatment; (2) bFGF-DDS; (3) Col alone; (4) Col with bFGF-DDS; (5) the OCP/Col composite alone; and (6) the OCP/Col composite with bFGF-DDS. bFGF-DDS was topically applied to the defect or the OCP/Col composite.

Each construct was covered with the remaining periosteum around the defect margin with 4-0 nylon. The skin incision was closed with 4-0 nylon. All rats were re-anesthetized with CO₂ 4 weeks after surgery to evaluate CT. Following an overdose with CO₂ and cervical dislocation, samples were retrieved 10 and 20 weeks after implantation (n=6 at each time point) and fixed in 10% formalin for CT, histological, and immunohistochemical (IHC) analyses (Fig. 1).

Radiography and CT evaluation: Radiography for OCP/Col was performed using a

soft X-ray generator (TRS-1005, SOFRON, Tokyo) under the conditions of 40 kV, 2.5 mA, and 90 sec, and calcification images were examined before implantation. Using a CT scan (LaTheta LCT-200, Hitachi Aloka Medical, Ltd., Tokyo), the microstructure of new bone was examined by generating CT images at 80 kV, 0.5 mA, and a slice thickness of 120 μm . Using bone structure analysis software (ImageJ, U.S. National Institute of Health, USA), three randomly selected regions of new bone formation underwent assessments of BV/TV (%), where BV was calculated as the percentage of bone volume (BV, μm^2) relative to tissue volume (TV, mm^2) in the measurement area, and shown as means and standard errors (SE).

Histological study: Samples were excised from the rat calvarium 10 and 20 weeks after implantation, dissected, and fixed by immersion in 10% neutral buffered formalin for one week. Fixed calvarial samples were decalcified with 10% ethylenediaminetetraacetic acid for 21 days and rinsed in PBS. Each fixed specimen was then dehydrated through a graded ethanol series, paraffin-embedded, and cut with a microtome into 6- μm -thick sections from the bisected face in the middle of the samples. Hematoxylin and eosin, Alizarin red, and Picrosirius red staining were used to assess general morphology, calcium, and collagen formation, respectively.

Immunohistochemical staining (IHC): Tissue sections were deparaffinized and antigens were unmasked by a microwave treatment (95°C, 15 minutes) in citrate buffer (pH 6.0) with subsequent washing in distilled water. Sections were incubated in endogenous peroxidase and alkaline phosphatase blocking solution (BLOXALL SP-6000, VECTOR Laboratories, Inc., Burlingame). After washing with PBS, sections were further incubated in blocking buffer (0.3% Triton-X and 5% normal goat serum in PBS) at room temperature for 60 min and then with primary antibodies in blocking buffer at 4°C overnight. The next day, slides were washed with Tris-buffered saline (TBS) 3 times for 5 min at room temperature and then incubated for 60 min with a horseradish peroxidase-conjugated polymer secondary antibody (MAX-PO MULTI 414191, Nichirei Biosciences Inc., Tokyo). After washing with TBS, color was developed using the chromogen diaminobenzidine (K3468; Dako North America, Inc., Carpinteria, CA). Slides were washed with TBS 3 times and counterstained with hematoxylin. A microscope (Nikon Eclipse Si, Nikon Corporation, Tokyo) was used to acquire images. Osteogenesis markers, such as bone sialoprotein (BSP) and osteocalcin (OCN), anti-rabbit BSP (1:50 dilution, PA5-79423, Invitrogen, Thermo Fisher Scientific, Waltham, MA), and anti-rabbit OCN (1:50 dilution, PA5-11849,

Invitrogen) were used as primary antibodies for staining. Regarding angiogenesis, CD34 (AF4117, R&D systems, Minneapolis, MN) and VEGF (AF564, R&D systems) were used as primary antibodies.

Statistical analysis: All experimental results are shown as means \pm SE. Statistical analyses used the Student's *t*-test for comparisons between two groups and a one-way analysis of variance and the Holm post-hoc test for comparisons among 3 or more groups. Data analyses were conducted using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA), and $P < 0.01$ and $P < 0.05$ were used as the criteria for significance.

Results

The OCP/Col composite: The OCP/Col composite was radiolucent. SEM images showed a porous structure in which OCP particles were embedded in a collagenous matrix (Fig. 2A).

