

Title	Synergistic effects of the dual release of stromal cell-derived factor-1 and bone morphogenetic protein-2 from hydrogels on bone regeneration.
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**COMBINED RELEASE OF STROMAL CELL-DERIVED FACTOR-1 AND BONE
MORPHOGENETIC PROTEIN-2 FROM GELATIN HYDROGELS SHOWS
SYNERGISTIC EFFECT ON BONE REGENERATION**

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Abstract

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5 The objective of this study is to evaluate the activity of gelatin hydrogels
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7 incorporating combined stromal cell-derived factor-1 (SDF-1) and bone morphogenetic
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9 protein-2 (BMP-2) on the *in vivo* bone regeneration at an ulna critical-sized defect and
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11 subcutaneous site of rats, and compared with that of those incorporating either SDF-1 or
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13 BMP-2. The similar release profile of SDF-1 and BMP-2 from the hydrogels was observed
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15 with or without the combination of BMP-2 and SDF-1, respectively. An enhanced bone
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17 regeneration by the hydrogels incorporating combined SDF-1 and BMP-2 was observed. In
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19 addition, the implantation of hydrogels incorporating combined SDF-1 and BMP-2 enhanced
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21 the expression level of CXC chemokine cell-surface receptor-4 (*Cxcr4*), Runt-related factor-2
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23 (*Runx2*), and *Osteocalcin* genes. The experiments with green fluorescent protein (GFP)-
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25 positive *Chimeric* mice revealed that the recruitment of bone marrow-derived cells was
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27 promoted and a vascular-like structure together with strong accumulation of CD31- and
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29 CD34-positive cells was observed at the site of hydrogels incorporating combined SDF-1 and
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31 BMP-2 implanted. In addition, a large fraction of CD29- and CD44-positive non-
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33 hematopoietic cells was detected. It is concluded that the combined release of SDF-1 and
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35 BMP-2 enhanced the recruitment of osteogenic cells and angiogenesis, resulting in the
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37 synergistic effect on bone regeneration.
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Key words

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49 Stromal cell-derived factor-1 (SDF-1), bone morphogenetic protein-2 (BMP-2), gelatin
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51 hydrogels, combined release, bone regeneration
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1. Introduction

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5 Tissue engineering is a newly emerging biomedical technology to promote the cell
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7 potentials of proliferation and differentiation and has been extensively studied to induce the
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9 regeneration and repairing of defective tissues over last 20 years. Many researches have been
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11 attempted to design the substitutes of natural microenvironment by providing the components
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13 of extracellular matrix (ECM), growth factors, cells, and the combination in different
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15 manners [1]. For bone tissue engineering, the combination of these multiple cues is required
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17 since the mechanism of bone repairing is complex and regulated by many factors, including
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19 the characteristics of ECM scaffolds, the type of angiogenic and osteogenic growth factors,
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21 and the osteogenic potential of cells [2]. In addition to tissue engineering-based regeneration
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23 therapy, the transplantation of various cells has been performed experimentally and clinically.
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25 However, the cell transplantation does not always obtain the good result of tissue
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27 regeneration because of the limitations of cell source, the low survival rate of cells
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29 transplanted [3,4], and the immunogenic responses of transplanted vs. host cells [5]. Also, the
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31 nature of cells cultured for transplantation might not be equal to that of cells present in the
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33 body. In this situation, recently, the body cells are focused as a potential source for tissue
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35 regeneration. Some researches have demonstrated that stem cells originating in the blood
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37 circulation play a crucial role in hematopoiesis, vascularization or mesenchymal tissue
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39 regeneration [6,7]. It is reported that bone marrow-derived osteoblast progenitor cells present
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41 in the circulating blood participated in BMP-2-induced ectopic bone formation [8,9]. The
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43 research gives us an idea that the appropriate supply of growth factors in a suitable fashion is
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45 effective in recruiting host circulating stem cells to the defect site, biologically functioning
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47 thereat, and inducing the bone regeneration.
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To achieve the recruitment of host circulating cells, stromal cell-derived factor-1 (SDF-1), scientifically designated as Chemokine (C-X-C motif) ligand 12 (CXCL12), was selected in this study. It is known that SDF-1 is critical to induce the natural migration and homing of stem cells to a targeted site within the body [10]. SDF-1 is also involved in the recruitment of inflammatory cells and stem cells committed to other types of tissue [11]. It is reported that SDF-1 mediates the homing of cells by binding to a G protein-coupled receptor, CXCR4, on the surface of circulating CXCR4-positive progenitor cells [12,13]. Aiuti *et al.* [14] have introduced that the SDF-1/CXCR4 interaction involved in the mobilization of CD34-positive progenitors to the peripheral blood circulation and in the homing of hematopoietic stem cells to their specific niches. Some researches have reported on the role of SDF-1 in the mobilization and recruitment of circulating bone marrow-derived hematopoietic (HSC) and mesenchymal stem cells (MSC), which contribute to cell-based vascularization and bone regeneration [15-17]. Our recent study has also demonstrated that a dose of SDF-1 released from gelatin hydrogels enhanced angiogenesis at the site implanted because of the enhanced recruitment of cells which are effective in angiogenesis [18].

Among osteogenic growth factors, bone morphogenetic protein-2 (BMP-2) is well-known as a strong inducer of bone tissue formation by enhancing the recruitment of osteoblast progenitor cells [8], angiogenesis [19], and promoting the osteogenic differentiation of mesenchymal stem cells [20,21]. The BMP-2 treatment induced complete bone regeneration at a bone defect [22] and formed bone tissues ectopically for the animal models [8,9]. In addition, it has an activity to enhance the cells mobilization and homing to the defect site [8]. Our previous study experimentally confirmed with the green fluorescent protein (GFP)-positive *Chimeric* mice that the recruitment of bone marrow-derived cells to the site of gelatin hydrogels incorporating BMP-2 implanted was enhanced [9]. This

phenomenon demonstrates ectopic bone formation due to the accumulation of osteogenic cells.

Angiogenesis is important for the process of bone regeneration and the combination of angiogenic factors further enhanced the bone regeneration induced by osteogenic factors [23,24]. Based on these findings, we hypothesized that a local release of combined SDF-1 and BMP-2 may facilitate the recruitment of host circulating stem cells to the site implanted, and achieve the consequent angiogenesis and bone regeneration. To this end, the controlled release system to prolong the *in vivo* half-life of growth factors is required [25]. In this study, gelatin hydrogels were selected as the controlled release carriers since our previous researches confirm that the controlled release of SDF-1 and BMP-2 from gelatin hydrogels successfully induced angiogenesis and bone regeneration, respectively [9,18]. The potential of combined SDF-1 and BMP-2 release from the gelatin hydrogels to induce bone regeneration has not been investigated yet.

This study is undertaken to evaluate the bone regeneration of gelatin hydrogels incorporating combined SDF-1 and BMP-2, comparing with that of hydrogels incorporating either SDF-1 or BMP-2. Bone regeneration was investigated at an ulna critical-sized defect and ectopically the back subcutis of rats in terms of radiological, micro-computed tomography (μ CT), peripheral quantitative computed tomography (pQCT), and histological examinations. In addition, the expression of osteogenic genes of cells in the hydrogels implanted was evaluated to compare it among the different hydrogels incorporating SDF-1, BMP-2, and the combination. We also examined the recruitment of GFP-positive-bone marrow-derived mesenchymal stem cells and endothelial positive cells into the site of hydrogels implanted. In addition, the number of live cells and the fraction type of non-hematopoietic cells in the hydrogels implanted were analyzed by flow cytometry.

2. Materials and Methods

2.1 Materials

A gelatin sample prepared by an acidic treatment of porcine skin collagen (isoelectric point (IEP) = 9.0) was kindly supplied by Nitta Gelatin Inc., Osaka, Japan. Recombinant human stromal cell-derived factor-1 (SDF-1, 350-NS/CF) was obtained from R&D systems Inc., Minneapolis, MN. Recombinant human bone morphogenetic protein -2 (BMP-2) was kindly supplied from Yamanouchi Pharmaceutical Co., Tokyo, Japan. Na¹²⁵I (NEZ-033H, >12.95 GBq/ml) and N'-succinimidyl-3-(4-hydroxy-3,5-di[¹²⁵I]iodophenyl)propionate or [¹²⁵I] Bolton-Hunter reagent (NEX-120H, 147 MBq/ml) were purchased from Perkin-Elmer Life Sciences Inc., Boston, MA. Glutaraldehyde (GA), glycine, and other chemicals were obtained from Wako Pure Chemical Industries, Ltd., Osaka, Japan and used without further purification. All primers (Table 1) were purchased from Invitrogen Corporation, Ltd., Carlsbad, CA.

2.2 Preparation of gelatin hydrogels

Chemically-crosslinked gelatin hydrogels with GA were prepared according to the method previously reported [9,18]. Briefly, gelatin aqueous solution (5 wt%, pH 5.0) was mixed with GA at a final concentration of 0.16 wt%, followed by leaving at 4 °C for 12 hr to allow gelatin crosslinking. The crosslinked gelatin hydrogels were treated with 0.1 M glycine solution to block the residual aldehyde groups. After three-time washing with double-distilled water (DDW), the hydrogels were freeze-dried. The hydrogels were sterilized by ethylene oxide before every experiment in this study. According to our previous researches, the water content of gelatin hydrogels prepared was 98.2 ± 0.1 wt% and the *in vivo* degradation time was approximately 4-5 weeks [9,18].

2.3 Isolation and culture of rat bone marrow-derived mesenchymal stem cells

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Bone marrow-derived mesenchymal stem cells (MSC) were isolated from the bone shaft of femurs of 3-week-old Wistar rats according to the procedure reported previously [26]. Briefly, after cutting off both ends of rat femurs, the bone marrow was flushed out with 1 ml of Alpha-modified Eagle minimal essential medium (α -MEM). Then, the bone marrow cells mixture was cultured in α -MEM supplemented with 15 vol% fetal bovine serum (FBS), and 100 U/ml penicillin and streptomycin at 37 °C in a 5% CO₂-95% air atmospheric condition. The medium was refreshed on the 4th day after isolation to remove non-adherent cells and continuous refreshed every 3 days thereafter. The cells of the second- and third-passages at the subconfluent condition were used for the following experiments.

2.4 In vitro MSC cultures with SDF-1 and BMP-2 and their functional evaluation

MSC were seeded onto 6-cm tissue culture plates (CORNING 430166, Corning Inc., Cambridge, MA) at 10⁵ cells/plate and cultured for 24 hr in the α -MEM containing 15 vol% FBS, and 100 U/ml penicillin and streptomycin at 37 °C in a 5% CO₂-95% air atmospheric condition. Then, the medium was changed to the α -MEM containing 0.5 vol% FBS and 500 ng/ml of SDF-1, combined SDF-1 and BMP-2 at concentration ratios of 500/300, 400/400, and 300/500 ng/ml or 300 ng/ml of BMP-2. The cells were cultured in α -MEM containing 0.5 vol% FBS as a control. After 24 hr culture, cells were collected, washed with 100 mM of phosphate-buffered saline solution (PBS, pH 7.4), and the total RNA was extracted by using RNeasy® Plus Mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Reverse transcription reaction was performed with the SuperScript II First-Strand Synthesis System (Invitrogen Corporation, Ltd., CA). Real-time polymerase chain reaction (PCR) was performed on a Prism 7500 real time PCR thermal cycler (Applied Biosystems, Foster City, CA) from 10 ng of cDNA in a total volume of 25 μ l containing Power SYBR Green PCR

1 Master Mix (Applied Biosystems) and 10 μ M of each primer (Table 1) to analyze the
2 expression level of CXC chemokine cell-surface receptor-4 (*Cxcr4*) and Runt-related factor-2
3 (*Runx2*). The reaction mixture was incubated for the initial denaturation at 95 °C for 10 min,
4 followed by 40 PCR cycles. Each cycle consisted of the following three steps; 94 °C for 15
5 sec, 57 °C for 15 sec and 72 °C for 1 min. Each mRNA level was normalized by the
6 expression level of 18S ribosomal RNA as an internal control. The activity of alkaline
7 phosphatase (ALP) for cells cultured similarly was assayed by *p*-nitrophenyl phosphate
8 method 3 days after culture [26]. Each experiment was independently performed for 3
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Gelatin hydrogels (1 x 6 x 6 mm³) incorporating PBS, 500 ng of SDF-1, 500 ng of
SDF-1 and 300 ng of BMP-2, and 300 ng of BMP-2 were prepared by dropping the solution
(20 μ l) onto the hydrogel freeze-dried, followed by leaving at 4 °C overnight to allow the
solution incorporation. Next, 50 μ l of cell suspension (10⁶ cells) was seeded into each
hydrogel for 6 hr by the agitation seeding technique reported previously [26]. Then, 1 ml of
 α -MEM containing 0.5 vol% FBS was added into each cells-seeded hydrogel and the cells
were cultured for 3 days without further medium change. The hydrogels were collected,
washed with PBS, and the total RNA was extracted by using RNeasy® fibrous tissue mini kit
(Qiagen, Valencia, CA) according to the manufacturer's instructions. The RT-PCR assay was
performed as described previously to evaluate the expression level of *Cxcr4* and *Runx2* genes
of MSC cultured in the hydrogels.

