Enhanced *in vivo* osteogenesis by nanocarrier-fused BMP4

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Running header: *in vivo* osteogenesis by CBD-BMP4

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Abstract

Purpose: Bone defects and non-unions are major clinical skeletal problems. Growth factors commonly are used to promote bone regeneration. However, the clinical impact is limited because the factors do not last long at the given site. The introduction of tissue engineering aimed to deter the diffusion of these factors is a promising therapeutic strategy. The purpose of the present study was to evaluate the *in vivo* osteogenic capability of an engineered bone morphogenetic protein-4 (BMP4) fusion protein.

Methods: BMP4 was fused with a nano-size carrier, collagen-binding domain (CBD) derived from fibronectin. The stability of the CBD-BMP4 fusion protein was examined *in vitro* and *in vivo*. Osteogenic effects of CBD-BMP4 were evaluated by computer tomography (CT) after intramedullary injection without a collagen sponge scaffold. Recombinant BMP-4, CBD or vehicle were used as controls. Expressions of bone-related genes and growth factors were compared among the groups. Osteogenesis induced by CBD-BMP4, BMP4 and CBD was also assessed in a bone defect model.

Results: *In vitro*, CBD-BMP4 was retained in a collagen gel for at least 7 days while BMP4 alone was released within 3 hours. *In vivo,* CBD-BMP4 remained at the given site for at least 2 weeks both with or without a collagen sponge scaffold, while BMP4 disappeared from the site by 3 days after injection. CBD-BMP4 induced better bone formation than did BMP4 alone, CBD alone and vehicle after the intramedullary injection into mouse femur. Bone-related genes and growth factors were expressed at higher levels in CBD-BMP4-treated mice than in all other groups including BMP4-treated mice. Finally, CBD-BMP4 potentiated more bone formation than did controls including BMP4 alone when applied to cranial bone defects without a collagen scaffold.

Conclusion: Altogether, nanocarrier-CBD enhanced retention of BMP4 in the bone, thereby promoting augmented osteogenic responses in the absence scaffold. These results suggest that CBD-BMP4 may be clinically useful in facilitating bone formation.
Keywords: BMP4, Bone repair, Bone tissue engineering, Osteogenesis.

Introduction

Bone defects and non-unions remain considerable problems caused by tumor and trauma, and their treatment constitutes a major challenge in orthopedic reconstitution surgery.[1] Autologous bone graft is a standard technique for inducing bone repair. However, clinical benefits are not ensured and collateral symptoms including persistent site-pain, nerve injury, hematoma, infection and fracture frequently occurr.[2] Recent advances in the treatment methods include the use of sophisticated biocompatible scaffolds, multi-potential cell populations and appropriate cellular stimulation at the affected sites. Currently, growth factors-based bone tissue engineering has been attracting increasing attention.

Many growth factors induce osteogenesis.[3] Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-β (TGFβ) protein superfamily and are known to play pivotal roles in the regulation of bone induction, maintenance and repair.[4, 5] FDA (Food and Drug Administration) has approved two BMPs, BMP2 and BMP7/OP1, which have accompanied orthopaedic surgery and are applied with an absorbable collagen sponge. The clinical benefits of BMP2 and BMP7 have been reported,[6, 7] however, several reports described heterotopic ossification associated with the use of BMP2 and BMP7.[8-10] To prevent heterotopic bone formation and induce successful site specific bone growth, requires an approach that limits the diffusion of factors to target tissues.

BMP4 induces osteogenic differentiation of osteoblasts and osteo-progenitors and promotes bone formation,[11] thus playing a crucial role in the onset of bone and cartilage development and fracture repair.[12] *In vivo* BMP4 gene therapy accelerated the repair of bone fractures[13] and performed as well or better when compared with BMP2.[14] Thus, BMP4 appears to be a viable other approach for treatment of bone defects and non-unions. BMP4 alone can be delivered by direct injection into the site of concern, but immobilized BMP4 can be localized and retained in the targeted site for longer

periods and thus extend the functional half-life of this factor. Very recently, we have created a novel collagen-PLGA (poly lactic-co-glycolic acid) hybrid scaffold with a BMP4 fused to an additional collagen-binding domain (CBD) derived from fibronectin (CBD-BMP4).[15] CBD-BMP4 exhibited stronger and more stable collagen binding activity than did wild type BMP4. This fusion protein, bound to a collagen-coated scaffold induced osteogenic differentiation of human mesenchymal stem cells when these cells were implanted into nude mice.[15]

