THE EFFECT OF SYNTHETIC OCTACALCIUM PHOSPHATE IN A COLLAGEN SCAFFOLD ON THE OSTEOGENICITY OF MESENCHYMAL STEM CELLS

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Abstract

Although the efficacy of the *in vivo* osteogenic capabilities of synthetic octacalcium phosphate (OCP) crystal implantation can be explained through its stimulatory capacity for the differentiation of the host osteoblastic cell lineage, direct evidence that OCP supports bone regeneration by osteogenic cells *in vivo* has not been shown. Mesenchymal stem cells (MSCs) isolated from 4-week-old male Wistar rat long bones were pre-incubated in osteogenic or maintenance medium in the presence or absence of basic fibroblast growth factor (bFGF). OCP/Collagen (OCP/Col) or collagen disks were seeded with MSCs that had been pre-incubated in osteogenic medium containing bFGF, which exhibited the highest differentiation induction, and then incubated for an additional day. The disks were implanted in critical-sized calvaria defects of 12-week-old male Wistar rats and the specimens were analysed radiographically, histologically, histomorphometrically, and by micro-computed tomography (CT) imaging at 4 and 8 weeks after the implantation. The OCP/Col·MSCs group rapidly induced more bone regeneration, even within 4 weeks, compared to the OCP/Col group without MSCs. The bone mineral density of the OCP/Col·MSCs group was also greater than the OCP/Col group. The Col·MSCs group did not exhibit prominent osteogenicity. These results indicate that OCP crystals in a collagen matrix efficiently promote exogenously introduced osteogenic cells to initiate bone regeneration if the cells are pre-treated in a suitable differentiation condition.

Keywords: Octacalcium phosphate, collagen, mesenchymal stem cells, basic fibroblast growth factor, bone regeneration.

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Introduction

In the field of tissue engineering, the number of basic and clinically related studies on the use of bone marrow stromal cells (BMSCs) for the repair of large bone defects without autologous bone transplantation has increased remarkably (Kawate *et al.*, 2006; Kuznetsov *et al.*, 2008). BMSCs can provide a source of mesenchymal stem cells (MSCs) that can differentiate into osteoblastic cells (Dennis *et al.*, 2007). MSCs incubated at suitable passage exhibit an excellent ability to implant at bone defect sites *in vivo* and could potentially replace autologous bone transplantation as a therapeutic intervention (Quarto *et al.*, 2001). In addition, it is becoming clear that the osteogenic ability of MSCs can be combined with suitable scaffold materials for *in vivo* bone regeneration (Grundel *et al.*, 1991; Werntz *et al.*, 1996).

Calcium phosphate ceramics, such as hydroxyapatite (HA; $Ca_{10}(PO_4)_6(OH)_2$) and β-tricalcium phosphate (β-TCP; $Ca_3(PO_4)_2$), have prominent osteoconductive properties and therefore have been used individually as bone substitute materials to assist in bone regeneration (Bucholz, 2002; LeGeros, 2002). These calcium phosphate ceramics have also been implanted together with MSCs in order to enhance bone regeneration in cases of severe bone atrophy or large bone defects where self-regeneration cannot occur. Calcium phosphate has been found to affect MSC functions in both *in vitro* and *in vivo* studies. Moreover, porous HA blocks have been shown to function as a scaffold when implanted in the subcutaneous tissues of nude mice together with human MSCs derived from bone marrow, even when the cells were obtained from patients with various bone-related diseases (Yoshikawa *et al.*, 2004). Ca-deficient HA ($Ca_{10-X}H_X(PO_4)_6(OH)_{2-X}$, $0 \leq X \leq 2$) (Winand, 1965) can support human MSC-mediated bone induction at ectopic sites (Kasten *et al.*, 2005). In addition, biphasic calcium phosphate (BCP) composed of HA and β-TCP has been shown to provide spaces for MSC interaction and displays bone-inducing abilities in various animal species, including mouse (Chevallier *et al.*, 2010), rat (Espitalier *et al.*, 2009), and dog (Kuznetsov *et al.*, 2008).

