

Enhanced bone formation using hydroxyapatite ceramic coated with fibroblast growth factor-2

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Abstract

Our object was to develop a bone substitute coated with fibroblast growth factor-2 (FGF-2) that subsequently releases FGF-2. We investigated the use of our system of bone substitutes to induce bone formation. Hydroxyapatite ceramic buttons (HAP-CBs) were coated with FGF-2 by precipitation in supersaturated calcium phosphate solution. HAP-CBs were coated with high or low doses of FGF-2, denoted as FGF-H and FGF-L. The release of FGF-2 from FGF-H and FGF-L was evaluated using its release profile and bioactivity. The efficacy of the subsequent bone formation was quantified using rats with round-shaped bone defects (5 mm in diameter) of the right parietal bone. Group 1 was treated only with HAP-CBs, Group 2 with HAP-CBs and drops of FGF-2 solution, Group 3 with FGF-L, and Group 4 with FGF-H. To detect the release of FGF-2 *in vivo*, the expression of bone morphogenic protein-2 (*BMP-2*) was measured in the defective bone tissue. FGF-2 was released *in vitro* from FGF-H and FGF-L and maintained its bioactivity. Rats treated with FGF-L showed better bone formation than rats from the other groups. *BMP-2* expression was detected in the defective bone tissues of Group 3 at 14 days, which might indicate *in vivo* FGF-2

release during this period. A specific FGF-2 concentration may be needed for bone formation, and our system can release FGF-2 at adequate concentrations to induce bone formation.

Key words: bone formation, fibroblast growth factor-2, hydroxyapatite ceramic button, releasing drug delivery system, cranioplasty

Running head: An FGF-2-releasing system induces bone formation

Introduction

Recently, many studies have reported that certain proteins, such as fibroblast growth factor-2 (FGF-2) [1-3], transforming growth factor- β (TGF- β) [4], and bone morphogenetic proteins (BMPs, which belong to the TGF- β superfamily) [5-8], can stimulate bone formation. In their use as clinical medicines, safety and quality are necessary conditions for using these proteins as drugs. FGF-2 is one of the most promising and practical options because FGF-2 has already been approved as a clinical pharmaceutical in Japan and is commercially available. When cytokines such as FGF-2 are administered *in vivo*, a large proportion of the applied cytokines are rendered inactive due to degradation or non-specific binding [5,9]. In general, the delivery system cannot maintain the *in vivo* effects of FGF-2 for very long. Therefore, a new delivery system is needed for the administration of FGF-2 to be useful [3].

The ability to hold and release various kinds of proteins, including FGF-2, has been studied [10,11]. In studies using cytochrome C (cyt C) as a harmless dummy protein, cyt C was effectively coated to a hydroxyapatite (HAP) ceramic, forming a cyt C/calcium phosphate composite layer in supersaturated calcium phosphate solutions

prepared by mixing several types of infusion fluids [10]. Because the molecular weight and isoelectric focusing point of FGF-2 are analogous to those of cyt C, an FGF-2/calcium phosphate composite layer would be expected to form on HAP ceramic [11] and then be slowly released from it.

HAP has already been applied to various clinically approved bone substitutes, such as the burr-hole buttons that are used to repair cranial defects. HAP causes minimal foreign-body reactions and acts as an osteoconductive material, binding easily to bone [12,13]. Therefore, HAP may be a good material to repair bone defects such as in cranioplasties, and it may also be a suitable substrate for binding FGF-2.

In this study, an FGF-2/calcium phosphate composite layer was formed on a HAP ceramic button (HAP-CB) using clinically approved pharmaceutical solutions and FGF-2. The amount of FGF-2 released and the biological activity of the resulting material were studied to confirm its abilities both *in vitro* and *in vivo*.