In the present study, we extend our previous work and demonstrate that CBD-BMP4 is retained longer at the targeted sites compared to BMP4 alone and induced augmented bone formation even when injected without scaffold. Considering that the 100 kDa of fibronectin fragment that includes CBD (45kDa) was reported to be about 24 nm,[16] CBD size can be regarded as a nano-carrier. When applied to cranial bone defects, CBD-BMP4 successfully induced new and accelerated bone formation as compared to BMP4 alone. Thus, CBD-BMP4 may be promising for the treatment of bone defects and non-unions.

Materials and methods

CBD-BMP4

CBD-BMP4 fusion protein was prepared as described.[15] In brief, the recombinant protein was produced by transgenic silkworms, which carried a chimeric gene encoding CBD of human fibronectin and human BMP4 (mature form). The enterokinase recognition sequence was inserted between the CBD and BMP4 sequences. The fusion protein produced by and secreted into cocoons was extracted with $CaCl_2$ and affinity-purified by Gelatin Sepharose 4B (GE Healthcare, Buckinghamshire, England). CBD protein (without BMP4 fusion) was prepared as described.[17] Control BMP4 was purchased from R&D systems (Minneapolis, MN, USA). For some experiments,

CBD-BMP4, BMP4 and CBD were labeled with HiLyte Fluor 555 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan), according to the manufacturers' instructions. In brief, NH_2-reactive HiLyte Flour 555 dissolved in 10 μl dimethylsulfoxide (DMSO) was mixed with CBD-BMP4, BMP4 or CBD solution (10 μg protein in 100 μl PBS). The protein-dye solution was incubated for 10 minutes at 37°C and unreacted free dye was removed by centrifugation (8000 × *g*, 10 minutes) using a provided filtration tube. The labeled protein was then recovered from the filter membrane.

Characterization of CBD-BMP4

Purified protein was digested with enterokinase (EK max, Life Technologies, Grand Island, NY), fractionated on SDS-polyacrylamide gel, and transferred to a polyvinylidene difluoride (PVDF) membrane. The gel was stained with Coomasie Brilliant Blue. The membrane was incubated with anti-BMP4 goat serum (R&D), followed by the incubation with HRP-linked anti-goat IgG antibody (Vector Laboratories, Burlingame, CA), and visualized using ECL system (GE Healthcare). To assess binding stability, CBD-BMP4 or BMP4 was mixed with 0.2% collagen solution in Eagle's MEM medium, pH 7.4 (Koken Co., Tokyo, Japan), and the mixture (30 μl) was gelled at 37°C for 1 hour. PBS (150 μl) was then added and the overlaid PBS was collected after 1 hour, 3 hours, 1 day, 3 days and 7 days. Protein released into PBS solution was dot-blotted to a nitrocellulose membrane, followed by immunostaining with goat anti-BMP4 and secondary antibody as above. The reaction was visualized using 4-Chloronaphthol as HRP substrate.

Animals

New Zealand white rabbits (Female, 2.0-2.5 kg) were purchased from Shimizu Laboratory Supplies (Kyoto, Japan). BALB/c mice (Female, 6-8 weeks) were obtained from Charles River Laboratories (Yokohama, Japan). Animals were housed in a

temperature-controlled environment with a 12-hour light/12-hour dark cycle under specific pathogen-free conditions and allowed free access to water and food. The animal care and use committee at Okayama University approved all animal experiments conducted in this study.