2.5 *In vivo* release test of SDF-1 and BMP-2 from gelatin hydrogels

SDF-1 and BMP-2 were radioiodinated according to the conventional chloramine T
method as previously described [27]. Briefly, 5 μ l of Na¹²⁵I was added into 200 μ l of SDF-1
or BMP-2 solution (150 μ g/ml) in 0.5 M potassium phosphate-buffered solution (pH 7.5)

1 containing 0.5 M NaCl. Then, 100 μ l of the same buffer containing 0.2 mg/ml chloramine-T
2 was added to the solution mixture. After vortex mixing at room temperature for 2 min, 100 μ l
3 of PBS containing 0.4 mg sodium metabisulfate was added to the reacting solution to stop the
4 radioiodination. The solution mixture was passed through a PD-10 desalting column (GE
5 Healthcare Life Sciences, Chalfont St Giles, UK) to remove the uncoupled, free 125 I
6 molecules from the 125 I-labeled SDF-1 or 125 I-labeled BMP-2 using PBS as an eluting
7 solution. PBS solution containing 125 I-labeled SDF-1 (4.3 μ g/ml) or 125 I-labeled BMP-2 (13.7
8 μ g/ml) was mixed with PBS containing non-labeled SDF-1 or BMP-2 to give the final
9 concentrations of 232 and 202 μ g/ml, respectively. The mixed solution containing 5 μ g of
10 SDF-1 and/or 3 μ g of BMP-2 (20 μ l) was then adsorbed onto a freeze-dried gelatin hydrogel
11 (1 x 6 x 6 mm³), followed by leaving at 4 °C overnight to obtain hydrogels incorporating 125 I-
12 labeled SDF-1 or 125 I-labeled BMP-2. The hydrogel samples used for the *in vivo* release test
13 are gelatin hydrogels incorporating 5 μ g of 125 I-labeled SDF-1, 5 μ g of 125 I-labeled SDF-1
14 and 3 μ g of non-labeled BMP-2, 5 μ g of non-labeled SDF-1 and 3 μ g of 125 I-labeled BMP-2,
15 and 3 μ g of 125 I-labeled BMP-2. Following the implantation into the back subcutis of 6-week-
16 old female C57BL/6 mice, the hydrogels were taken out at different time intervals to count
17 the radioactivity remaining by the gamma counter (Auto Well Gamma System ARC-380 CL,
18 Aloka Co., Ltd, Tokyo, Japan). Each experiment was independently performed for 3 samples
19 at each sampling time.
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50 2.6 *In vivo* degradation test of gelatin hydrogels

51 To evaluate the *in vivo* degradation profile of gelatin hydrogels, the implantation of
52 125 I-labeled hydrogels was performed according to the method previously reported [18].
53 Briefly, 20 μ l of [125 I]Bolton–Hunter reagent solution in benzene was completely evaporated
54 at room temperature. The resultant solid reagent was redissolved into 1 ml of PBS and the
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1 resulting solution (20 μ l) was adsorbed onto a freeze-dried gelatin hydrogel, followed by
2 leaving at 4 °C overnight to introduce 125 I into the amino groups of gelatin. The
3 radioiodinated hydrogels were washed with DDW thoroughly to exclude the uncoupled, free
4 125 I molecules till to make the DDW radioactivity to the background level. Following the
5 implantation into the back subcutis of mice, the hydrogels were taken out at different time
6 intervals to count the radioactivity remaining by the gamma counter. Each experiment was
7 independently performed for 3 samples at each sampling time.
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20 *2.7 Evaluation of in vivo bone regeneration at ulna critical-sized defect*

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22 A bone defect model of rat ulna was prepared to evaluate the bone regeneration of
23 hydrogels incorporating SDF-1 and/or BMP-2. The surgery was made for 12-week-old male
24 Wistar rats (n = 12) under standard sterile conditions according to the procedure previously
25 reported [28]. Briefly, the rats were anesthetized with an intraperitoneal injection of
26 pentobarbital sodium solution (35-40 mg/kg body weight). After shaving the hair and
27 disinfection with 70 vol% ethanol, a longitudinal incision was made along the forearm skin of
28 rats. The periosteum was incised circumferentially to approach to the ulna bone. A critical
29 defect of 6 mm length was then created at the middle position of ulna bone using a side-
30 cutting diamond disk and a high-speed micromotor under an abundant irrigation with sterile
31 saline solution [29]. The hydrogels were implanted into the defects while the periosteum and
32 overlying muscle were repositioned with an absorbable polydioxanone suture (Ethicon 7-0,
33 NJ). Then, the wound was closed with a non-absorbable polypropylene suture (Ethicon 4-0,
34 NJ). All the animal experiments were performed according to the Institutional Guidance of
35 Kyoto University on Animal Experimentation and under permission by animal experiment
36 committee of Institute for frontier Medical Science, Kyoto University. The hydrogel samples
37 used for the defect experiment are gelatin hydrogels incorporating PBS, 5 μ g of SDF-1, 5 μ g
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1 of SDF-1 and 3 μg of BMP-2, and 3 μg of BMP-2. Each rat received the implantation of 2
2 hydrogels randomly in both left and right of forearm's ulna. Each experiment was
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4 independently performed for 6 samples.
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7 8 9 10 *2.8 Evaluation of in vivo ectopic bone formation at back subcutis*

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12 Ectopic bone formation by gelatin hydrogels incorporating SDF-1 and/or BMP-2 was
13 assessed for 12-week-old male Wistar rats ($n = 12$) under standard sterile conditions. Briefly,
14 the rats were anesthetized, shaved the hair, and disinfected with 70 vol% ethanol. A vertical
15 incision was created down the midline of the back. The hydrogels ($1 \times 6 \times 6 \text{ mm}^3$) were
16 inserted subcutaneously away from the incision. The wound was sutured and disinfected with
17 betadin. The 4 types of hydrogels similar to the bone defect model were randomly implanted.
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19 To evaluate gene expression by the RT-PCR assay, the hydrogels implanted were taken out
20 with a surgical scalpel. The total RNA was extracted by using RNeasy fibrous tissue mini kit
21 (Qiagen, Valencia, CA) according to the manufacturer's instructions. The RT-PCR assay was
22 performed as described previously to evaluate the expression level of *Cxcr4* and *Runx2* genes
23 of cells in the hydrogels implanted. Expression level of *Cxcr4* gene was assessed 3 days after
24 implantation, and that of *Runx2* and *Osteocalcin* genes of osteogenic marker was done 2 and
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26 4 weeks after implantation.
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46 *2.9 Radiological and histological examinations of bone tissue regenerated*

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48 The bone tissue regenerated at the defect and subcutaneous site, 4 weeks later, were
49 radiologically examined by the soft x-ray machine (Hitex-100, Hitachi Ltd., Tokyo, Japan) at
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51 56 kV and 2.5 mA for 20 sec. Then, the samples were fixed with 3 wt% paraformaldehyde in
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53 PBS at room temperature for 48 hr, decalcified with PBS containing 9 wt% ethylenediamine
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55 tetraaceticacid disodium salt and 10 wt% ethylenediamine tetraaceticacid tetrasodium salt
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1 (EDTA) solution at room temperature for 7 days. The EDTA solution was changed every
2 other day. After decalcification, the samples were equilibrated in PBS containing 15 wt%
3 sucrose for 24 hr, and then in PBS containing 30 wt% sucrose for further 24 hr, embedded in
4 Tissue-Tek OCT Compound (Sakura Finetek Inc., Tokyo, Japan), and frozen on liquid
5 nitrogen. For the histological examination, the 6 μm -thick sections were cut at the center of
6 samples, followed by staining with hematoxylin and eosin (H&E) and Masson trichrome to
7 observe the cell infiltration and collagen newly formed. The images were taken under a
8 microscope (AX80 Provis, Olympus Ltd., Tokyo, Japan) and the area percentage of matured
9 collagen to collagen newly formed was measured by Image J software (Version 1.41, Java™,
10 USA) from each image (20X magnification) randomly selected. Each measurement was
11 performed for 6 images.
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29 *2.10 Micro-computed tomography (μCT) and peripheral quantitative computed tomography* 30 *(pQCT) scan examinations of bone regenerated* 31 32 33

34 Three-dimensional images of bone regenerated in the ulna defects were visualized
35 with the CT scans (X-RAY CT System, SMX-100CT-SV3 TYPE, Shimadzu Ltd., Kyoto,
36 Japan). Samples were scanned over a fixed length of bone with a 20 mm sample holder at a
37 resolution of 20 μm , energy of 30 kV, current of 25 μA , and exposure time of 300 ms. The 2-
38 dimensional images were reconstructed and submitted to the VGStudio MAX 1.2 software
39 (Volume Graphics GmbH, Heidelberg, Germany) for processing to produce the 3-
40 dimensional images of bone regenerated. The bone mineral density (BMD) of whole cortical
41 compartment of bone tissue regenerated was analyzed by using a highly accurate multi-slice
42 pQCT (XCT Research SA+, Stratec Medizintechnik, GmbH, Pforzheim, Germany) at a
43 resolution of 0.08 mm voxel size and a threshold value of 267 mg/cm^3 with a scanning protocol
44 of 3 slices with a thickness of 460 μm and a slice interval of 1 mm, at a defined distance.
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1 Analysis of the regional cortical BMD was carried out for each of 3 tomographic slices at a
2 square region of interest (ROI) generated.
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7 *2.11 Evaluation of bone marrow-derived cells recruitment to gelatin hydrogels with GFP-*
8 *positive Chimeric model*
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11 C57BL/6 transgenic mice that ubiquitously express enhanced green fluorescent
12 protein (GFP) under the Cytomegavirus (CMV) early enhancer/chicken β actin (CAG)
13 promoter were provided by the RIKEN BioResource Center (Tsukuba, Japan) through the
14 National Bio-Resource Project of the Ministry of Education, Culture, Sports, Science and
15 Technology (MEXT, Japan). *Chimeric* mice were prepared according to the procedure
16 reported previously [9,17]. Briefly, 8- to 10-week-old female C57BL/6 mice were irradiated
17 lethally with 10 Gy of gamma-ray. For the total bone marrow transplantation (BMT), 5×10^6
18 of bone marrow cells prepared from 8- to 10-week-old male GFP transgenic mice was
19 intravenously administered to recipient irradiated mice [30]. After the transplantation, the
20 mice were bred for 5 months to complete the replacement of bone marrow-derived cells to
21 GFP-positive cells. The replacement ratio of GFP-positive-bone marrow-derived cells was
22 $98.1 \pm 2.8 \%$ when evaluated by the fluorescence-associated cell sorter method (FACS
23 Calibur, BD Bioscience Ltd., Franklin Lakes, NJ).
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44 Various gelatin hydrogels were subcutaneously implanted into the back of *Chimeric*
45 mice in the similar procedure mentioned above. The hydrogels including the surrounding
46 tissue were extracted 2 and 4 weeks post-operatively, and the fluorescent images were taken
47 by a digital microscope (AxioCam MRc 5, Carl Zeiss Inc., Jena, Germany). For
48 immunohistochemistry staining, the hydrogel samples were fixed with 3 wt%
49 paraformaldehyde in PBS at 4 °C for 24 hr, equilibrated in PBS containing 15 wt% sucrose at
50 4 °C for 24 hr, and then in PBS containing 30 wt% sucrose at 4 °C for further 24 hr. The
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1 samples were embedded and sectioned (8 μm thickness) as described above. To stain
2 osteocalcin-positive cells, the sections were washed with PBS, blocked with a normal goat
3 serum for 1 hr at room temperature before incubation with a rabbit polyclonal anti-mouse
4 osteocalcin antibody (1:250; Takara Bio Inc., Shiga, Japan) for 1 hr at room temperature.
5 Then, the sections were stained with a Alexa Fluor 546-conjugated goat anti-rabbit IgG (8
6 $\mu\text{g}/\text{ml}$, Invitrogen Corporation, Ltd., CA) for 45 min at room temperature and cell nuclei
7 were finally stained with Hoechst 33258 (Nacalai Tesque Inc., Kyoto, Japan). After washing,
8 the sections were mounted with Vectashield® (Vector Laboratories, Burlingame, CA). The
9 fluorescence images were taken on a fluorescent microscope (Apotome, Imager.Z1, Carl
10 Zeiss, Jena, Germany).