Materials & Methods

Preparation of HAP-CB

Pure, stoichiometric HAP powder was supplemented with 3% (wt) polyvinyl alcohol and 1% (wt) polyethylene glycol and sieved to select only particles under 75 μm in size and then formed into disks at 98 MPa and sintered at 1150°C for one hour. The resulting shape of HAP-CB is shown in Figure 1, with a surface area of 15.94 mm^2 and a mean thickness of 1.00 mm per button. The HAP-CB was designed for a round cranial bone defect 5 mm in diameter, and its sides were cut bilaterally to permit bone formation into the space that was created by cutting.

FGF-2 solution

An FGF-2 solution was prepared by dissolving FGF-2 (Fiblast[®], Kaken Pharmaceuticals, Japan) in a sterilized physiological salt solution at the final concentration of 100 $\mu\text{g}/\text{mL}$. Fiblast is a pharmaceutical human recombinant FGF-2 containing an undisclosed amount of sucrose, ethylene diamine tetraacetic acid, and a pH adjustment agent.

Preparation of the other solutions

A calcium-containing solution, a phosphate-containing solution, and an alkalinizer (Table 1) were prepared by dissolving reagent-grade KCl, $\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$, NaHCO_3 , KH_2PO_4 , Xylitol (Wako Pure Chemical Industry Ltd., Japan), NaCl, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$, and $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (Nacalai Tesque Co., Japan) in ultra-pure water. These solutions are equivalent in their chemical composition to the clinically available infusion fluids. The calcium-containing solution corresponds to Ringer's solution (Otsuka Pharmaceutical Co., Ltd., Japan), including 147 mM Na^+ , 4.00 mM K^+ , 2.25 mM Ca^{2+} , and 157 mM Cl^- at pH 6.43. The phosphate-containing solution corresponds to Klinisalz[®] B (Irom Pharmaceutical Co., Ltd., Japan) and contains 45.0 mM Na^+ , 25.0 mM K^+ , 2.50 mM Mg^{2+} , 45.0 mM Cl^- , 10.0 mM H_2PO_4^- , 20.0 mM CH_3COO^- , and 333 mM Xylitol at pH 5.87. The alkalinizer is a NaHCO_3 solution corresponding to Bifil[®] (AJINOMOTO PHARMA Co., Ltd., Japan) and contains 166 mM Na^+ , and 166 mM HCO_3^- at pH 8.28.

Formation of a calcium phosphate composite

The above-mentioned solutions were mixed to prepare supersaturated calcium phosphate solutions that included the FGF-2 solution. The supersaturated calcium phosphate solution with FGF-2 was filtered using a membrane with a pore size of 0.22 μm . HAP-CBs were immersed in 2 mL of the supersaturated calcium phosphate solution for 24 hours at 25°C. Two conditions were chosen for the FGF-2 coating with precipitation: a high-dose FGF-2 coating, which was named “FGF-H,” and a low dose FGF-2 coating, which was named “FGF-L.” An HAP-CB was also prepared under FGF-H conditions but without FGF-2, and this condition was named “HAP-N”. All of the HAP-CBs were used immediately after washing with 2 mL of phosphate-buffered saline (PBS). The surfaces of the HAP-CB, FGF-L, FGF-H, and HAP-N were observed using a scanning electron microscope (SEM; JSM-5500LV, JEOL Ltd., Japan).

***in vitro* FGF-2 release assay**

FGF-L and FGF-H buttons were immersed in 2 mL of Dulbecco’s modified Eagle’s medium (DMEM; Gibco-BRL, Grand Island, NY) without fetal bovine serum

(FBS) and allowed to sit at 37°C for up to 16 days. DMEM was used to simulate the *in vivo* setting because it contains various minerals at concentrations close to those found in the body fluid. At each measured time point, 0.15 mL of DMEM was added after collecting 0.15 mL of sample from each well. The FGF-2 concentration in these samples was measured with an ELISA (human Fibroblast Growth Factor-2 ELISA kit, Calbiochem, EMD bioscience, CA), and the amounts of the released FGF-2 were calculated.