Release of bFGF from gelatin hydrogels: The release of bFGF from gelatin hydrogels was assessed *in vitro* by measuring residual radioactivity (Fig. 2B). When bFGF was impregnated into gelatin hydrogels at 37°C, its release rate plateaued at 31.9% after a 1-hour incubation. This result indicated that bFGF permeated between gelatin molecules and also that interactions between bFGF and gelatin hydrogels became stronger during the 1-hour incubation period, suggesting the more sustained release of bFGF from gelatin hydrogels than from PBS solution.

The scratch assay after 24 hours of the bFGF treatment: In order to investigate how cell maturity influences bFGF effect using hBMSC and hOB, the scratch assay was performed. It revealed that cell migration into scratch wounds was significantly increased by the administration of bFGF in the hBMSC and hOB groups (Fig. 3).

Calvarial bone defect healing *in vivo*: Serial CT analyses were performed in several timepoint after implantation. By four weeks, a hard structure was not observed in the groups without the OCP/Col composite (Groups 1 and 2). New bone formation was primarily noted along the margins of the calvarial bone defect in the Col group (Group

3), but not in the Col/bFGF-DDS group (Group 4). In the OCP/Col composite groups (Groups 5 and 6), bone defects were covered by bone-like hard tissue. During the observation period of 20 weeks, the area in which OCP/Col was implanted became dense over time; however, the junction between the OCP/Col composite and donor calvarium was not connected at 20 weeks in the OCP/Col composite group (Group 5). In contrast, the OCP/Col/bFGF-DDS group (Group 6) developed a high-density area that initially extended towards the peripheral edges of the bone defect and finally integrated with the donor calvarium after 10 and 20 weeks, respectively. The high-density area and reductions in the bone defect area were not detected in the groups without the OCP/Col composite (Groups 1 and 2) or in the Col/bFGF-DDS group (Group 4). No apparent inflammatory tissue changes or signs of infection were observed in any rat (Fig. 4).

BV/TV of defects without the OCP/Col composite was low ($< 20\%$) in Groups 1 and 2. BV/TV was significantly lower in the Col/bFGF-DDS group (Group 4) than in the Col group (Group 3) (Group 3: Group 4 = $21.2 \pm 7.0 : 56.2 \pm 1.7$ at 10 weeks, $23.1 \pm 7.9 : 48.1 \pm 7.9$ at 20 weeks, respectively), which is indicative of the accelerated degradation of Col by bFGF-DDS. The OCP/Col composite with bFGF-DDS (Group 6) resulted in significantly higher BV/TV than the OCP/Col composite alone (Group 5) at 20 weeks (Group 5: Group 6 = $61.5 \pm 2.9 : 76.8 \pm 3.6$) (Fig. 5).

The area and extent of bone regeneration was histologically examined using hematoxylin and eosin, Alizarin red, and Picrosirius staining. New bone formation was not observed in Group I (Blank) or 2 (Blank/bFGF-DDS). In the Col group (Group 3), newly formed bone increased with time and was observed from the margins toward the center of the bone defect after implantation. In contrast, in the Col/bFGF-DDS group (Group 4), bone formation was not observed during the observation period (Fig. 6). In the OCP/Col group (Group 5), bone regeneration was only detected around residual OCP particles, particularly at the peripheral region of the composite at 20 weeks (Fig. 7, above). However, large numbers of capillaries and newly formed bone were observed around OCP/Col particles in the interstitial spaces at 10 weeks in the OCP/Col/bFGF-DDS group (Group 6). The bone defect area was markedly reduced and the junction between the OCP/Col composite and donor calvarium became indistinct at 20 weeks (Fig. 7, below). The decrease of Picrosirius staining stainability at 20 weeks in both Group 5 and 6 may suggest that maturation of woven bone occurred in time (Fig. 7).

Immuno-histochemical staining (IHC): IHC revealed a few positive cells with

BSP and OCN antibody in Group 5. However, there were many BSP+ and OCN+ cells around the OCP particles in the OCP/Col/bFGF-DDS group (Group 6) at 20 weeks (Fig. 8). Additionally, expression of VEGF was found within the OCP granules that were absorbed over time and replaced with newly formed bone in Group 5 and 6. CD34 was expressed inside and outside of the OCP granules in Group 5, which extended into the OCP in Group 6 (Fig. 9).

Based on these results, the OCP/Col composite treated with bFGF-DDS (Group 6) showed a significantly enhanced osteogenesis and higher amount of newly formed bone than the defects treated without bFGF-DDS.