24 For the immunohistochemical staining with CD31 and CD34 of endothelial marker,
25 the sections were incubated with a fluorescein isothiocyanate (FITC) anti-mouse CD31
26 (1:100, BioLegend Inc., CA) or a FITC anti-mouse CD34 (1:100, BD Biosciences
27 Pharmingen Inc., CA) for 1 hr at room temperature. After washing, the sections were stained
28 with a Simple Stain Mouse MAX-PO (Histofine®, Nichirei Biosciences Inc., Tokyo, Japan)
29 for 45 min at room temperature. For bright-field microscopy, bound primary antibodies were
30 detected using DAKO EnVision- Horseradish Peroxidase and 3, 3'-diaminobenzidine (DAB)
31 substrate kit (Vector Laboratories, Burlingame, CA), and counter-stained with Hematoxylin
32 to visualize the cell nuclei. Then, the sections were mounted with an aqueous mounting
33 medium (MGK-S, Matsunami Glass Ind. Ltd., Osaka, Japan) and the images were taken on a
34 microscope (AX80 Provis, Olympus Ltd., Tokyo, Japan).

2.12 Evaluation of the fraction type of non-hematopoietic stem cells in gelatin hydrogels implanted by flow cytometry

The fraction type of non-hematopoietic stem cells in gelatin hydrogels incorporating SDF-1 and/or BMP-2 implanted was analyzed by flow cytometry after staining with 3 monoclonal antibodies including anti-CD29, anti-CD44, and anti-CD45 (BioLegend Inc., CA) [31]. Similarly to the *in vivo* experiments described above, various gelatin hydrogels were subcutaneously implanted into the back of 6-week-old female C57BL/6 mice. The hydrogels were taken out 4 weeks post-operatively, minced into small pieces, and incubated in collagenase solution (400 units/ml) at 37 °C for 40 min for tissue digestion. The tissues remained were separated from cells by using cell strainer (70 µm, BD Falcon™, BD Biosciences Inc., MA) and the cells were collected by centrifugation at 2,000 rpm, 4 °C, and 5 min (Microcentrifuge 5417R, Eppendorf, Hamburg, Germany). Then, the cells were washed with PBS, blocked with 2.4G2 (anti-FcRII/III) antibody on ice for 15 min, and stained with monoclonal antibodies in PBS containing 2 vol% FBS and 0.1 vol% sodium azide on ice for 15 min. Propidium iodide was used to distinguish dead cells from viable cells. The cells immunostained were analyzed on FACSCanto II flow cytometer (BD Biosciences Inc., MA). Analysis was performed by BD FACSDiva software (BD Bioscience Inc., MA) and FLOWJO software (Tree Star, San Carlos, CA). Each analysis was performed for 3 samples.

2.13 Statistical analysis

All the results were statistically analyzed by the unpaired student's t test and $p < 0.05$ was considered to be statistically significant. Data were expressed as the mean \pm the standard deviation.

3. Results

3.1 Gene expression of MSC cultured with SDF-1 and/or BMP-2

Figures 1A and B show the expression level of *Cxcr4* and *Runx2* genes for MSC cultured with SDF-1 and BMP-2 at different mixing ratios. Irrespective of the mixing ratio, combined SDF-1 and BMP-2 or BMP-2 alone significantly up-regulated the *Cxcr4* and *Runx2* expression. On the other hand, the expression level of SDF-1-incubated cells was similar to that of non-treated MSC. Figure 1C shows the ALP activity of MSC cultured with SDF-1 and/or BMP-2. The effect of SDF-1 and BMP-2 on the ALP activity was similar to that of gene expressions, though MSC cultured with SDF-1 showed a significant increase in the ALP activity compared with the non-treated MSC.

Figures 2A and B show the expression level of *Cxcr4* and *Runx2* genes for MSC cultured in the gelatin hydrogels incorporating SDF-1 and/or BMP-2. MSC cultured in the gelatin hydrogels incorporating combined SDF-1 and BMP-2 or BMP-2 showed significantly up-regulated expression of both *Cxcr4* and *Runx2* genes. A significant higher level of *Runx2* expression of MSC cultured in the hydrogels incorporating combined SDF-1 and BMP-2 than that of the hydrogels incorporating BMP-2 was observed.

3.2 Controlled release of SDF-1 and BMP-2 from gelatin hydrogels and hydrogel degradation

Figure 3A shows the time profiles of SDF-1 and BMP-2 released from gelatin hydrogels. An initial burst in SDF-1 release was seen for the first day, and 70 and 80 % of radioactivity were released out from the hydrogels incorporating SDF-1 and combined SDF-1 and BMP-2, respectively. Within the first 3 days, the SDF-1 release was significantly accelerated by the combination of BMP-2. However, this effect became less after 7 days of

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release test. On the contrary, irrespective of the SDF-1 combination, BMP-2 showed less burst release than SDF-1 and the radioactivity remaining were around 55 and 45 % for the hydrogels incorporating BMP-2 and combined SDF-1 and BMP-2, respectively. At 14 days, about 10-15 % of SDF-1 was remained in the hydrogels, while the percent remaining of BMP-2 was approximately 22 %. After 28 days, the radioactivity of SDF-1 and BMP-2 remained in every hydrogel was around 5-10%. On the other hand, gelatin hydrogels were gradually degraded over 28 days. Figure 3B shows the relationship of remaining radioactivity between SDF-1 or BMP-2 incorporated in gelatin hydrogels and the hydrogels of release carriers. Irrespective of the type of growth factors, a linear relationship of remaining amount between SDF-1 or BMP-2 and the hydrogels was observed. The time profiles of SDF-1 and BMP-2 release were correlated well with the degradation of the hydrogels ($R^2 \approx 0.93$).

3.3 Bone regeneration in an ulna critical-sized defect

Figure 4 shows the soft x-ray and histological images of bone regenerated. New bone tissues were regenerated in the defects implanted with the hydrogels incorporating combined SDF-1 and BMP-2 or BMP-2, although the extent of the former was greater than that of the latter. On the contrary, bone regeneration was not observed in the defects implanted with the hydrogels incorporating SDF-1 or PBS. Similarly to soft x-ray images, histological sections showed abundant collagen formation for the hydrogels incorporating SDF-1 and BMP-2 or BMP-2. On the contrary, a slight formation of collagen was observed in the defect implanted with the hydrogels incorporating SDF-1 or PBS.

Figures 5A and B show the μ CT images of bone regenerated in the defects implanted with the hydrogels incorporating combined SDF-1 and BMP-2 or BMP-2. For both groups, the bone union was formed. A new bone was regenerated from the edge toward the center of the defects while a low bone density was observed around the center of the defects. Figure 5C

1 shows the pQCT analysis data. A significantly higher BMD was found in the bone
2 regenerated by the hydrogels incorporating combined SDF-1 and BMP-2 than that of the
3 hydrogels incorporating BMP-2.
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9 3.4 Gene expression of cells in the hydrogels incorporating SDF-1 and/or BMP-2 implanted

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12 Figure 6A shows the expression level of *Cxcr4* gene of cells in the hydrogels
13 subcutaneously implanted. The presence of SDF-1 and BMP-2 enhanced the level of *Cxcr4*
14 gene compared with the hydrogels incorporating PBS. Figures 6B and C show the expression
15 level of *Runx2* and *Osteocalcin* genes (osteogenic genes) of cells in the hydrogels implanted.
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17 Similarly to the results of *Cxcr4* expression, a significantly up-regulated *Runx2* expression
18 was found in every hydrogel group 2 and 4 weeks after implantation. Implantation of
19 hydrogels incorporating combined SDF-1 and BMP-2 resulted in a significantly up-regulated
20 *Runx2* gene than that of the hydrogels incorporating PBS or BMP-2 4 weeks later.
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22 Apparently, the *Osteocalcin* expression of cells in the hydrogels incorporating combined
23 SDF-1 and BMP-2 implanted was significantly higher than that of every hydrogel group both
24 the 2 (50-fold increase) and 4 weeks (1,000-fold increase) after implantation.
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41 3.5 Ectopic bone tissue formation

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44 Figure 7 shows the soft x-ray and histological images of bone tissue formation at the
45 subcutaneous site. The ectopic formation of bone tissues was observed for hydrogels
46 incorporating combined SDF-1 and BMP-2 or BMP-2. The combined SDF-1 and BMP-2
47 incorporated in the hydrogels generated a larger area of bone tissue formation than the BMP-
48 2-incorporated hydrogels. In contrast, only a slight amount of calcification (white area) was
49 observed in the hydrogels incorporating SDF-1 or PBS. Comparing with the corresponding
50 soft x-ray images, histological sections showed that the hydrogels incorporating combined
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1 SDF-1 and BMP-2 induced the larger formation of matured bone tissue (stained as dark pink
2 and blue), followed by the BMP-2 group. On the other hand, a low density of collagen was
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4 observed in both the hydrogels incorporating SDF-1 and PBS. Figure 7E shows the area
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6 percentage of matured collagen to collagen newly formed. The percentage of matured
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8 collagen was significantly higher for the hydrogels incorporating SDF-1 and BMP-2 than that
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10 of hydrogels incorporating BMP-2.
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14 *3.6 Cell recruitment to the tissue around the site of hydrogels incorporating SDF-1 and/or* 15 16 17 *BMP-2 implanted* 18 19 20

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22 Figure 8 shows immunohistochemical staining images of cells recruited into the
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24 tissue around the site of hydrogels implanted. At 2 weeks, more GFP-positive-bone marrow-
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26 derived cells were recruited and accumulated around the interface of hydrogels incorporating
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28 SDF-1, BMP-2, and combined SDF-1 and BMP-2, in contrast to that of hydrogels
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30 incorporating PBS. Figure 8E shows the percentage of GFP-positive cells to total cells
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32 recruited into the tissue around hydrogels implanted. Only the hydrogels incorporating
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34 combined SDF-1 and BMP-2 showed significantly higher percentage of GFP-positive cells to
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36 the total cells, when compared with that of the hydrogels incorporating PBS. At 4 weeks, in
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38 addition to the GFP-positive cells, osteocalcin-positive cells were also found inside and
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40 around the hydrogels incorporating combined SDF-1 and BMP-2 or BMP-2 implanted. A
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42 good merge of GFP- and osteocalcin-positive cells localization (yellow) was detected for
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44 both the hydrogel groups. However, the higher density of osteocalcin-positive cells was
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46 found in the hydrogels incorporating combined SDF-1 and BMP-2 than that of the hydrogels
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48 incorporating BMP-2.
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56 Figure 9 shows the immunohistochemistry staining images of CD31- and CD34-
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58 positive cells recruited into the tissue around hydrogels implanted. Around the hydrogels
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1 incorporating combined SDF-1 and BMP-2 or BMP-2, CD31- and CD34-positive cells were
2 accumulated. In addition, a vascular-like structure was observed for both the hydrogel
3 groups, particularly around the hydrogels.
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10 *3.7 The fraction type of non-hematopoietic stem cells recruited in gelatin hydrogels*
11 *incorporating SDF-1 and/or BMP-2 implanted*
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14 Figure 10A shows the flow cytometric profiles and histograms of cells detected in
15 gelatin hydrogels 4 weeks after implantation. The high density of CD45-positive cells were
16 found in every hydrogel implanted whereas the CD45-negative and CD44-positive cells were
17 remarkably seen only in the hydrogels incorporating combined SDF-1 and BMP-2 (square
18 gates). In addition, in the fraction of CD45-negative and CD44-positive cells, a number of
19 CD29-positive cells were obviously detected in the hydrogels incorporating combined SDF-1
20 and BMP-2. Figures 10B - D show the number and percentage of targeted cells analyzed
21 from the profiles and histograms above. The similar trend was found for the total number of
22 live cells, the percentage of CD45-negative and CD44-positive cells to total live cells, and the
23 percentage of CD45-negative, CD44-positive, and CD29-positive cells to total live cells.
24 Gelatin hydrogels incorporating combined SDF-1 and BMP-2 tended to increase the number
25 of live cells, and showed significantly enhanced fraction number of CD45-negative, CD44-
26 positive, and CD29-positive cells, compared with those incorporating PBS, SDF-1, and
27 BMP-2, respectively. The largest number percentage of CD45-negative and CD44-positive
28 cells to total live cells was observed for the hydrogels incorporating combined SDF-1 and
29 BMP-2 among other groups.
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4. Discussion