Collagen sponge scaffold model

Rabbits were anaesthetized with an intramuscular injection of ketamine (80 mg/kg). Both knees were shaved and draped in a sterile fashion, and a medial incision was made. A bone hole was made in the distal diaphysis of the femur with a φ5.0 mm drill, and a collagen sponge (3-mm cylinder, Atelocollagen sponge, MIGHTY; Koken Co. Tokyo, Japan) was intramedullary implanted though the hole. The collagen sponge was pre-soaked with CBD-BMP4, BMP4 or CBD solution and contained 1 μg of each protein. A sponge soaked with vehicle PBS alone was used as a control. These 4 groups of collagen sponge were randomly implanted into rabbit femurs (10 rabbits, 20 femurs, 5 femurs per each group). Rabbits were then housed in each cage without knee immobilization until the time of evaluation. Rabbits were sacrificed 4 weeks later, and the collagen sponge was retrieved from the femur, fixed in 10% formalin, decalcified in 10% EDTA, embedded in paraffin, and the sections were stained with hematoxylin-eosin (HE). Histological sections were digitalized under a microscope, and the ossification area in the sponge was measured by image-analyzing software, WinROOF (Mitani Co. Fukui, Japan). Peripheral area of each sponge was deselected in order to exclude bone ingrowth by spontaneous healing. To assess the retainment of CBD-BMP4, BMP4 or CBD *in vivo*, a collagen sponge containing 1 μg of each protein fluorescenctly-labeled was implanted as described above. Sponges soaked with vehicle PBS alone were used as controls (6 rabbits, 12 femurs, 3 femurs per each group). On day 1, 3 and 14 after the implantation, rabbits were killed and the sponge was removed, frozen in SCEM compound (SECTION-LAB Co. Hiroshima, Japan) and cut with

tungsten blade at -20°C, as described.[18] The sections were stained with Calcein-AM (40 μg/ml, Dojindo) for bony calcium detection and evaluated under a fluorescence microscope.

Intramedullary injection model

Mice were anesthetized with ketamine (100 mg/kg). Both knees were shaved and the skin was cleaned with 70% ethanol. A bone hole was made in the distal diaphysis of the femur with a 24G needle, and CBD-BMP4, BMP4 or CBD (100 ng in 10 μl PBS) was injected into the medullary cavity of the right femur with a 27G needle-tipped syringe. The left femur injected with 10 μl PBS was used as a control (5 mice per each group). At 4 weeks after the injection, the mice were sacrificed and the femurs were resected. The femurs were scanned by micro-CT (Latheta LCT-200, Hitachi Aloka, Tokyo, Japan) using 48 μm slices (0.3 mm interval), and the bone mineral density (BMD) of individual trabecular bone area was calculated by accompanying image-analyzing software, LaTheta v1.20. In some mice, the bone marrow was washed with saline and the cells were stored at -80°C for mRNA expression study. To assess the retainment of CBD-BMP4, BMP4 or CBD *in vivo,* each protein fluorescenctly-labeled (1 μg in 10 μl PBS) or vehicle (10 μl PBS) was directly injected into the femur as described above (3 mice per group). Mice were sacrificed on day 1, 3 and 14 after the injection and non-decalcification femurs were cut with a tungsten blade at -20°C, and the sections were stained with Calcein-AM and evaluated under a fluorescence microscope.

Bone defect model

Bone defects were made in the cranial bone, as previously described.[19] Briefly, mice were anesthetized with ketamine (100 mg/kg) and the cranial bone was exposed. Bone defects were made in the parietal bone with a φ3.0 mm drill. After washing with saline, CBD-BMP4, BMP4, CBD (100 ng in 5 μl PBS) or vehicle (5 μl PBS) was applied to

the defects, and the scalp was closed (5 mice per each group). At 2 weeks after the surgery, the mice were killed and the cranial bone was scanned by micro-CT at 48 μm slices. To analyze ossification area, regions of interest (ROIs) were set on bone defect area, and the accumulation of dots (counts per pixel) in the selected ROI was measured using image-analyzing software (WinROOF). Three-dimensional (3D) images were reconstructed by an image processing software, OsiriX (Pixmeo SARL, Bernex, Switzerland). Subsequently, the defect area was resected, fixed in 10% formalin, decalcified in 10% EDTA, embedded in paraffin, and the sections were stained with hematoxylin-eosin (HE).