Synthetic octacalcium phosphate (OCP: $Ca_8H_2(PO_4)_6 \cdot 5H_2O$) is another potential scaffold for MSCs, because OCP and OCP-polymer complexes, such as OCP/Collagen (OCP/Col), enhance bone regeneration and

tend to be replaced with newly formed bone if implanted in experimentally created bone defects or if introduced as onlay grafts on cortical bone in various animal models (Suzuki *et al.*, 1991; Sugihara *et al.*, 1995; Sugihara *et al.*, 1996; Kamakura *et al.*, 1999; Kamakura *et al.*, 2006; Kawai *et al.*, 2009). OCP is known to be a potential bone apatite precursor phase (Brown *et al.*, 1962) and is more soluble than HA or β-TCP at a physiological pH (Brown *et al.*, 1981; Chow, 2001). Although the composition of biological tissue fluid is considered to be almost saturated with respect to OCP crystals (Driessens *et al.*, 1978; Eidelman *et al.*, 1987), OCP tends to convert to HA (Ca-deficient HA) during bone regeneration *in vivo* (Suzuki *et al.*, 1991; Suzuki *et al.*, 2006b). This conversion occurs because OCP is composed of apatite layers stacked together with hydrated layers, and has a triclinic crystal system that can be converted to HA by hydrolysis of the hydrated layers in OCP (Brown *et al.*, 1962). OCP is converted into HA through (1) a dissolution–reprecipitation mechanism and (2) topotaxial conversion by ion diffusion within the crystal lattice (Brown *et al.*, 1981), which consumes calcium ions from the surrounding milieu and releases phosphate ions from the crystals (Brown *et al.*, 1981; Suzuki *et al.*, 2006a). The capacity of OCP to enhance bone regeneration could be associated with its ability to activate host osteoblasts and induce their acquisition through cell surface interactions with surrounding cells, tissue fluid, and serum proteins (Suzuki *et al.*, 1993), which is caused by the OCP-HA conversion process (Suzuki *et al.*, 2006b). OCP supports osteoblastic cell differentiation (Suzuki *et al.*, 2006b) and has been shown to increase the *in vitro* expression of mRNA of associated genes, such as alkaline phosphatase (ALP) and osterix (Osx), in a dose-dependent manner (Anada *et al.*, 2008). OCP also increases the ALP activity of host osteoblastic cells *in vivo* (Kikawa *et al.*, 2009; Miyatake *et al.*, 2009). Based on the experimental evidence to date, we hypothesised that OCP may promote MSCs to initiate bone regeneration around the implanted OCP, which has been observed with host osteoblastic cells. To date, the question of whether OCP can induce bone regeneration *in vivo* when implanted with MSCs prepared using tissue engineering techniques has not been examined.

The aim of the present study was to investigate whether seeding an OCP/Col composite with MSCs induces better bone formation than a composite without MSCs when implanted in critical-sized calvaria defects in rats.

Materials and Methods

Preparation of octacalcium phosphate, collagen disks, and OCP/Col disks

OCP was prepared by mixing calcium and phosphate solutions as previously described (Suzuki *et al.*, 1991). Sieved OCP granules (particle sizes 300-500 μm) obtained from the dried OCP were sterilised by heating them at 120 °C for 2 h. As shown in our previous study, this heating technique does not affect the physical properties, such as the crystalline structure or specific surface area of the OCP granules (Suzuki *et al.*, 1993; Suzuki *et al.*, 1995), although it has been reported that increasing the

temperature above 100 °C can induce the collapse of the OCP structure due to dehydration (Fowler *et al.*, 1966; Nelson and McLean, 1984). A 1 % solution of collagen was purchased (NMP collagen PS; Nippon Meat Packers, Tsukuba, Ibaraki, Japan) and adjusted to pH 7.4 in order to form a gel of collagen fibrils, which was then condensed by centrifugation. A 3 % collagen suspension was then prepared from the condensed collagen suspension. OCP granules with a 300-500 μm diameter were added to the concentrated collagen and mixed. The weight percentage of OCP in the OCP/Col mixture was 77 %. The mixture was then lyophilised, and disks were moulded to a 9 mm diameter and 1 mm thickness. The moulded OCP/Col and collagen disks were subjected to a dehydrothermal treatment at 150 °C for 24 h in a vacuum drying oven (DP32, Yamato Scientific, Tokyo, Japan) and were then sterilised using electron beam irradiation (5 kGy). The OCP/Col disk had a relatively small pore size of 48 μm. The porosity was evaluated by mercury intrusion and determined to be 92 %, and the elastic modulus was 0.4 MPa, as estimated by mechanical testing (Suzuki *et al.*, 2009; Matsui *et al.*, 2010).

Isolation and culture of MSCs

MSCs were isolated from the bone shaft of femurs and tibias of 4-week-old male Wistar rats (SLC, Hamamatsu, Shizuoka, Japan). After both ends of the femurs or tibias were removed from the epiphysis, the bone marrow was flushed out with 10 mL of maintenance medium consisting of Dulbecco's modified Eagle medium (DMEM) supplemented with 10 % foetal bovine serum (FBS) and 1 % penicillin/streptomycin (Invitrogen-Gibco, Carlsbad, CA, USA) through a syringe with a 25-gauge needle. The cells were maintained in a humidified atmosphere of 95 % air with 5 % CO_2 at 37 °C. As a separate method, the MSCs were also isolated with an osteogenic differentiation medium consisting of DMEM with 10 % FBS, 1 % penicillin/streptomycin, 10^{-7} M dexamethasone, 10 mM β-glycerophosphate, 50 μg/mL ascorbate 2-phosphate, and 2 mM L-glutamine (MSCs isolated with osteogenic differentiation medium; osteogenic MSCs). The MSCs were maintained in 10 mL of medium at 37 °C in a 5 % CO_2 environment and were detached from the dish 3-4 d after the cell isolation.