***in vitro* biological activity induced by FGF-2**

BHK-21 cells (RIKEN BioResource Center, Tsukuba, Japan), which are fibroblastic cells derived from hamster and can respond to FGF-2, were used to evaluate the biological activity of FGF-2. MG-63 cells (RIKEN BioResource Center), which are osteoblastic cells derived from humans, were used to evaluate the FGF-2 concentrations for bone formation. The BHK-21 cells were maintained in DMEM containing 10% FBS, and the MG-63 cells in modified Eagle's medium (MEM; Gibco-BRL) with 10% FBS. Cells (1×10^4 / well) were seeded on 24-well culture plates. In some wells,

FGF-2 was added to the medium at each concentration, and FGF-H or FGF-L buttons were placed on the bottom of the other wells. The cells were cultured for three days at 37°C in a humidified CO₂ incubator with 1 mL of medium without FBS. After this step, the cells were detached via a trypsin treatment and counted with a counting chamber slide. The wells grown with 10% FBS were used as a positive control. In some wells, bone morphogenetic protein-2 (*BMP-2*) expression was evaluated by the Mini Opticon real-time PCR system (Bio-Rad Laboratories Inc.).

Animal experiments

During all of the experiments that were approved by the Institutional Animal Care and Use Committee, the animals were housed and handled in accordance with the guidelines of the National Institutes of Health. Seven- to eight-week-old male Wistar rats were purchased (Japan Crea Co., Ltd., Japan). Under anesthesia, a round craniotomy (5 mm in diameter) was drilled into the right parietal bone. The rats were divided into four treatment groups. In Group 1, the cranioplasty was performed with HAP-N buttons alone. In Group 2, the cranioplasty was performed with HAP-N

buttons with drops of the FGF-2 solution containing 500 ng of FGF-2 applied directly to the HAP-N buttons. Groups 3 and 4 were treated with FGF-L buttons and FGF-H buttons, respectively. Rats were sacrificed at two, four, and eight weeks after the procedures, and the skull bones were removed together with the defects. The samples were fixed in 10% formaldehyde in PBS for four days and then demineralized in 10% ethylene diamine tetraacetic acid solution at 4°C for three days, embedded in paraffin, and cut into 10- μ m-thick sections. The samples were cut into the center of the skull defect, or at the nearest possible site, at a right angle across the lengthwise axis of the HAP-CB (Fig. 2). These sections were stained with hematoxylin and eosin and were viewed using an IX71 microscope system equipped with the DP-Controller imaging software (Olympus, Japan). In cranial bone healing, it is reported that bone formation occurs in the periphery of the bone defect [14] and on the dural membrane side [15]. Bone formation was quantified by measuring the length of the new bone extension into the inside of the bone defect and the thickness of the edges of the bone defect using the IX71 microscope system (Olympus). In some rats, the tissue inside of the bone defects was removed and homogenized. Total RNA was isolated, and cDNA was synthesized.

The expression levels of *BMP-2*, alkaline phosphatase (*ALP*), and osteocalcin (*OC*) were evaluated with the Mini Opticon real-time PCR system (Bio-Rad Laboratories).

The detection of *Bmp-2*, *ALP*, and *OC* expression with real-time PCR

The *in vitro* cell samples were washed three times with PBS. The *in vivo* tissue samples were homogenized and centrifuged, and the supernatant was used to extract RNA. Total RNA was extracted from some samples with an RNA extraction kit (QIAGEN). One microgram of total RNA was reverse transcribed in a buffer containing 1 μ L of oligo-dT primers (2.5 μ M), 250 μ M deoxynucleotides, 10 U RNasin (Promega), and 100 U Superscript II (Gibco-BRL). This mixture was incubated for 75 minutes at 42°C and for 5 minutes at 75°C. The expression levels of *BMP-2*, *ALP*, *OC*, and *GAPDH* were detected using the following primers: forward primer 5'-AAGGCACCCTTTGTATGTGG-3' and reverse primer 5'-CATGCCTTAGGGATTTTGG-3' for *BMP-2* [16]; forward primer 5'-GAGCAGGAACAGAAGTTTGC-3' and reverse primer 5'-GTTGCAGGGTCTGGAGAGTA-3' for *ALP* [16]; forward primer