Quantitative real-time PCR

Samples were homogenized in Lysis buffer (Quickgene, Fujifilm, Tokyo, Japan), and total RNA was isolated according to the manufacturer's instructions. First-strand cDNA was constructed from 2 μg of total RNA with oligo $(dT)_{12-18}$ as primers, and the cDNAs were used as a template for PCR. Quantitative real-time PCR was performed with SYBR PCR master mix (Agilent Technologies, Santa Clara, CA) and specific primers. To validate the SYBR Green PCR products, a dissociation step was done to verify the T_m (annealing temperature) of the SYBR Green PCR product after the PCR were run. The expression levels of each mRNA were normalized by the expression of a housekeeping gene hypoxanthine phosphoribosyltransferase (HPRT). The primers used in this study are listed in Table 1.

Statistical analysis

Statistical analyses were performed using Student's t-test for paired samples and ANOVA for multiple samples. All data were expressed as mean ± SEM. $p<0.05$ was considered statistically significant.

Results

Characterization of CBD-BMP4

Recombinant CBD-BMP4 was purified from cocoons of transgenic silkworms. Calculated molecular sizes of CBD-BMP4, CBD and BMP4 moieties were 52.5 kDa, 39 kDa and 13 kDa, respectively. As confirmed by enterokinase digestion, this fusion protein consisted of CBD and BMP4. BMP4 moiety released from CBD was immunoreactive with anti-BMP4 antibody (Fig. 1A). In *in vitro* experiments, CBD-BMP4 in a collagen gel was entrapped and localized for at least 7 days while unfused BMP4 diffused out of the gel as early as 1 hour and was undectable after 1 day (Fig. 1B), indicating that CBD-BMP4 showed much higher collagen-binding affinity than did BMP4.

Enhanced bone formation in collagen sponge scaffold

We next asked whether CBD-BMP4 could display enhanced collagen-binding capacity *in vivo* and promote bone formation more effectively than BMP4 alone. To examine this, collagen sponges containing 1 μg of CBD-BMP4, BMP4 or CBD were placed in the rabbit femur as described in methods. The immunohistological data in Figure 2 demonstrated that BMP4 was found localized on day 1 but was no longer visible on day 3 after the implantation. A scant calcification was seen on day 14 (Fig. 2). In case of CBD-BMP4, a stronger signal was seen on day 1 relative to BMP4 and the signal intensity did not diminish by day 3. Results with CBD alone were similar to those of the fusion protein at day 3. Of note was the finding that only CBD-BMP4 appeared to induce robust calcification in the site at day 14 (Fig. 2). In other set of experiments, histological examination four weeks later revealed that CBD-BMP4 induced a thicker trabecular bone formation than BMP4 did (Fig. 3A). Further, the ossification area formed by CBD-BMP4 injected animals was significantly larger than seen in BMP4

injected animals (Fig. 3B). CBD induced slightly augmented bone formation as compared to PBS control. These data clearly showed that CBD-BMP4 is retained longer at the given site *in vivo* and induced bone formation more effectively than BMP4, CBD or PBS when the fusion protein was implanted with scaffold.

Augmented bone formation without scaffold

Collagen is the major constituent of bone matrix.[20] The above data prompted us to investigate whether CBD-BMP4 could remain at the given site without scaffold, leading to an augmented bone formation. To address this, we first confirmed the retainment of CBD-BMP4, BMP4 and CBD after a direct injection of fluorescence-labeled proteins (1 μg in 10 μl PBS) into the medullary cavity of mouse femur. The data in Figure 4 demonstrated that BMP4 was not found on day 3 after the injection whereas CBD-BMP4 was observed even after day 14. CBD remained in a similar fashion. Apparent calcification was seen only next to CBD-BMP4 on day 14 (Fig. 4), suggesting enhanced new bone formation by CBD-BMP4.

We then assessed bone mineral density (BMD) by measuring micro-CT images (Fig. 5A). In mice treated with CBD-BMP4, BMD 4 weeks later was increased compared to the BMP4, CBD and PBS groups. There was no difference between BMP4 and PBS groups. BMD in CBD groups was higher than was seen in PBS groups (Fig. 5B). The BMD in CBD-BMP4 and BMP4 groups were also measured at 8 weeks after the injection, and showed that BMD in CBD-BMP4 groups was higher than that observed in BMP4 groups (Fig. 5C). BMD in CBD-BMP4 groups at 8 weeks was increased as compared to that at 4 weeks (737.1 ± 8.1 mg/cm^3 vs. 703.5 ± 5.9 mg/cm^3, respectively, $p<0.05$, 5 mice each) whereas no change was found in BMP4 groups (Fig. 5C), suggesting prolonged osteogenic activity with CBD-BMP4. These data indicate that a single injection of CBD-BMP4 augments bone formation even without scaffold.