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5 Natural bone regeneration is a complex process in which hematopoietic and bone
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7 progenitor cells are regulated by a number of environmental stimuli to coordinately function
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9 for osteogenesis. In this study, the system of gelatin hydrogels for combined release of SDF-1
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11 and BMP-2 synergistically enhanced bone regeneration. SDF-1 has an inherent ability to
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13 enhance the recruitment of hematopoietic cells [14-17] and BMP-2 has a potential to promote
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15 the osteogenic differentiation of bone progenitor cells [20,21] and to recruit the cells [8,9]. If
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17 the release of two protein factors works well, it is theoretically possible that the recruitment
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19 of hematopoietic and bone progenitor cells will be promoted. The former cells will generate
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21 new blood vessels while the latter cells will be differentiated by BMP-2 action, resulting an
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23 enhanced bone regeneration. The present study experimentally confirmed that the story works
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25 well through the combined release of SDF-1 and BMP-2 with the gelatin hydrogels.
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31 Our previous researches have demonstrated that the hydrogels prepared from gelatin
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33 with IEP 9.0 could release SDF-1 or BMP-2 in a controlled fashion [9,18]. Therefore, in this
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35 study, the same gelatin hydrogels were selected for the combined release of SDF-1 and BMP-
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37 2. The *in vivo* release test revealed that the initial release of SDF-1 was accelerated by the
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39 BMP-2 combination, but the BMP-2 release was not influenced by the SDF-1 combination.
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41 This phenomenon is explained by the viewpoint of interaction forces between the molecules
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43 to be released and the material of release carrier. The interaction between the BMP-2 and
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45 gelatin is stronger than that of SDF-1 and gelatin. The BMP-2 combination would weaken the
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47 interaction force of SDF-1. In the other words, the co-presence of BMP-2 in the hydrogels
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49 decreases the site of gelatin to interact with SDF-1, resulting in a large amount of initial
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51 diffusional SDF-1 release. Several studies have reported on difference in the release behavior
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53 between a single and combined molecules [32,33]. It is found that the release of
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1 indomethacin from a heparin-conjugated polymeric micelle was suppressed by the
2 combination of basic fibroblast growth factor (bFGF) whereas the indomethacin combination
3 did not affect the profile of bFGF release [32]. This result was also explained in terms of the
4 interaction force. In addition, after 14 days of implantation, the remaining amount of BMP-2
5 in the hydrogels was more than that of the SDF-1, implying a more controlled manner of
6 BMP-2 than SDF-1, which corresponded to the results reported previously [9,18]. When the
7 release profile of SDF-1 or BMP-2 was plotted against that of gelatin hydrogel degradation, a
8 good correlation between the two profiles was observed. It is conceivable that the
9 mechanisms of SDF-1 and BMP-2 release from the gelatin hydrogels were in the diffusion or
10 carrier degradation manners. SDF-1 and BMP-2 would be initially burst released from the
11 hydrogels by the diffusion mechanism. After that, they were released by the degradation of
12 gelatin hydrogels. The carriers with an optimal degradation profile will contribute to the
13 optimal sustained release of growth factors incorporated to achieve growth factor-induced
14 bone regeneration. In addition to the profile of growth factors release, bone tissue formation
15 is also influenced by the degradation profile of carrier itself [34]. Slow-degraded carriers
16 physically hindered the process of bone formation and biochemically impaired the matrix
17 deposition, resulted in minimal bone formation, while the extent of bone formation could be
18 augmented by the carriers with an appropriate degradability [35]. Based on the finding in this
19 study, the gelatin hydrogels with *in vivo* biodegradation for 4-5 weeks was selected for bone
20 regeneration [9].

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To investigate the biological effects of SDF-1 and BMP-2 for MSC, the gene
expression was evaluated. No influence of SDF-1/BMP-2 mixing ratio on the level of *Cxcr4*
and *Runx2* gene expression and the ALP activity was observed (Figure 1). Any combination
of SDF-1 and BMP-2 or BMP-2 up-regulated both the *Cxcr4* and *Runx2* expression. This
corresponds the finding that BMP-2 played a role in migration (via the activation of *Cxcr4*

1 pathway) and osteogenic differentiation (via the activation of *Runx2* pathway) of MSC *in*
2 *vitro* [36]. SDF-1 is one ligand of *Cxcr4* on the cell surface, so it does not change the level of
3 *Cxcr4* expression [37]. Our previous researches reported that a single delivery of 5 μ g of
4 SDF-1 and 3 μ g of BMP-2 was effective in enhancing *in vivo* angiogenesis and bone
5 regeneration, respectively [9,18]. Thus, in our combined SDF-1/BMP-2 system, it is highly
6 possible to consider that the mixing ratio of SDF-1/BMP-2 affects the biological activity.
7 However, a significant difference among the mixing ratios was not found in this study. Based
8 on the previous findings, the 500/300 mixing ratio of SDF-1/BMP-2 was then selected for the
9 hydrogel incorporation system. For MSC cultured in the hydrogels incorporating combined
10 SDF-1 and BMP-2, the cell response was quite different from that of the factors without the
11 hydrogel incorporating. A significantly high up-regulation of *Runx2* gene was observed
12 compared with the hydrogels incorporating BMP-2. It may be explained that the gelatin
13 hydrogels was effective in prolonging the half-life of SDF-1 and BMP-2 and the
14 consequently enhanced their biological activities for cells [38].

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34 Gelatin hydrogels incorporating combined SDF-1 and BMP-2 revealed a significant
35 great potential to induce bone regeneration at both the bone defect and subcutaneous site. The
36 level of *Osteocalcin* gene expression of cells in the hydrogels implanted was substantially up-
37 regulated (> 1,000-fold) (Figure 6E). Osteocalcin-positive cells were accumulated around the
38 hydrogels implanted (Figure 8C). In addition, the fraction of non-hematopoietic cells (CD45-
39 negative, CD44-positive, and CD29-positive) which may contain mesenchymal stem cells,
40 was apparently detected (Figure 10). Taken together, it is likely that the combined release of
41 SDF-1 and BMP-2 enhanced the number of cells recruited and their osteogenic differentiation
42 thereat. Consequently, the biological event would result in enhanced bone regeneration. The
43 molecular mechanism regulating the osteogenic differentiation and bone regeneration by the
44 combined SDF-1 and BMP-2 delivery system is not clear at present. However, there are some
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1 reasons to support this synergistic phenomenon. The first possible reason is the enhancement
2 of cell recruitment by both the SDF-1 and BMP-2 through SDF-1/CXCR4 or other signals
3 [12-14,39]. It is well recognized that SDF-1 plays a critical role in the recruitment of bone
4 marrow-derived cells including hematopoietic and mesenchymal stem cells, together with
5 inflammatory cells to the site of SDF-1 present [10-16]. The recruited cells would secrete a
6 number of cytokines and growth factors which are necessary to proceed the natural process of
7 tissue regeneration and healing [40]. BMP-2 is also reported to have a property to induce the
8 recruitment of many types of cells [39,41]. It has been demonstrated that BMP-2 released
9 from the gelatin hydrogels could recruit bone marrow-derived cells via the activation of
10 placental growth factor (PIGF) pathway [9]. The enhanced expression of PIGF consequently
11 promoted the recruitment of progenitor cells from the bone marrow [42-44]. In this study, it
12 is experimentally confirmed on the chemotactic effect of combined SDF-1 and BMP-2
13 system on the GFP-positive-bone marrow-derived stem cells in the *Chimeric* mouse model.
14 In addition, the higher number of live cells and non-hematopoietic stem cells was detected in
15 the hydrogels incorporating combined SDF-1 and BMP-2 implanted, compared with that of
16 other hydrogels (Figures 10B - D). Taken together, the enhanced recruitment of bone
17 marrow-derived mesenchymal stem cells together with the other types of cells by the
18 combined SDF-1 and BMP-2 system would be one of the key factors contributing to the
19 accelerated bone regeneration.
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46 The second reason would be the enhanced angiogenesis as a result of the enhanced
47 recruitment of hematopoietic stem cells, which may be committed to endothelial cells. It is
48 well known that the local presence of blood vessels is necessary for wound healing and bone
49 regeneration [45]. Many researchers have demonstrated that angiogenesis was enhanced by
50 vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) family, and other
51 growth factors or chemokines [46,47], while SDF-1 is reported to be induced by the
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1 angiogenic growth factors [48,49]. Schantz *et al.* [50] examined the homing of mesenchymal
2 stem cells by SDF-1 treatment with an infusion pump *in vivo*. It was shown that the
3 combination of VEGF, SDF-1, and BMP-6 was effective in inducing vascularization in a
4 poly(caprolactone) scaffold. In addition, a recent research has reported on the role of SDF-1
5 in the modulating inflammation response by the participation of the recruited stem cells,
6 providing a suitable environment for the materials implanted and subsequent guiding an
7 angiogenesis and wound healing [51]. We have demonstrated that the gelatin hydrogels
8 enabled SDF-1 to locally release, resulting in an enhanced vascularization at the site
9 implanted [18]. In this study, an enhanced number of CD31- and CD34-positive cells
10 accumulated around gelatin hydrogels incorporating SDF-1, in remarked contrast to the
11 hydrogels incorporating PBS. The combined SDF-1 and BMP-2 or BMP-2 release strongly
12 increased the accumulation of positive cells. It is reported that BMP-2 also exhibits an
13 angiogenic nature [52-54]. Several researches claim that BMP-2 promoted angiogenesis
14 through the stimulation of Smad 1/5, Erk 1/2, and Id expression [55,56]. The Id family of
15 transcription factors to regulate the neovascularization has been identified as one of the main
16 BMP-2 signaling pathways [57]. In addition, BMP-2 and BMP-4 induced the production of
17 VEGF by different cells, contributing to the angiogenic response [57,58]. Furthermore, BMP-
18 2 might enhance angiogenesis through the chemotaxis of monocytes, which can secrete
19 cytokines that promote the formation of blood vessels [59]. Considering the angiogenic
20 property of SDF-1 and BMP-2 reported, it may be reasonable to show the highest expression
21 of endothelial markers around the tissue of hydrogels incorporating SDF-1 and BMP-2
22 implanted because of the combined angiogenic effect of SDF-1 and BMP-2.
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53 Third possible mechanism is the activation of osteogenic differentiation through the
54 co-requirement of the SDF-1 and BMP-2 signaling axis. Zhu *et al.* [23] have demonstrated
55 that a SDF-1 signaling is required in *in vitro* osteogenic differentiation induced by BMP-2 for
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1 C2C12 and ST2 cells. The blocking of SDF-1 signaling before BMP-2 stimulation strongly
2 suppressed the BMP-2-induced osteogenic differentiation. They revealed that the interaction
3 between SDF-1 and BMP-2 signaling was mediated via the intracellular Smads and MAPK
4 activation. In addition, it was shown that C2C12 cells treated with SDF-1 before BMP-2
5 stimulation significantly enhanced osteocalcin synthesis. Hosogane *et al.* [24] have also
6 reported a similar regulatory role of SDF-1 in BMP-2-induced osteogenic differentiation for
7 human and mouse MSC. It is suggested that the SDF-1 effect was mediated via the
8 intracellular Smad and Erk activation. The present study clearly indicated that the enhanced
9 osteogenic differentiation of combined SDF-1 and BMP-2 was due to the highly up-regulated
10 osteogenic gene expression, particularly *Osteocalcin* gene, of cells in the hydrogels
11 subcutaneously implanted. Additionally, in the *Chimeric* mouse model, a number of
12 osteocalcin-positive cells were found in the hydrogels incorporating combined SDF-1 and
13 BMP-2. Furthermore, the same localization of GFP- and osteocalcin-positive cells was
14 observed. This indicated that the combined SDF-1 and BMP-2 released by the hydrogels
15 promoted the recruitment of bone marrow-derived mesenchymal stem cells and the
16 subsequent enhancement of their osteogenic differentiation. In addition, the combined SDF-1
17 and BMP-2 showed the synergistic effect to induce the presence of high expressing CD44
18 and CD29 non-hematopoietic cells including the fraction of mesenchymal stem cells [31]. It
19 is thus conceivable that the co-requirement of SDF-1 and BMP-2 for osteogenic
20 differentiation may be a significant factor to achieve bone regeneration.