Discussion

It has been well known that bioceramics have excellent biocompatibility and bind well to surrounding bone tissue through an apatite layer formed on the ceramic surface. It was reported that the pore size around 200 to 400 μm in a porous bioceramics allows excellent osteoconduction, while the optimal porosity with bone formation is 50 to 60%.¹⁴ Another report also revealed that a minimum pore size of 100 μm is required for osteoblast migration into the bioceramics.¹⁵ However, bioactive ceramic is hardly absorbed and could cause delayed union, fracture and infection. Bioabsorbable ceramic, on the other hand, is easily decomposed and enables the soft tissues to penetrate the structure, that negatively effect on bone repair.²

Octacalcium phosphate ($\text{Ca}_8\text{H}_2(\text{PO}_4)_6 \cdot 5\text{H}_2\text{O}$, OCP) is a calcium phosphate and precursor material of HA.¹⁶ It is resorbed by osteoclast-like cells and is more resorbable than HA and β -TCP because of the large number of water molecules present in its structure.¹⁷ OCP has higher osteogenic and osteoconductive abilities because OCP particles enhance the migration of osteoblasts more *in vivo* than HA or β -TCP; however, it is not yet widely used in clinical practice due to its poor molding ability and brittleness. To overcome these weak points, OCP particles (diameter: 300 ~ 500 μm) are conjugated with porcine collagen (types I and III) to form an OCP/Col composite (micropore: 0.3 ~ 48 μm , weight % of OCP in Col: 77wt%, porosity: 92%).

In the present study, we examined the osteoinductive capacity of the OCP/Col composite and compared it with the Col in a rat calvarial defect model. In comparisons between the Col group (Group 3) and the OCP/Col composite group (Group 5), an area of bone formation was observed at the peripheral margin of the scaffold in the Col group, but was centrally located in the OCP/Col composite group. In magnified views, bone

formation was noted around OCP particles, and OCP was later absorbed in the process of new bone formation. Based on these results, OCP appears to induce bone formation through an osteoinduction/conduction mechanism by promoting osteogenic cell proliferation around OCP particles in the OCP/Col composite. The peripheral margin of the OCP/Col composite did not integrate with the host calvarium, indicating insufficient bone formation that necessitated another method to promote osteogenesis.

bFGF has been shown to exert strong cell proliferation effects while maintaining the differentiation potential of mesenchymal stem cells *in vitro*.^{18,19} Our scratch assay revealed a significant increase in cell migration following the administration of bFGF, suggesting that the migration of cells into OCP granules were more accelerated with bFGF regardless of the cell maturity. Previous study demonstrated that a single administration of bFGF does not have sufficient pharmacological effects *in vivo*.²⁰ And drug delivery system using gelatin hydrogels was consequently developed.¹⁸ In bFGF-DDS, basic FGF is absorbed in acidic gelatin hydrogels and immobilized electrostatically. According to the degradation rate of gelatin, bFGF is released slowly and the pharmacological activity of bFGF can be maintained for approximately 14 days with the average tissue concentration of 5 µg/day *in vivo* ^{2,18}, where the effective tissue concentration of bFGF has been reported to be approximately 2-10 µg/cm²/day. As reported previously, the strongest angiogenesis *in vivo* was observed at the initial concentration of 1 mg/mL. In the present study, the same concentration was applied in the OCP/Col composite with bFGF-DDS for the purpose of creating more vascular networks and promoting osteogenesis. Based on the result of radioactivity study, it was confirmed that initial binding of the bFGF with gelatin hydrogels was completed with 1 hour incubation, that allowed continuous release rate of 31.9%. This result was coincided with the previous report.^{18,19}

Newly formed bone was hardly observed in the bone defects treated by the Col/bFGF-DDS (Group 4). The collagen used in this study was produced under the condition of thermal dehydration cross-linking at 150°C for 24 hours. As the collagen is mechanically weak and the degree of cross-linking is low, it is degraded in the body within a week. Hence, when collagen was implanted with bFGF-DDS, degradation of collagen might have occurred sooner than osteogenesis due to enhancement of angiogenesis by bFGF. On the other hand, when collagen was combined with OCP, the composite increased bone regeneration capacity and early degradation of its structure did not interfere the process of bone formation. From this result, it was speculated that the combination of the OCP/Col and bFGF-DDS is optimal for promoting osteogenesis. Our CT study further demonstrated that the osteogenesis was more accelerated in the

OCP/Col/bFGF-DDS (Group 6). And the structural analysis demonstrated that its BV/TV fraction was significantly higher than the bone defect treated with OCP/Col alone (Fig.3, $p < 0.01$). It was also found that the newly formed bone was bridged and integrated with the host calvarium bone and this process is indicative of the appositional growth over the cortical bone.