5'-AGCTCAACCCCAATTGTGAC-3' and reverse primer
5'-AGCTGTGCCGTCCATACTTT-3' for *OC* [16]; and forward primer
5'-AACTCCCATTCCTCCACCTT-3' and reverse primer
5'-GAGGGCCTCTCTCTTGCTCT-3' for *GAPDH* [16]. Each primer (12.5 pM) was
added to a solution containing 12.5 μ L of iQ SYBR green supermix (Bio-Rad
Laboratories) along with 0.5 μ L of template sample (final volume, 25 μ L). The Mini
Opticon real-time PCR system (Bio-Rad Laboratories Inc., Hercules, CA) was used.
The expression levels of *BMP-2*, *ALP*, and *OC* were indicated as the delta cycle times
(Δ cycle(t)). Some of the *BMP-2* expression levels were reported as the fold increase
(Δ - Δ C(t)), in which values normalized to *GAPDH* expression were compared with the
BMP-2 expression in HAP-N.

Statistical analysis

The experimental results are expressed as the mean \pm the standard deviation.
All data were analyzed using Student's t-test, and probability values less than 0.05 were
considered to be statistically significant.

Results

SEM observation

Each HAP grain was covered with fine particulate precipitations (Fig. 3B, 3C, 3D). No particle with a diameter of more than 1 μm was observed at the surface of the HAP, which indicated that homogenous nucleation did not occur in the supersaturated calcium phosphate solution.

Evaluation of the cell responses to soluble FGF-2

BHK-21 cells were incubated for three days in each concentration of FGF-2 and counted. The measured values were indicated as the average and standard deviation of three independent experiments. BHK-21 cell proliferation was responsive to FGF-2 concentrations between 1 and 100 ng/mL (Fig. 4A). MG-63 cells were incubated for three days in each concentration of FGF-2. MG-63 cells proliferated better at FGF-2 concentrations between 1 and 10 ng/mL (Fig. 4B).

To look at gene expression levels, *BMP-2* expression was evaluated using BHK-21 and MG-63 cells. *BMP-2* expression was induced in osteoblasts through the administration of FGF-2 [17,18]. *BMP-2* expression was indicated as fold increase

($\Delta\Delta C(t)$), in which values normalized to *GAPDH* expression were compared with the *BMP-2* expression levels in wells with 0 ng/mL FGF-2. *BMP-2* expression in cells cultured with 10% FBS was taken as the positive control because FBS includes several types of growth factors. The measured values were indicated as the average and standard deviation of three independent experiments. *BMP-2* expression in MG-63 cells peaked when they were cultured with concentrations of FGF-2 between 3 and 10 ng/mL, and it decreased at concentrations over 30 ng/mL (Fig. 4C). *BMP-2* expression did not change in BHK-21 cells, likely because these cells may be fibroblasts. An adequate concentration of FGF-2 may be needed to activate osteoblasts such as MG-63 cells.

***in vitro* release of FGF-2 from FGF-L or FGF-H buttons**

FGF-L and FGF-H buttons were immersed in 2 mL of DMEM without FBS. FGF-2-release from the FGF-L and FGF-H buttons was observed over time (Fig. 5). The measured values were indicated as the average and standard deviation of three independent experiments. The FGF-H buttons released approximately 26 ng/mL of

FGF-2, and the FGF-L buttons released around 5.8 ng/mL of FGF-2. The FGF-L and FGF-H buttons continued to release FGF-2 over 16 days.

***in vitro* biological activity of the released FGF-2**

The biological activity of FGF-2 was evaluated by monitoring the proliferation of BHK-21 cells. The measured values were indicated as the average and standard deviation of three independent experiments. The cell counts in the wells that included the FGF-H buttons were higher than those in the wells that included the FGF-L buttons (Fig. 6A). Because the FGF-H buttons could release more FGF-2 than the FGF-L buttons, the difference in the observed cell proliferation is reasonable. These findings indicate that the FGF-2 coated onto and released from the HAP-CBs maintained its biological activity. In MG-63 cells, the cell counts in the wells that included the FGF-H buttons were lower than those in wells with the FGF-L buttons (Fig. 6B). The dose of FGF-2 released from the FGF-H buttons might be too high to stimulate these osteoblast cells.