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Based on three mechanisms proposed, the synergistic effect of combined SDF-1 and
BMP-2 release on the bone regeneration may be explained. This finding provides a novel
controlled release system that simultaneously stimulates bone regeneration through the
mechanism of cell recruitment, angiogenesis, and osteogenic differentiation of body-originating
cells. However, it should be noted that the extent of bone formation can be optimized and

1 augmented by modifying the release kinetics of multiple growth factors. For example, if
2 multiple growth factors can release in a controlled and sequential manner, the release
3 technology will be able to imitate the process of natural bone healing.
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9 **5. Conclusions**

10 The present study demonstrated that the combined release of SDF-1 and BMP-2 from
11 gelatin hydrogels induced bone regeneration at both the critical-sized bone defect and
12 subcutaneous site to a significantly great extent compared with a single release of SDF-1 or
13 BMP-2. The synergistic effect of combined SDF-1 and BMP-2 release on the bone
14 regeneration was explained through the mechanism of cell recruitment, angiogenesis, and
15 osteogenic differentiation of body-originating cells.
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Figure 1. Gene expression of (A) *Cxcr4* and (B) *Runx2* and (C) ALP activity of MSC cultured with SDF-1, BMP-2, and the combination at different mixing ratios for 24 hr. The gene expression is normalized with the level of non-treated MSC. * $p < 0.05$, significant against the value of non-treated MSC.

Figure 2. Gene expression of (A) *Cxcr4* and (B) *Runx2* of MSC cultured in the gelatin hydrogels incorporating PBS, SDF-1, BMP-2, and the combination for 3 days. The gene expression is normalized with the level of MSC cultured in the hydrogels incorporating PBS. * $p < 0.05$, significant against the value of gelatin hydrogels incorporating PBS, † $p < 0.05$, significant against the value of gelatin hydrogels incorporating BMP-2.

Figure 3. (A) *In vivo* release profiles of SDF-1 and BMP-2 from gelatin hydrogels incorporating ^{125}I -labeled SDF-1 and/or ^{125}I -labeled BMP-2 after implantation into the back subcutis of C57BL/6 mice. the hydrogels incorporating 5 μg of ^{125}I -labeled SDF-1 (Δ), 5 μg of ^{125}I -labeled SDF-1 and 3 μg of non-labeled BMP-2 (\blacktriangle), 5 μg of non-labeled SDF-1 and 3 μg of ^{125}I -labeled BMP-2 (\blacksquare), and 3 μg of ^{125}I -labeled BMP-2 (\square) or ^{125}I -labeled gelatin hydrogels (\bullet). * $p < 0.05$, significant against the value of gelatin hydrogels incorporating ^{125}I -labeled SDF-1. (B) Relationship of remaining radioactivity between ^{125}I -labeled SDF-1 or ^{125}I -labeled BMP-2 incorporated in gelatin hydrogels and ^{125}I -labeled hydrogels of release carriers.

Figure 4. Soft X-ray and histological (H&E and Masson Trichrome staining) images of bone regenerated in the ulna critical-sized defects of Wistar rats implanted with the gelatin hydrogels incorporating (A) PBS, (B) 5 μg of SDF-1, (C) 5 μg of SDF-1 and 3 μg of BMP-2, and (D) 3 μg of BMP-2 4 weeks post-operatively. (C: collagen, M: muscle, scale bar = 200 μm).

Figure 5. Three-dimensional CT images of bone regenerated in the ulna critical-sized defects of Wistar rats implanted with gelatin hydrogels incorporating (A) 5 μg of SDF-1 and 3 μg of BMP-2 and (B) 3 μg of BMP-2 4 weeks post-operatively. (C) Bone mineral density of bone regenerated 4 weeks post-operatively, analyzed by pQCT. * $p < 0.05$, significant against the value of gelatin hydrogels incorporating BMP-2.

Figure 6. Gene expression of (A) *Cxcr4*, (B, C) *Runx2*, and (D, E) *Osteocalcin* of cells in the hydrogels incorporating PBS, 5 μg of SDF-1, 5 μg of SDF-1 and 3 μg of BMP-2, and 3 μg of BMP-2 3 days (A), 2 (B, D) and 4 weeks (C, E) after implantation into the back subcutis of Wistar rats. The gene expression is normalized with the level of sham group. * $p < 0.05$, significant against the value of sham group. † $p < 0.05$, significant against the value of gelatin hydrogels incorporating PBS. ‡ $p < 0.05$, significant against the value of gelatin hydrogels incorporating BMP-2.

Figure 7. Soft X-ray and histological (H&E and Masson Trichrome staining) images of bone tissue formation at the back subcutis of Wistar rat implanted with gelatin hydrogels incorporating (A) PBS, (B) 5 μ g of SDF-1, (C) 5 μ g of SDF-1 and 3 μ g of BMP-2, and (D) 3 μ g of BMP-2 4 weeks post-operatively. (C: collagen, MC: matured collagen, scale bar = 200 μ m). (E) Percentage of matured collagen to collagen newly formed. * $p < 0.05$, significant against the value of gelatin hydrogels incorporating BMP-2.

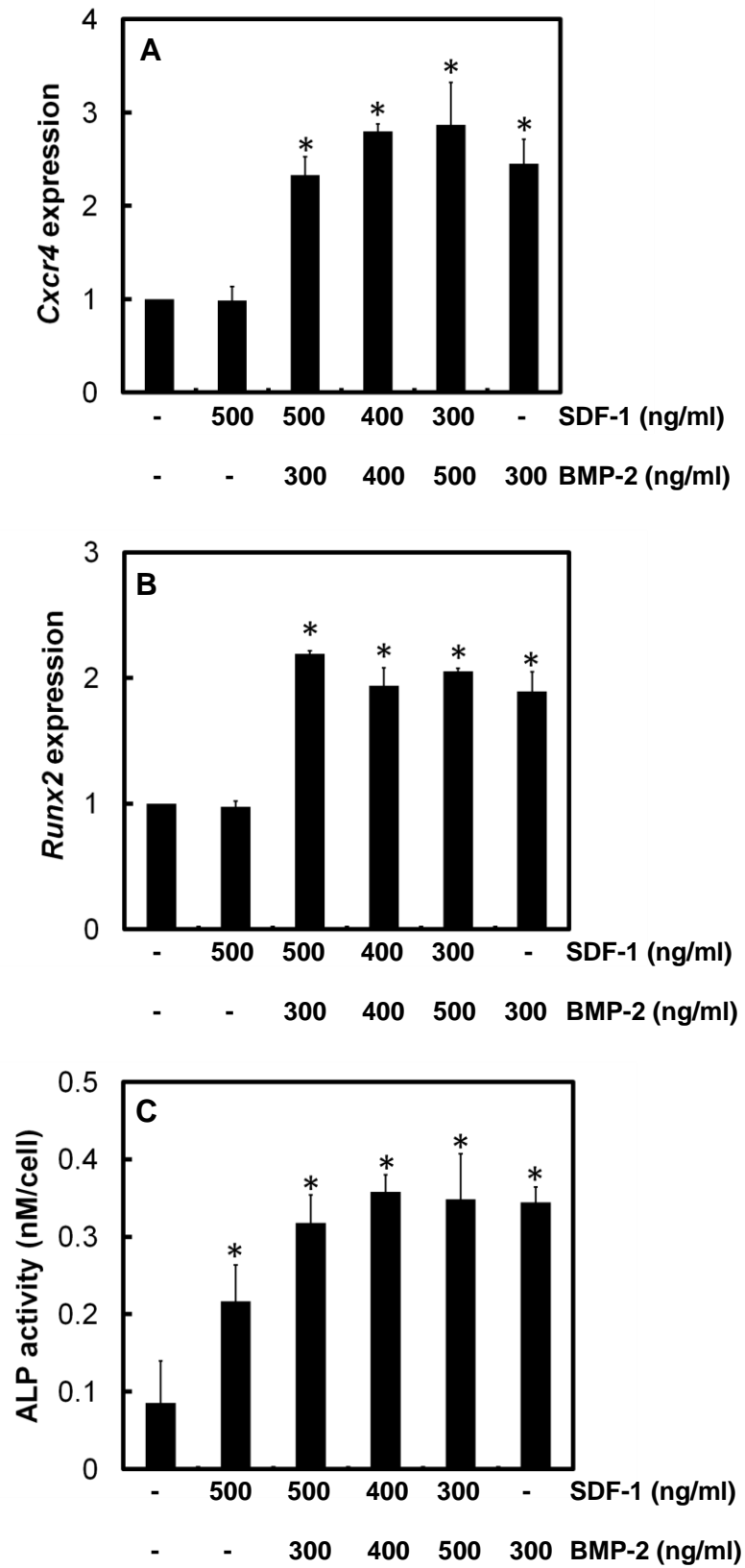
Figure 8. Immunohistochemical staining images of cells recruited into the tissue around the site implanted with gelatin hydrogels incorporating (A) PBS, (B) 5 μ g of SDF-1, (C) 5 μ g of SDF-1 and 3 μ g of BMP-2, and (D) 3 μ g of BMP-2 implanted into the back subcutis of GFP-positive *Chimeric* mice. The images were taken 2 (left and middle) and 4 weeks after implantation (right) (GFP-positive cells: green, osteocalcin-positive cells: red, scale bar = 200 μ m). (E) Percentage of GFP-positive cells to total cells recruited into the tissue around hydrogels implanted. * $p < 0.05$, significant against the value of gelatin hydrogels incorporating PBS.

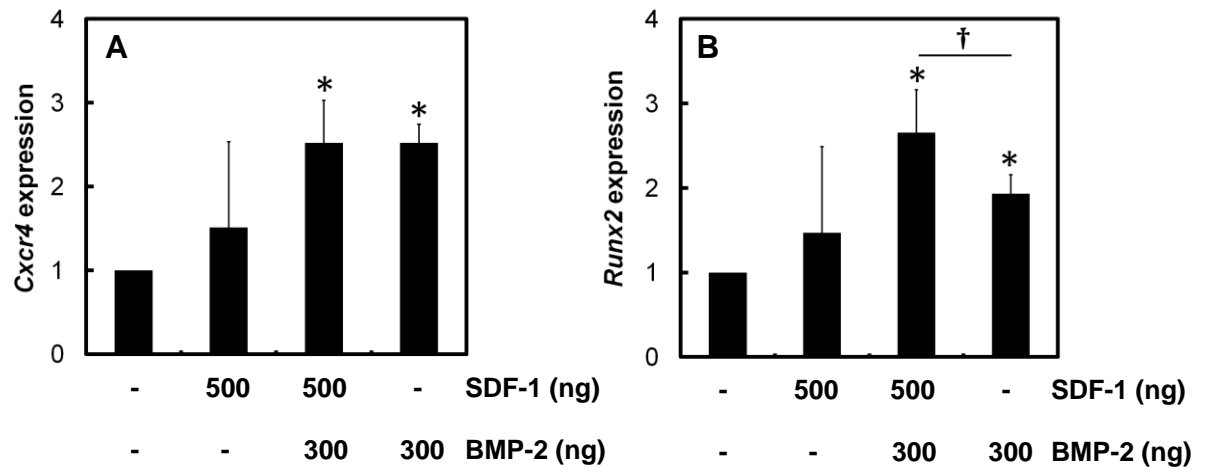
Figure 9. Immunohistochemical staining images of CD31- (left) and CD34-positive cells (right) recruited into the tissue around the site implanted with gelatin hydrogels incorporating (A) PBS, (B) 5 μ g of SDF-1, (C) 5 μ g of SDF-1 and 3 μ g of BMP-2, and (D) 3 μ g of BMP-2 2 weeks after implantation into the back subcutis of GFP-positive *Chimeric* mice (CD31- and CD34-positive cells: brown, cell nuclei: purple, scale bar = 100 μ m).

Figure 10. (A) Flow cytometric profiles of sort gates of CD45-negative and CD44-positive cells and histograms of CD45-negative, CD44-positive, and CD29-positive cells (B) Total number of live cells, (C) Percentage of CD45-negative and CD44-positive cells to total cells, and (D) Percentage of CD45-negative, CD44-positive, and CD29-positive cells to total cells in gelatin hydrogels incorporating PBS, 5 μ g of SDF-1, 5 μ g of SDF-1 and 3 μ g of BMP-2, and 3 μ g of BMP-2 implanted into the back subcutis of C57BL/6 mice 4 weeks. * $p < 0.05$, significant against the value of gelatin hydrogels incorporating PBS. † $p < 0.05$, significant against the value of gelatin hydrogels incorporating SDF-1. ‡ $p < 0.05$, significant against the value of gelatin hydrogels incorporating BMP-2.