Osteogenic gene expressions by CBD-BMP4

To better understand the molecular mechanisms underlying the augmented osteogenesis by intramedullary CBD-BMP4 treatment, bone marrow cells were harvested from the femurs at 4 weeks after therapy injection and mRNA expression of osteogenic factors were examined. Osteoblast-associated factors including alkaline phosphatase (ALP), bone sialoprotein (BSP), osteocalcin, osterix and Runt-related transcription factor 2 (Runx2) were expressed at higher levels in CBD-BMP4 group than in other groups including BMP4 group (Fig. 6A). In contrast, there was no difference between the BMP4 group and the PBS control. Interestingly, the CBD group showed increased ALP, BSP and osteocalcin expression relative to the control PBS (Fig. 6A). Endogenous expression of growth factors was next examined, which demonstrated that the CBD-BMP4 group, but not the BMP4 group, up-regulated expression of BMP2 and BMP4 as compared to PBS control. Augmented expression of BMP4 was also found in the CBD group. No change was found in the expression level of TGFβ (Fig. 6B). Thus, enhanced bone formation in the CBD-BMP4 group was associated with augmented expressions of osteogenic factors and growth factors. The BMP4 alone group failed to augment these factors during this time period (4 weeks after the injection), possibly due to the diffusion from the site (Fig. 4). CBD, which was also present at the targeted site over a longer period, might stimulate bone formation by inducing endogenous BMP4.

Accelerated bone formation in cranial bone defect model

To further strengthen the *in vivo* osteogenesis induced by CBD-BMP4, we applied CBD-BMP4, BMP4, CBD or vehicle in a cranial bone defect model without scaffold. BMP4 treatment demonstrated new bone formation on day 14 after the treatment (Fig. 7A). Obviously, CBD-BMP4 showed substantial ingrowth of new bone formation. As shown in Figure 7B, the ossification area induced by CBD-BMP4 and BMP4 was statistically increased as compared to that by PBS control. Importantly, there was more

new bone formation in the CBD-BMP4 treated group than that in the BMP4 group. Ossification area by CBD was similar to that of the PBS control (Fig. 7B). Histological examination consistently demonstrated new bone formation lined by a layer of osteoblasts in CBD-BMP4- and BMP4-treated group, which was more prominent in CBD-BMP4 group (Fig. 7C).

Discussion

Immobilized growth factors targeting to extracellular matrix can be more effective than diffusible, free growth factors.[21] The goal of engineering a fusion protein was to deliver a functional substance that had limited diffusion over a prolonged period of time. Our novel BMP4 fusion protein with a nano-size carrier, namely, the CBD seems to fulfill this requirement.[15] The stable binding of CBD to collagen led us to investigate whether this novel CBD-BMP4 by itself could enhance bone formation using bone matrix collagen as an innate scaffold.

As expected, CBD-BMP4 induced stronger bone formation than did by BMP4. CBD-BMP4 remained at the injected site for at least 2 weeks possibly through the CBD binding to bone matrix collagen, and the results demonstrated that new bone formation continues to be observed even after 8 weeks. There exist several kinds of gene-engineered binding growth factors including BMPs.[21] Our CBD-BMP4 has advantages over other engineered fusion proteins. First, CBD-BMP4 induced entopic bone formation without using scaffold. As far as we know, this is the first report demonstrating an accelerated bone formation by a fusion protein on its own. Previous studies reported that collagen-binding BMP2 demonstrated ectopic bone formation when applied with bone-derived matrix as a scaffold.[22, 23] Bone-derived matrix contains many factors including native BMPs and the complex is not fully defined, suggesting potential difficulties in clinical use. Second, CBD-BMP4 was effective even in a single low dose. In the present study, we injected 100 ng of CBD-BMP4 (≈2 pM), as