BMP-2 expression was evaluated in samples incubated with HAP-N, FGF-L, or

FGF-H buttons. The measured values were indicated as the average and standard deviation of three independent experiments. When BHK-21 cells were incubated with HAP-N, FGF-L, or FGF-H buttons, there was no difference in *BMP-2* expression (Fig. 6C). When MG-63 cells were incubated with HAP-N, FGF-L, or FGF-H buttons, the FGF-L buttons increased the expression of *BMP-2* more than the other treatments (Fig. 6C). These findings indicate that the FGF-L buttons may release an effective concentration of FGF-2 to stimulate osteoblast cells. Thus, we predict that FGF-L buttons would be more effective for *in vivo* bone formation.

***in vivo* bone formation**

Eighteen rats were in each group and six rats were sacrificed at two, four, and eight weeks after the craniotomy procedures. We observed new bone formation as extension into the open space on both sides of HAP-CB (indicated by the 'extension' value in Figure 2 and 7A) and increased thickness in the space between the edge of the bone defect and the dura membrane (indicated by the 'thickness' value in Figure 2 and 7B). Figures 7A and 7B show the histological sections from Group 3 rats treated with

FGF-L and 7C shows rats from Group 1 treated with HAP-N. In Group 3, which was treated with the FGF-L buttons, bone formation was enhanced significantly more than that in Groups 1 or 2 (Fig. 8A and 8B). There was no significant difference between Group 4 and Groups 1 or 2. These findings suggest that only a regionally adequate concentration of released FGF-2 may be capable of stimulating for bone formation.

Two or four weeks after the procedures, some rats were sacrificed (n=3 in each group at each point), and the gene expression in the tissue of the bone defects was evaluated. The measured values were indicated as the average and standard deviation of three independent experiments. Increased *BMP-2* expression levels were detected in Group 3, and *BMP-2* expression was low in Groups 1, 2, and 4 at two weeks (Fig. 9A). Because osteoblasts express *BMP-2* in response to an adequate concentration of FGF-2, the FGF-L buttons in Group 3 appeared to maintain an adequate *in vivo* release of FGF-2 for about two weeks. At four weeks, *BMP-2* expression decreased in Group 3. Increased expression of *ALP* and *OC* was observed in Group 3 at four weeks (Figs. 9B and 9C). These expression patterns suggested the progression of bone formation in Group 3.

Discussion

In this study, we show that an FGF-2-releasing system can function *in vivo* to promote bone formation. The enhancement of bone formation was observed in the periphery of the cranial bone defect and on the dural membrane side, as it was reported in other articles [14,15]. In many reports, polymer or collagen-gel devices have been used as *in vivo* delivery systems for cytokines [1-8]. However, there is no mechanical strength in these materials, and the cross-linkers that are used to make the gels may be toxic [3,4]. In this case, we tried to incorporate an FGF-2-releasing system into bone substitutes to repair the bone defects and to create a system that would use only government-approved medicines. This FGF-2 releasing system was applied to cranioplasties, although a cranioplasty does not require mechanical strength. Given that mechanical strength would be important for certain future applications, this releasing system could be applied to apatite as a substitute for its use in other operations without cranioplasty. In addition, this release system could easily be modified for other applications. Another advantage of this delivery system is that FGF-2 and other fluids are approved for use as medicines by the Japanese government, meaning that their

safety is guaranteed. Therefore, if the handling of this mixture of fluids and drugs is performed in accordance with “good manufacturer practice” regulations (GMP regulations), the quality and the safety of the final FGF-2-coated product can be guaranteed. In our laboratory, we use a designated clean bench at which we can handle these materials in accordance with the GMP regulations to perform clinical studies.