Table 1. Sequence of primers used for RT-PCR assay

Gene	Primer/probe	Sequence
<i>Cxcr4</i>	Forward	CGAGCATTGCCATGGAAATATA
	Reverse	AGGTGCAGCCGGTACTTGTC
<i>Runx2</i>	Forward	CAAGTGGCCAGGTTCAACGA
	Reverse	GGGACCGTCCACTGTCACCTTAATA
<i>Osteocalcin</i>	Forward	TGCAAAGCCCAGCGACTCT
	Reverse	TTGAGCTCACACACCTCCCTGT
18S	Forward	ACTCAACACGGGAAACCTCA
	Reverse	AACCAGACAAATCGCTCCAC

Figure 1. (Ratanavaraporn *et al.*)

Figure 2. (Ratanavaraporn *et al.*)

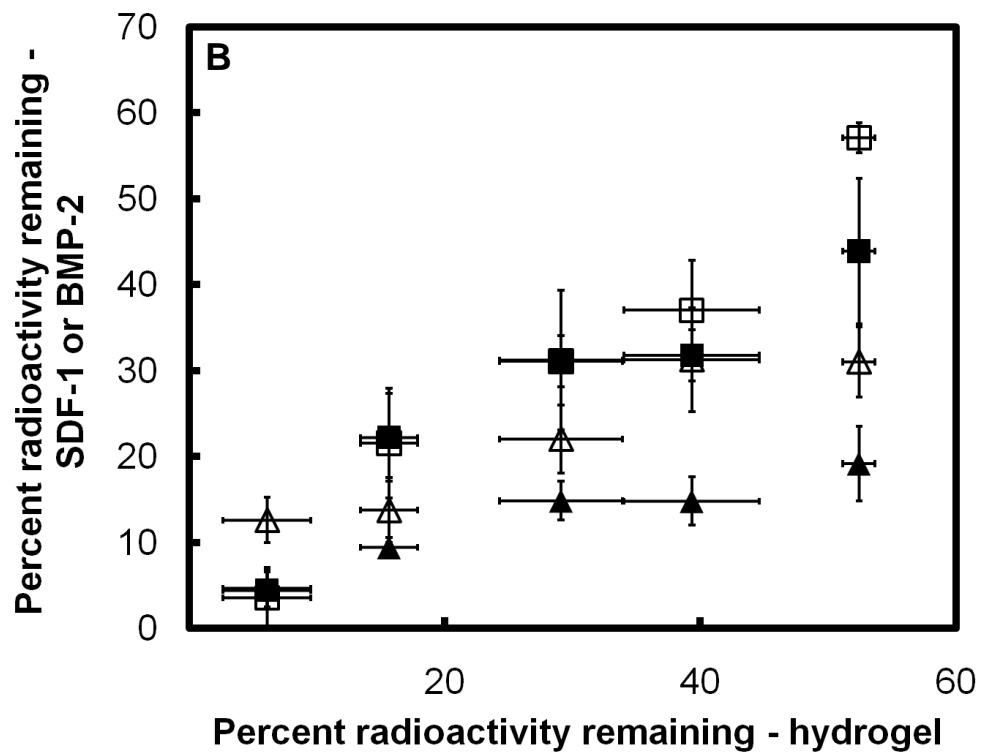
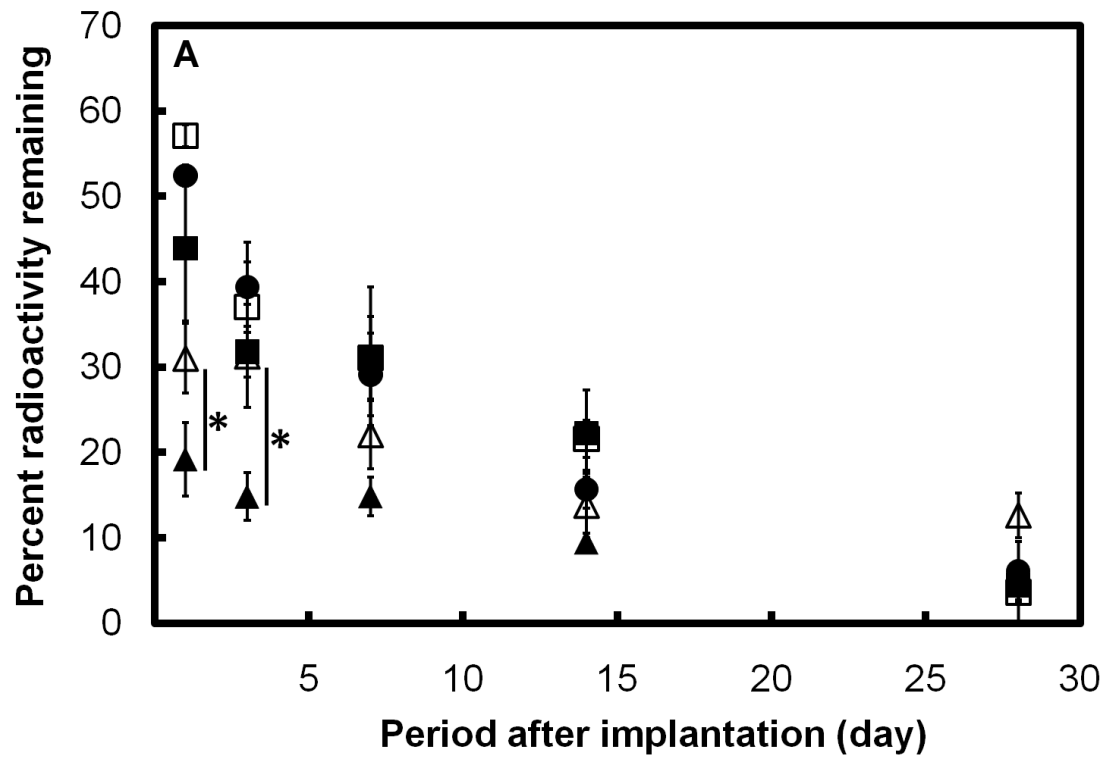
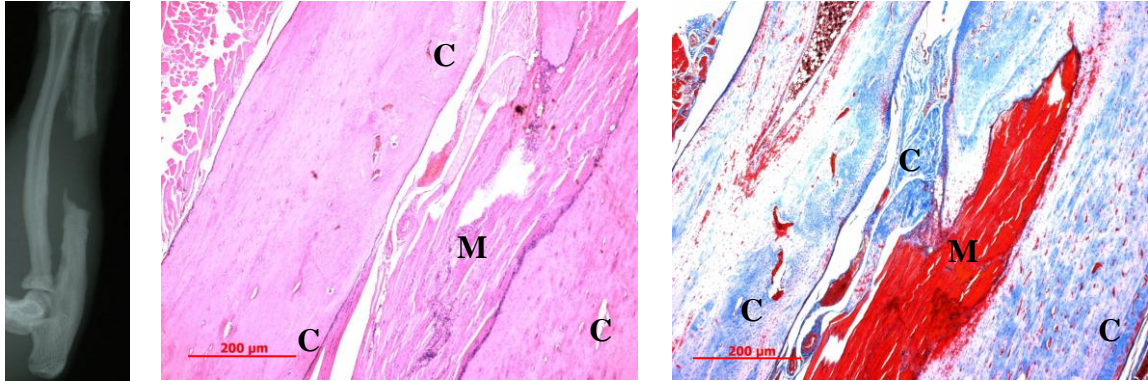
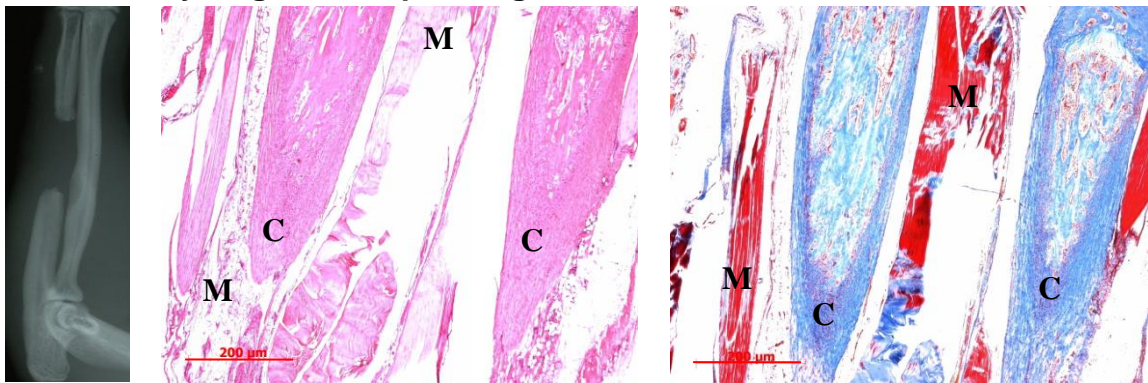


Figure 3. (Ratanavaraporn *et al.*)