According to the previous reports, bFGF activates the MAPK pathway by binding to the FGF receptor on the cell membrane and stimulates the release of vascular endothelial growth factor (VEGF).^{6,7} VEGF serves as a chemotactic factor to further stimulate migration and differentiation of osteoblastic lineage cells for mineralized bone^{8, 21, 22} together with vascular endothelial cells for new blood vessel formation. That is, VEGF plays critical role for angiogenesis and osteogenesis during bone repair. Indeed, higher percentage of osteoblast lineage cells differentiate into fibroblasts when VEGF levels are reduced.⁸ Thus, angiogenic response as well as osteogenesis occurs by the VEGF-mediated intramembranous ossification mechanism.^{6,7,8,21,22} Immunohistochemical analysis in the present study revealed that VEGF expression and CD34+ cells were detected within OCP granules which were later absorbed and replaced with newly formed bone. Moreover, this process was enhanced by bFGF administration (Fig. 9). In vitro scratch assay also demonstrated that the migration of osteoblast lineage cell was accelerated with bFGF (Fig. 3). Based on these findings, it was suggested that exogenous bFGF stimulated the release of VEGF that was firstly recruited in OCP granules. Then OCP granules acted as a primary ossification center stimulating the migration and differentiation of the osteoblastic lineage cells and the endothelial cells in the bone marrow to induce both osteogenesis and angiogenesis, while OCP granules were absorbed and replaced in time by the newly formed bone.

Furthermore, BV/TV ratio reached a plateau at 4 weeks after implantation in Group 6. This was similarly found at 10 weeks in Group 4. On the other hand, newly formed bone continued to increase during the course of osteogenesis in Group 5. From these results, it was considered that OCP/Col was a useful scaffold and bFGF promoted bone formation following the implantation of the scaffold. To the best of our knowledge, this is the first report which has shown evidence that the OCP/Col composite with bFGF-DDS has a strong influence on both angiogenesis and osteogenesis in a rat model. Further studies on the long-term effects of bFGF-DDS are warranted.

Conclusion

In the present study, we selected a gelatin hydrogel-based bFGF-DDS into the OCP/Col composite to examine its usefulness in inducing bone regeneration. As a result, the bone regeneration process was accelerated in the OCP/Col composite with bFGF-DDS. It was suggested that the OCP/Col/bFGF-DDS is an indispensable method for early induction of bone regeneration in the rat calvarial defect model.

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Figure Legends

Figure 1 Experimental protocol

Figure 2 (A) Gross morphology, X-ray, and SEM images of the OCP/Col composite
(B) Release rate from gelatin hydrogel-based bFGF-DDS
n=6 in each group. Values are expressed as means \pm SE. Significance was defined at the * $p < 0.05$ (*) level for specimens in comparisons of bFGF+ Gelatin vs. bFGF + PBS.

Figure 3 (A) Scratch assay after 24 hours treatment by bFGF
(B) % Scratch closure
n=6 in each group. Values are expressed as means \pm SE. Significance was defined at the * $p < 0.05$ (*) level for specimens in comparisons of hBMSC+ bFGF vs. hBMSC and hOB +bFGF vs. hOB.

Figure 4 CT findings at 4, 10, and 20 weeks after implantation in Groups 1-6
(n=6, representative images are shown)

Figure 5 Bone structure analysis using BV/TV ratio
n=6 in each group. Values are expressed as means \pm SE. Significance was defined at the $p < 0.01$ (*) level for specimens in comparisons of Group 5 vs. Group 6 and at the $p < 0.05$ (†) level for specimens in comparisons among Group 3 vs. Groups 4, 5, and 6.

Figure 6 Histological examination of Groups 1-4, at 10, and 20 weeks after
| implantation.
(Hematoxylin & eosin staining, n=6, representative images are shown)

Figure 7 Histological examination of Groups 5 and 6 at 10 and 20 weeks after
implantation (Alizarin Red, Picrosirius, and hematoxylin & eosin staining,
n=6, representative images are shown)

Figure 8 Immunohistochemical images of biomarkers, bone sialoprotein (BSP) and
osteocalcin (OCN), in the central region of Groups 5 and 6 at 10 and 20
weeks after implantation (n=6, representative images are shown)

Figure 9 Immunohistochemical images of VEGF in Groups 5 and 6 at 20 weeks
after implantation (n=6, representative images are shown)