It has been reported that the releasing ability of apatite is rather low [19]. In our system, a few micrograms of FGF-2 were coated onto HAP-CBs, and about 52 ng (26 ng/mL x 2 mL/well) could be discharged in an *in vitro* releasing assay. When considering a biologically effective concentration of FGF-2, 52 ng of released FGF-2 might be enough to induce biological effects. In our other studies, about 2-3 µg of FGF-2 was released from 3.06 cm² of HAP disk with the FGF-2 coating in a physiological salt solution [10,11]. Calculating the surface area of HAP-CBs, the FGF-H buttons should have released about 150 ng of FGF-2. Daculsi et al. reported that a dissolution-precipitation process of apatite occurred in settings with high calcium concentrations, such as *in vivo* [20]. In an *in vitro* releasing assay, the

dissolution-precipitation process might occur, and the released FGF-2 might be reabsorbed into the apatite coating layer because the DMEM used in the assay contained a high dose of calcium. This could make it difficult to estimate the available dose of the released FGF-2. Although we wanted to evaluate the *in vivo* release of FGF-2, it appears to be more difficult for a few reasons: (1) there is an *in vivo* clearance system for FGF-2, and (2) the presence of heparan sulfate in the extracellular matrix could bind to FGF-2 and sequester it and/or enhance the activity of FGF-2 [21,22]. Instead, we used *BMP-2* expression in osteoblast cells, which is induced by FGF-2, to evaluate the *in vivo* release of FGF-2. Our data indicated that our system released FGF-2 *in vivo* over the course of two weeks and that there appeared to be an adequate *in vivo* concentration of FGF-2 for bone formation, consistent with the *in vitro* data [23,24]. Some studies have reported that a high FGF concentration *in vivo* is not conducive to bone formation [25,26]. In our study, the FGF-H buttons, which released a high-dose of FGF-2, did not result in an increased level of bone formation compared to the FGF-L buttons. Although the FGF-2 dose released from the FGF-H buttons might be just four or five times higher than that from the FGF-L buttons, there were significant differences

in the bone formation between the FGF-H and FGF-L buttons. These findings suggest that the FGF-2 concentration must be within a certain range to stimulate osteoblast cells. Strict control of the released FGF-2 dose might be needed for bone formation, as increasing the concentration of FGF-2 from 10 to 30 ng/mL resulted in a remarkable decrease in FGF-2-induced *BMP-2* expression *in vitro*. To strictly control the *in vivo* concentration of FGF-2, we think that one must be able to adjust the amount of coated FGF-2 on the HAP-CBs.

When gel-releasing systems are used as delivery systems, it might be difficult to strictly control the amount of FGF-2 that is applied because it is easy for clinical doctors to apply too much medicine. Some studies have reported that FGF-2 gel-releasing systems were not effective because the concentration of released FGF-2 was too high to stimulate osteoblast differentiation [25-27]. When considering the ability to manage the amount of applied FGF-2, our system may have an advantage over these other methods.

Enhanced bone formation after cranioplasty may be more meaningful for pediatric patients than for adult patients [28,29] because the failure of a cranioplasty can

result in deformities of the cranium, a growing skull fracture, or the inconvenience of wearing headgear. Just like the above-mentioned effects, enhanced bone formation may also play a large role in cervical operations, such as cervical anterior fusion or laminoplasty. Although many instruments for cervical operations have been developed and are widely used, some doctors believe that cervical fixation by bone formation is more natural and preferential for some patients.

Generally, techniques for enhanced bone formation have a huge potential in clinical medicine. However, our release system needs to be optimized before it can be applied in a clinical setting, including determining the adequate *in vivo* dose of FGF-2 for human bone formation. We believe that our system is one of the most promising methods that can be used to enhance bone formation.

Conclusion

A specific FGF-2 concentration may be required for bone formation. Our coating system can precisely release FGF-2 at concentrations adequate for inducing bone formation *in vivo*. Because our system consists of approved medicine and solutions, it may have significant potential in clinical medicine.

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Figure legends

Table 1: Preparation of the solutions and agents used in this study. All of the solutions and agents are approved for clinical usage in Japan.