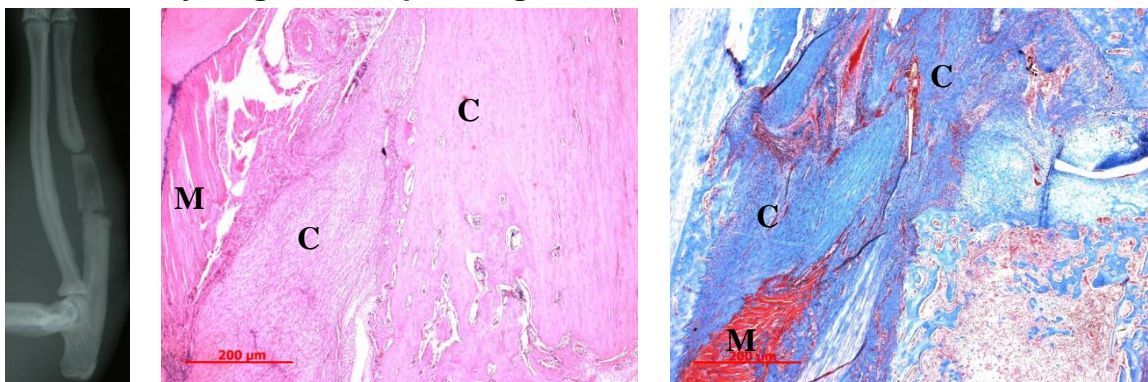
A. Gelatin hydrogels incorporating PBS



B. Gelatin hydrogels incorporating SDF-1



C. Gelatin hydrogels incorporating SDF-1 and BMP-2



D. Gelatin hydrogels incorporating BMP-2

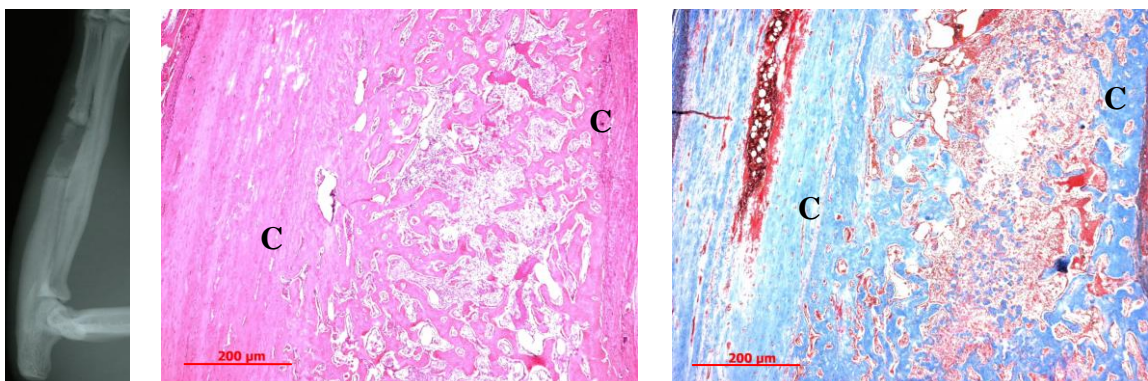
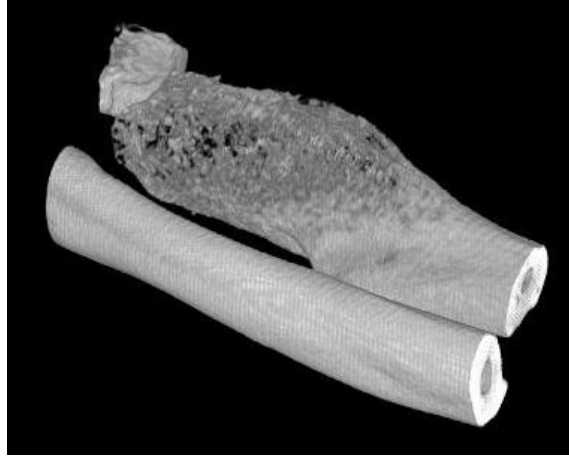
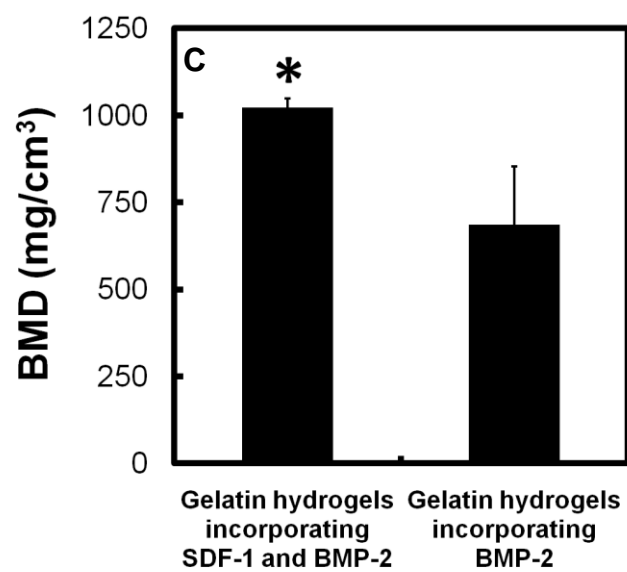
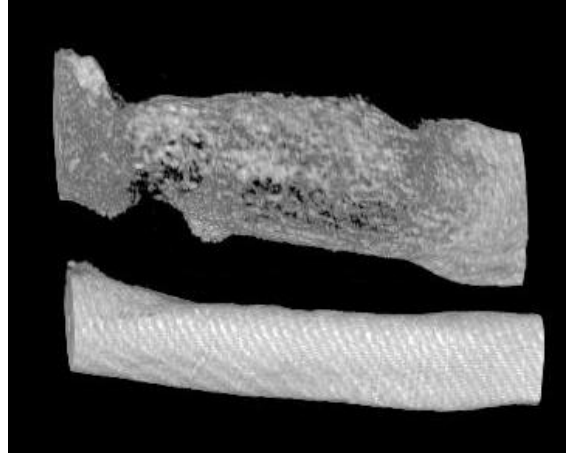
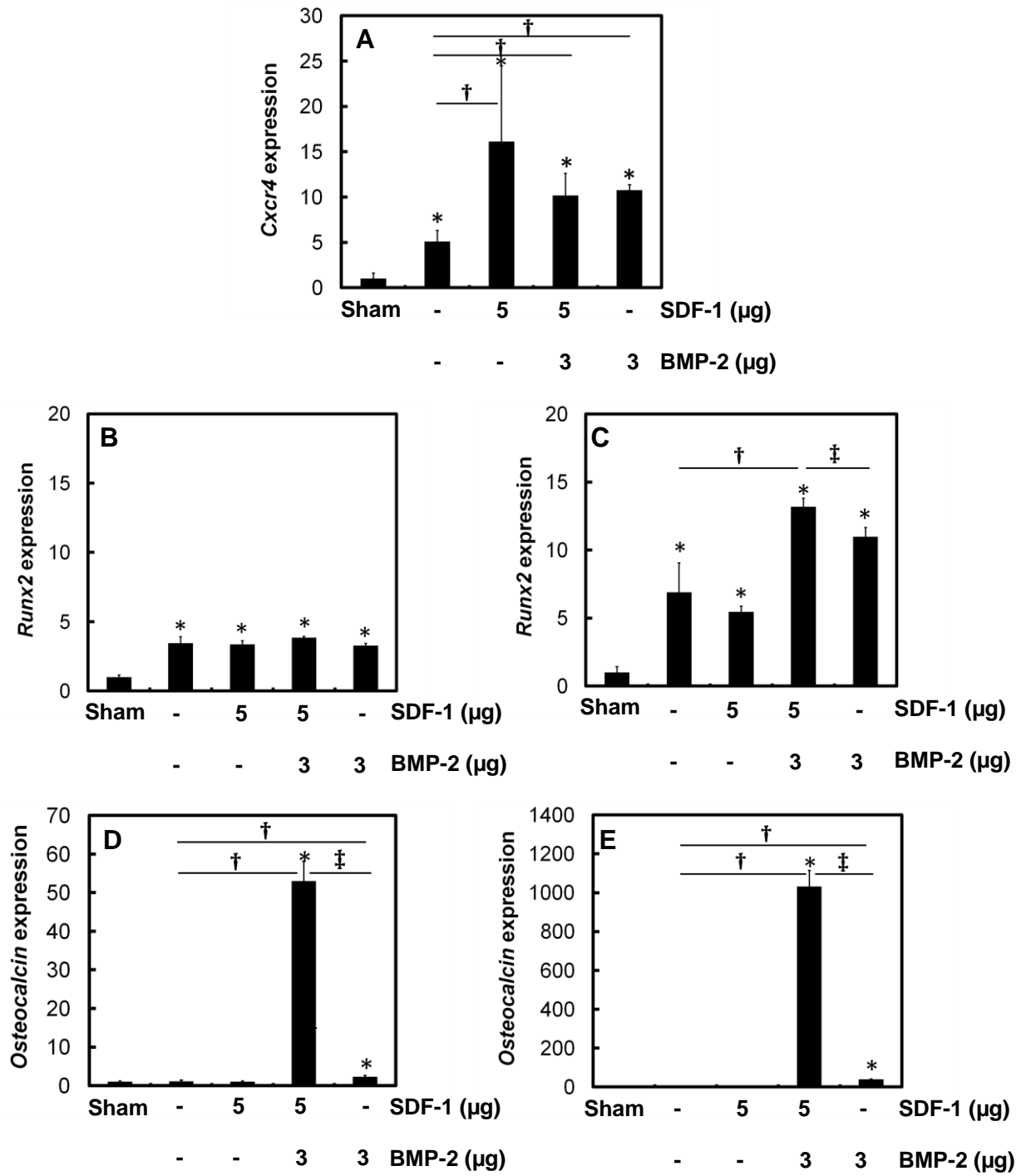
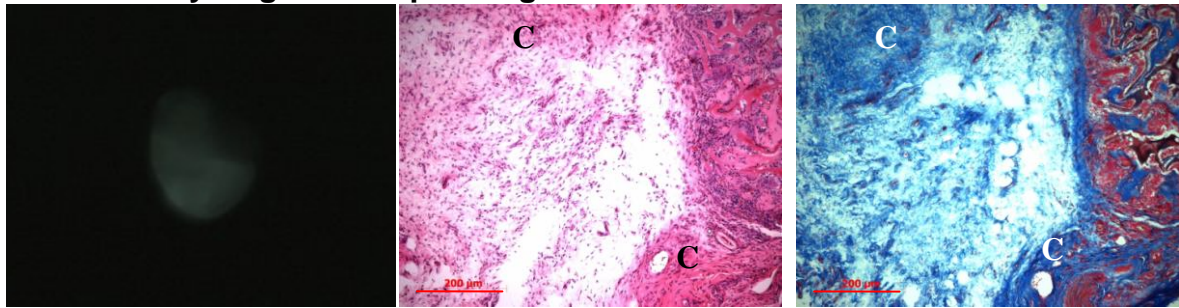


Figure 4. (Ratanavaraporn *et al.*)

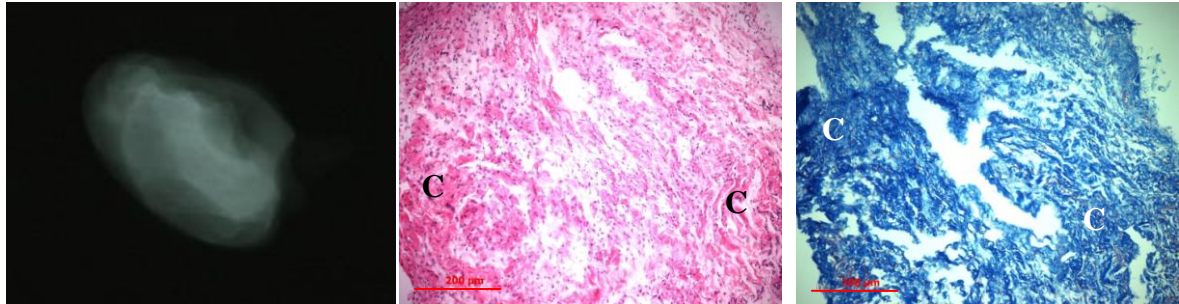
A. Gelatin hydrogels incorporating SDF-1 and BMP-2**B. Gelatin hydrogels incorporating BMP-2**Figure 5. (Ratanavaraporn *et al.*)

Figure 6. (Ratanavaraporn *et al.*)

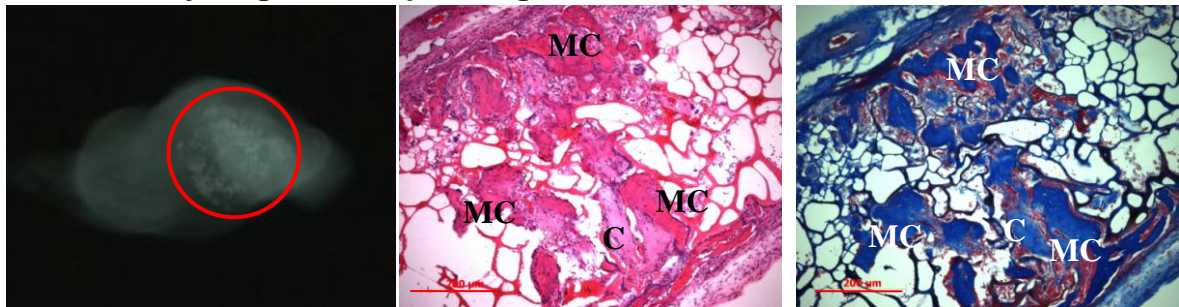
A. Gelatin hydrogels incorporating PBS



B. Gelatin hydrogels incorporating SDF-1



C. Gelatin hydrogels incorporating SDF-1 and BMP-2



D. Gelatin hydrogels incorporating BMP-2

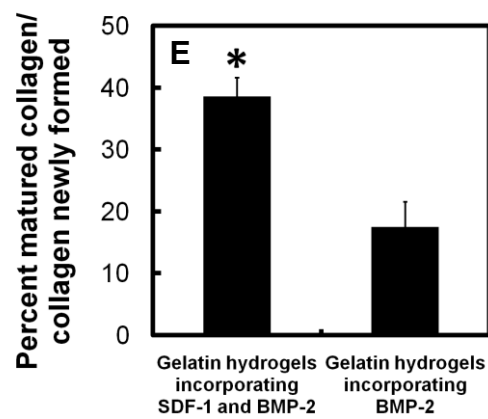
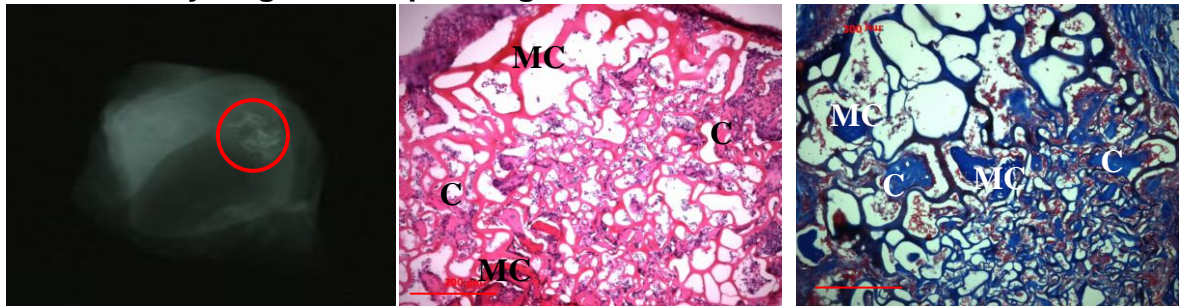
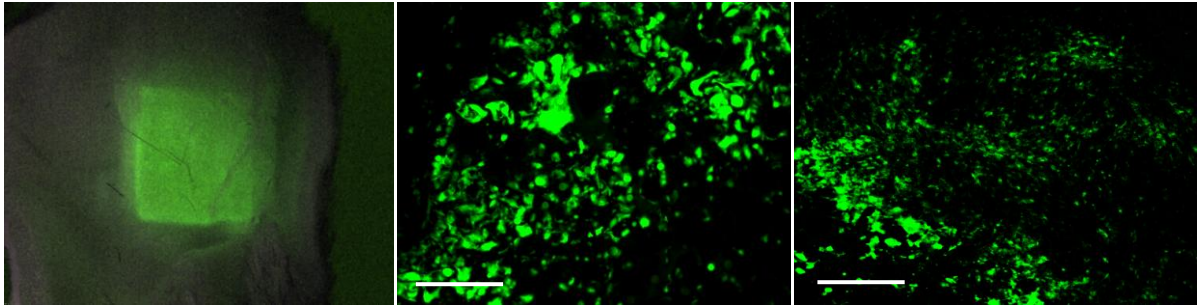
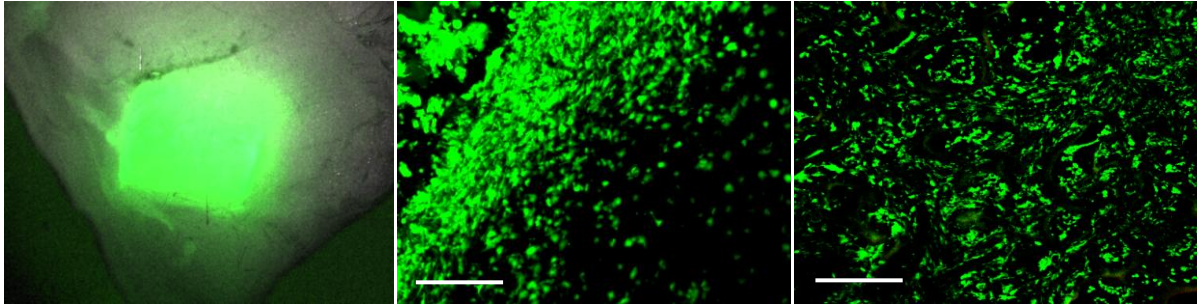