preliminary experiments showed that BMP4 at this dose failed to induce appreciable bone formation at 4 weeks after the injection (not shown). Others used 8 nM CBD-BMP2 for ectopic bone formation model and 0.5 nM for bone defect model.[24] Although verification of whether higher amounts of CBD-BMP4 could induce stronger bone formation in a critical-sized bone defect remains to be done, we believe that our results predict that CBD-BMP4 will be very efficient with larger doses. In a bone defect model, 2.5 to 5 μg of recombinant BMP4 was applied with collagen sponge or beta-tricalcium phosphate.[11] We believe our initial results also predict that compared to other reagents, the CBD-BMP4 fusion protein will efficaciously require reduced amounts of protein to induce bone formation, and thus limit possible clinical complications.

It appears that CBD-BMP4 both directly and indirectly induced bone formation. We have demonstrated that CBD-BMP4 induced osteogenic differentiation using three-dimensional cultures of human bone marrow-derived mesenchymal stem cells.[15] As shown in this study, CBD-BMP4 treatment up-regulated expression of all relevant osteogenic genes examined in the target site, even after 4 weeks post-injection possibly due to the continued functional localization of the fusion protein. ALP is a major biomarker of bone formation and plays a key role in bone mineralization.[25] BSP is up-regulated as osteoblasts mature at sites of de novo bone formation.[26] Osteocalcin is an extracellular matrix (ECM) protein and among the most specific markers for osteoblast maturation.[27] Osterix is a bone-related transcription factors that functions genetically downstream of Runx2, which regulates the differentiation and/or function of osteoblasts.[28, 29] In addition, CBD-BMP4 increased the expression of endogenous BMP2 and BMP4. Thus, CBD-BMP4 stimulated de novo expressions of osteogenic genes and bone-growth factors for a longer period and initiated the calcification of the ECM, leading to the prolonged bone formation.

Of note was the finding that CBD by itself somewhat induced bone formation when intramedullary injected. Similar to CBD-BMP4, CBD remained at the injected site. The CBD used in this fusion protein was from fibronectin,[17] which is an ECM component. ECMs and growth factors function cooperatively to stimulate osteoblast differentiation. There is evidence to suggest a role for fibronectin in the early stages of bone formation.[30, 31] Recently, it has been demonstrated that ECMs including fibronectin modified the growth patterns and induced the osteoblast differentiation of human myeloid stem cells, as evidenced by increased expressions of ALP, osteocalcin, osterix and Runx2.[32] Accordingly, we showed in this study that CBD up-regulated the expressions of osteogenic genes such as ALP, BSP and osteocalcin, and growth factor BMP4. Although CBD is a segment (amino acids 260–599) of the original fibronectin,[15] CBD may contain fibronectin's active site leading to osteogenesis. Thus, the osteogenic capacity of CBD-BMP4 may be partly due to the activity of CBD. A question arises whether simultaneous injection of CBD and BMP4 could represent additive or synergistic osteogenic effects, which was not examined in this study. CBD-BMP4 had no osteogenic effects when applied to the defective section of cranial bone. The disparity may be due to the different location of the given site (bone marrow vs. cortical bone).

There are other several concerns that were not confronted in this study. Although we believe that CBD-BMP4 binds to bone matrix collagen at the injection site we have not shown direct evidence. It is not clear how CBD-BMP4 interacts with the BMP4 receptor at the site and how CBD-BMP4 itself affects the surrounding cells either when acting as a complex or as individual components. CBD-BMP4 has a higher molecular weight than BMP4, which may contribute to the physics of diffusion. Further studies are necessary to explore these precise mechanisms.

Conclusion

In conclusion, our engineered CBD-BMP4 is a novel fusion protein with an exquisite ability to promote *in vivo* osteogenesis even by a single injection at the targeted site both with and without scaffold. They are many ways to enhance the release of BMP4 using chemical conjugation, particle vehicle or gene delivery, which are low cost, verified and convenient. We believe that our fusion protein is better than others as CBD-BMP4 can localize longer at the designated site, resulting in stronger bone formation by a single low dose of complex. This novel CBD-BMP4 may be promising clinically for the treatment of unresolved fractures, bone defects and other bone tissue engineering and regeneration related scenarios.