Figure 1: Three-dimensional views of the hydroxyapatite ceramic button (HAP-CB), which was made for cranial repair (cranioplasty) in rats. Both sides of the HAP-CB were cut in order for bone formation to extend into the space around the bone defect.

Figure 2: The HAP-CB implanted in the bone defects (burr holes). The lower panel demonstrates how bone formation was measured. Bone formation was quantified by measuring the length of the new bone extension into the inside of the bone defect and the thickness of the edges of the bone defect.

Figure 3: SEM photos of the HAP-CB (A), FGF-L (B), FGF-H (C), and HAP-N (D). Particulate precipitations were observed on the HAP grains of the FGF-L (B), FGF-H (C), and HAP-N (D). Magnification: 20000X.

Figure 4: Cell proliferation and bone morphogenetic protein-2 (*BMP-2*) expression are induced by fibroblastic growth factor-2 (FGF-2). BHK-21 cells (fibroblasts) and MG-63 cells (osteoblasts) were incubated with increasing concentrations of FGF-2 for three days. A) The number of BHK-21 cells after incubation with FGF-2. B) The number of MG-63 cells after incubation with FGF-2. C) Real-time PCR results of *BMP-2* expression in BHK-21 cells (empty bars) and MG-63 cells (solid bars). *BMP-2* expression is indicated as fold increase ($\Delta\Delta C(t)$), in which the values normalized to *GAPDH* expression were compared with the *BMP-2* expression in wells with 0 ng/mL of FGF-2. The results shown are the average and standard deviation of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 5: FGF-2-release curves from the FGF-L (round) or FGF-H (square) buttons in phosphate-buffered saline. The results shown are the average and standard deviation of three independent experiments.

Figure 6: Cell proliferation and *BMP-2* expression were stimulated by the HAP-N, FGF-L, and FGF-H buttons. BHK-21 cells and MG-63 cells were incubated in medium including HAP-N, FGF-L, or FGF-H buttons for three days. A) The number of BHK-21 cells after incubation with each of the three buttons. B) The number of MG-63 cells after incubation with each of the three buttons. C) Real-time PCR results of *BMP-2* expression in BHK-21 cells (empty bars) and MG-63 cells (solid bars). *BMP-2* expression is reported as fold increase ($\Delta\Delta C(t)$), in which values normalized to *GAPDH* expression were compared with the *BMP-2* expression in HAP-N. The results shown are the average and standard deviation of three independent experiments.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 7: Histological sections of the bone defects were stained with hematoxylin and eosin after demineralization. The yellow dotted lines show the area of bone formation indicated by BF. A) A representative section from Group 3 treated with FGF-L indicates the extension (arrows) of the bone formation into the open space left by the bone defect. The lining cells in surroundings of bone formation seem osteoblasts. B)

A representative section from Group 3 indicates the bone formation (arrows) between the cranium and the dura membrane, resulting in increased cranial thickness. C) A representative section from Group 1 treated with HAP-N, which shows the poor response of the bone formation under these conditions. ILC: inner layer of the cranium. Magnification: 100X.

Figure 8: Bone formation was quantified in each group. Group 1 was treated with the HAP-N button alone (cross lines), Group 2 with the HAP-N button and drips of the FGF-2 solution (triangle lines), Group 3 with the FGF-L button (square lines), and Group 4 with the FGF-H button (round lines). A) The extension of new bone formation into the space left by the bone defect. B) The increased thickness of the cranium due to the bone formation. The results shown are the average and standard deviation of six samples. * $p < 0.01$, ** $p < 0.001$.

Figure 9: The *in vivo* expression of *BMP-2*, alkaline phosphatase (*ALP*), and osteocalcin (*OC*) in the bone-defect tissues at two (empty bars) or four (solid bars) weeks after the

procedure. A) The expression of *BMP-2*. B) The expression of *ALP*. C) The expression of *OC*. The gene expression is reported as fold increase ($\Delta\text{-C}(t)$), where the values were compared with *GAPDH* expression. The results shown are the average and standard deviation of three samples. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.