Figure 7. (Ratanavaraporn *et al.*)

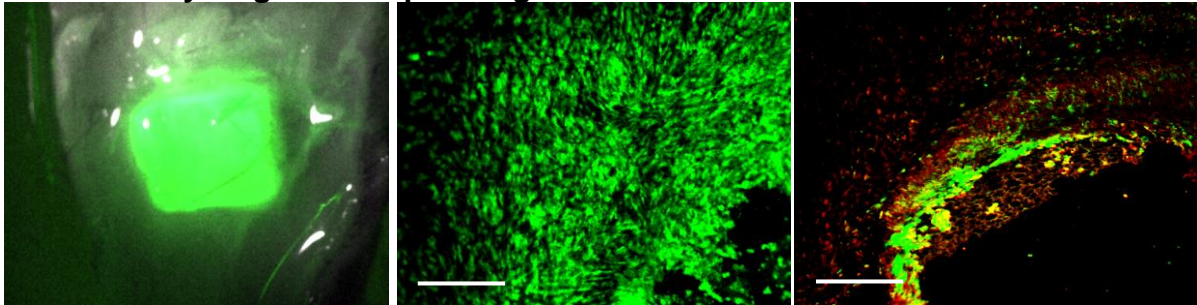
A. Gelatin hydrogels incorporating PBS



B. Gelatin hydrogels incorporating SDF-1



C. Gelatin hydrogels incorporating SDF-1 and BMP-2



D. Gelatin hydrogels incorporating BMP-2

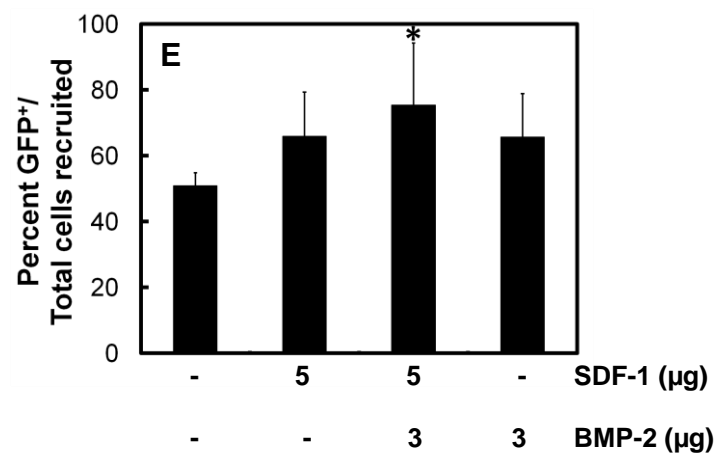
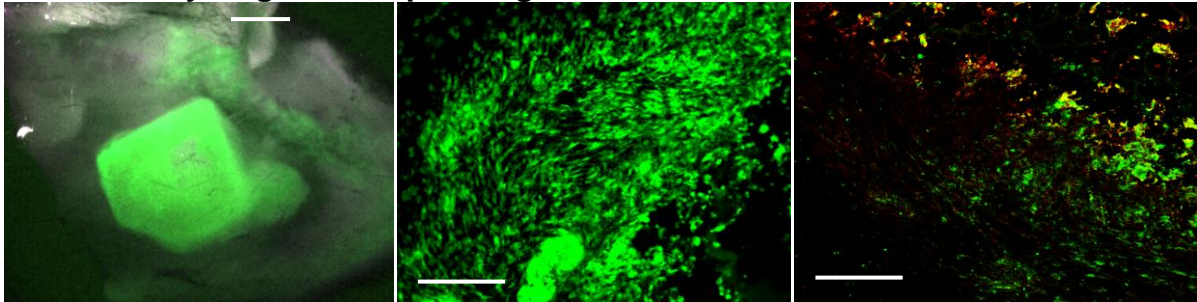


Figure 8. (Ratanavaraporn *et al.*)

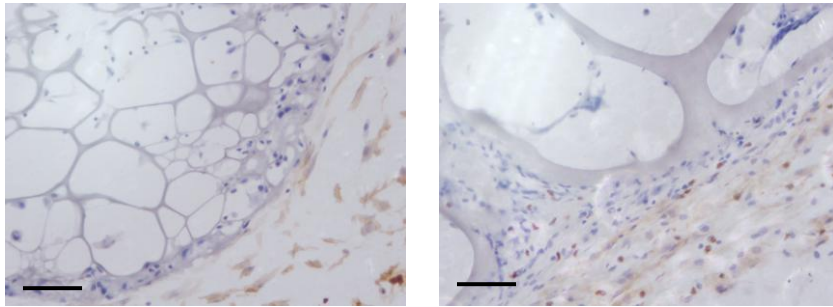
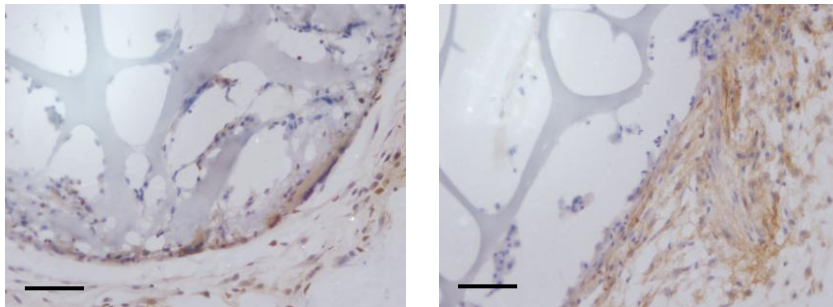
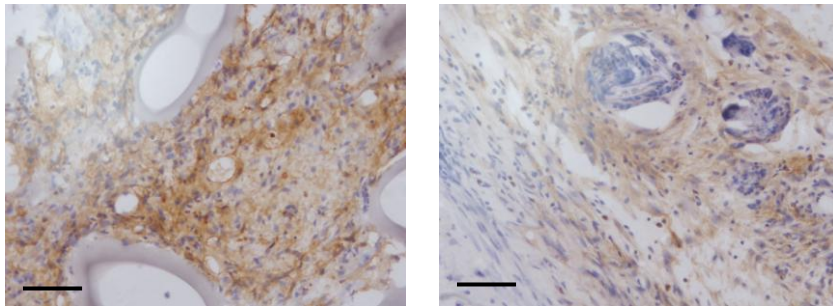
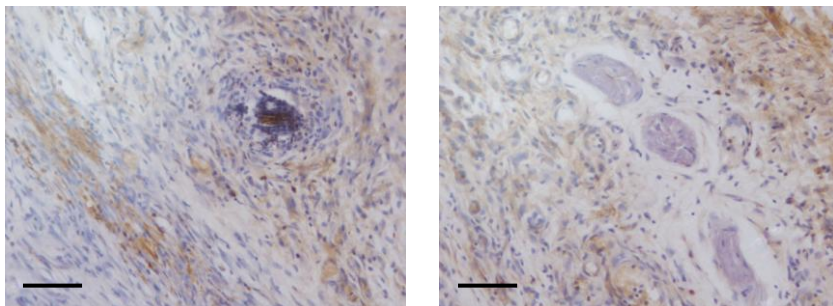
A. Gelatin hydrogels incorporating PBS**B. Gelatin hydrogels incorporating SDF-1****C. Gelatin hydrogels incorporating SDF-1 and BMP-2****D. Gelatin hydrogels incorporating BMP-2**

Figure 9. (Ratanavaraporn *et al.*)

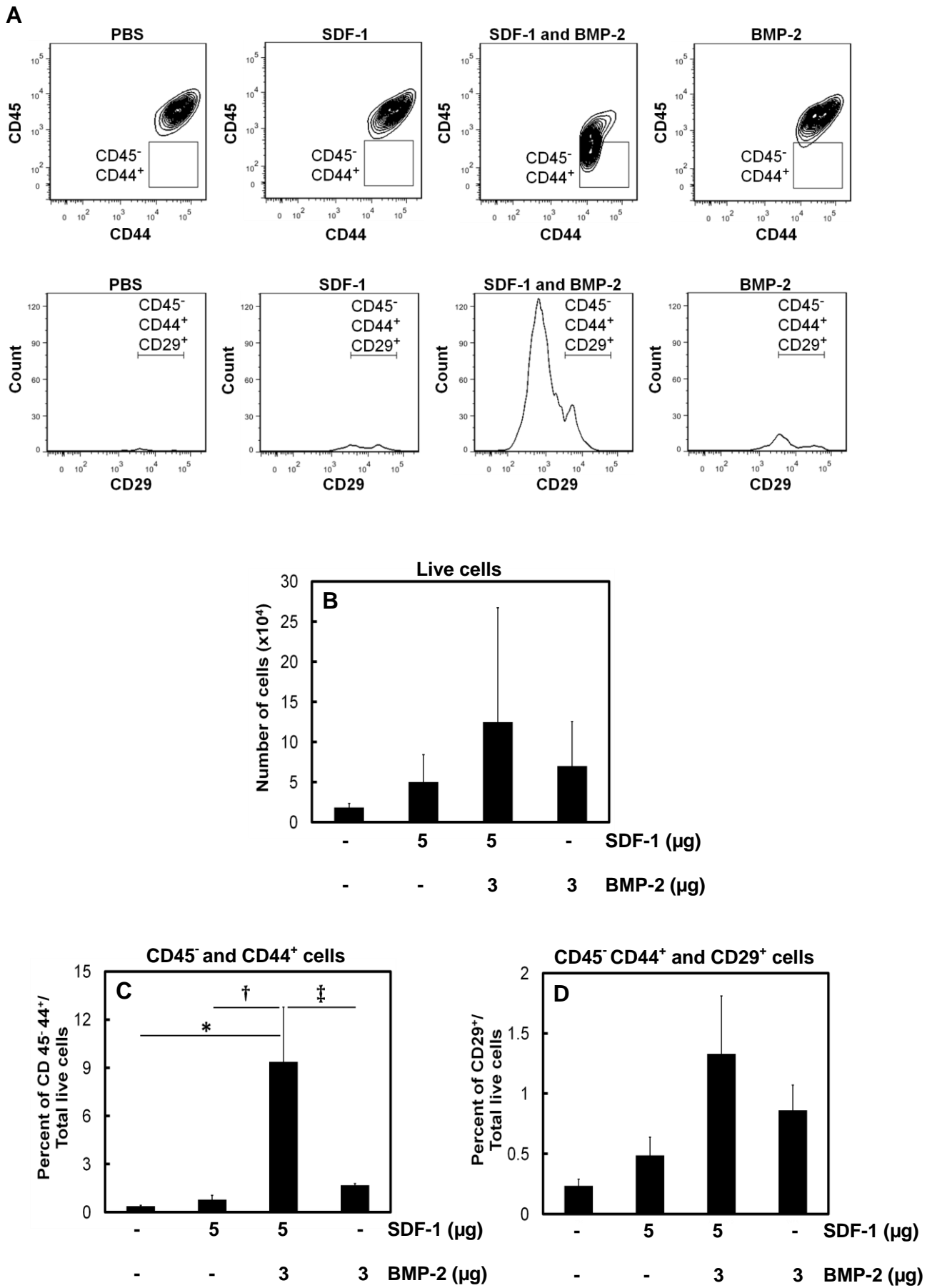


Figure 10. (Ratanavaraporn *et al.*)