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Disclosure

The authors report no conflicts of interest in this work.

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Figure Legends

Figure 1 Characterization of CBD-BMP4. (A) Purified CBD-BMP4 was digested with enterokinase, fractionated on SDS-polyacrylamide gel, and transferred to a PVDF membrane. The gel was stained with Coomasie Brilliant Blue (left), and the membrane was immunoblotted with anti-BMP4 (right). (B) BMP4 or CBD-BMP4 was mixed with collagen solution, and the mixture was gelled at 37°C for 1 hour. Protein released into PBS solution was blotted to a nitrocellulose membrane and immunodetected with anti-BMP4.

Figure 2 Fluorescence images of a collagen sponge. Collagen sponges containing fluorescence-labeled CBD-BMP4, BMP4 or CBD were implanted into rabbit femur. Sponges soaked with vehicle PBS were used as a control (3 femurs per each group). On day 1, 3 and 14 after the implantation, the sponges were retrieved and the sections were examined under a fluorescence microscope. HiLyte Flour 555-labeled CBD-BMP4/BMP4/CBD is shown in red. Bone stained by Calcein-AM is shown in green. Shown were representative photographs from each group.

Figure 3 CBD-BMP4 augments new bone formation when delivered by collagen sponges. Collagen sponges containing 1 μg of CBD-BMP4, BMP4 CBD were implanted into rabbit femurs. Sponges soaked with vehicle PBS were used as a control (5 femurs per each group). Four weeks later, the sponge was retrieved, fixed, decalcified and the sections were stained with HE. (A) Histological sections of the collagen sponge. Representative photographs of each group were shown. ❖: new bone (note osteocyte). ◆:collagen sponge. (B) Ossification area in the sponge was calculated (5 femurs per each group). §$p<0.05$, †$p<0.01$, ¶$p<0.0001$. Maginification is shown by bar (100 μm).

Figure 4 Fluorescence images of mouse femur. Fluorescence-labeled CBD-BMP4, BMP4 or CBD (1 μg in 10 μl PBS) were injected into mouse femurs. PBS alone (10 μ) was used as a control (3 mice per each group). On day 1, 3 and 14 after the injection, the femurs were resected, frozen, cut and examined under a fluorescence microscope. HiLyte Flour 555-labeled CBD-BMP4/BMP4/CBD is shown in red. Bone stained by Calcein-AM is shown in green. Bone hole is shown in a dashed line. Shown were representative photographs from each group. Maginification is shown by bar (500 μm).

Figure 5 CBD-BMP4 accelerates bone mineral density. CBD-BMP4, BMP4, CBD (100 ng in 10 μl PBS) or vehicle PBS (10 μl) were directly injected into mouse femurs (5 mice per each group). At 4 weeks after the injection, the femurs were scanned by micro-CT. (A) Representative images from each group. (B, C) Bone mineral density was calculated by micro-CT at 4 weeks (B) and 8 weeks (C) after the injection. §p<0.05, †p<0.01.

Figure 6 CBD-BMP4 increases osteogenic gene expression. Bone marrow cells were harvested at 4 weeks after the injection of CBD-BMP4, BMP4, CBD (100 ng in 10 μl PBS) or vehicle PBS (10 μl) (5 mice per each group). mRNA expression for (A) osteoblast-associated factors and (B) bone growth factors were quantitated by RT-PCR. The expression levels of mRNA were normalized to HPRT. §p<0.05, †p<0.01.

Figure 7 CBD-BMP4 enhances bone formation in a cranial bone defect model. CBD-BMP4, BMP4, CBD (100 ng in 5 μl PBS) or vehicle PBS (5 μl) was applied to the defective section of cranial bone (5 mice per each group). At 2 weeks after the injection, the mice were killed and the bone defects were scanned by micro-CT. (A) Representative 3D-CT images from each group. (B) Ossification area was calculated by micro-CT. §p<0.05, †p<0.01. (C) Representative tissue sections from each group are

shown (HE staining). Arrowheads indicated the layer of osteoblasts.