Measurement of proliferation and alkaline phosphatase (ALP) activity of MSCs

MSCs (3×10^4) were suspended in 250 μL of maintenance medium and seeded in a 48-well tissue culture plate. The MSCs were cultured in maintenance medium containing 3 ng/mL of basic fibroblast growth factor (bFGF) for 7 d, which was almost 10 d after MSC isolation. After this culturing period, the medium was changed to an osteogenic medium. This culture process is referred to as the "normal method" (Fig. 1AIII). Osteogenic MSCs (3×10^4) were then suspended in 250 μL of osteogenic medium and seeded in a 48-well tissue culture plate. The osteogenic MSCs were cultured in osteogenic medium with bFGF (osteogenic (FGF+)) or without bFGF (osteogenic (FGF-)) (Fig. 1AI, II). For prolonged incubation, the culture medium was exchanged with fresh medium every 3 d.
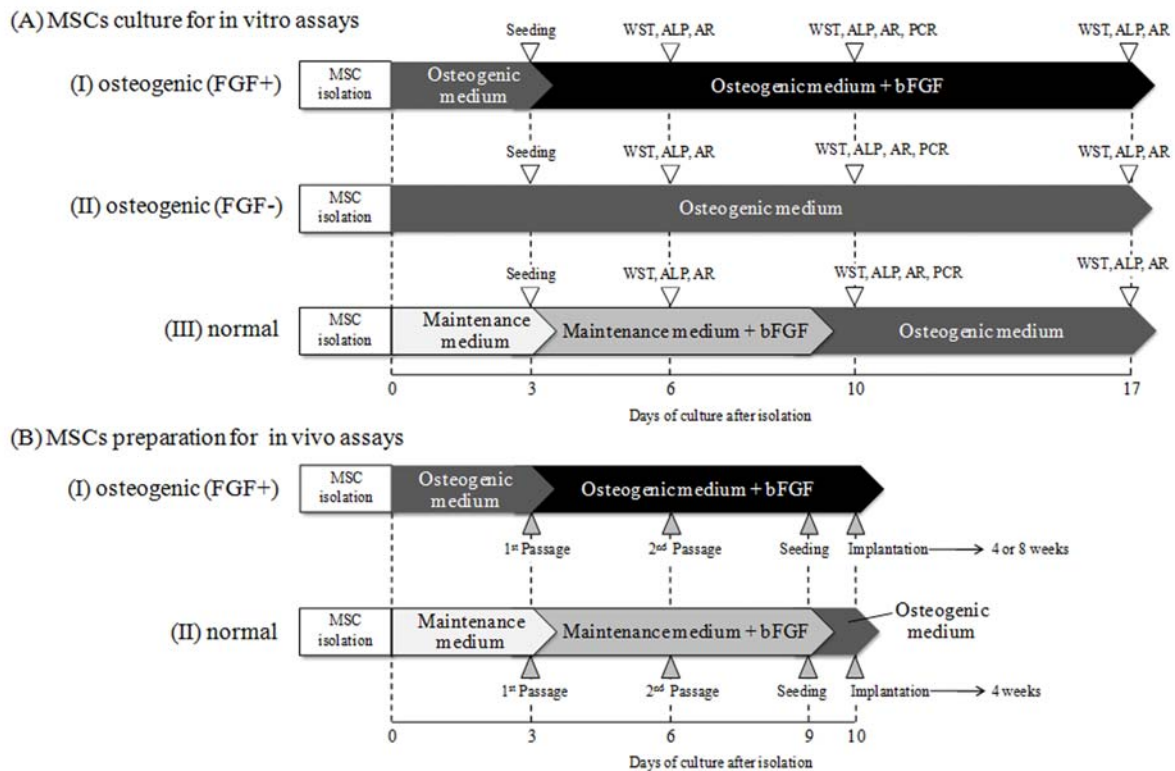
Fig. 1. Cell culture conditions of the present study. (A) MSCs culture for *in vitro* assays (I) MSCs were cultured in osteogenic medium from MSC isolation and with bFGF at 3 d (osteogenic (FGF+)). (II) MSCs were cultured in osteogenic medium from MSC isolation without bFGF (osteogenic (FGF-)). (III) As the normal method, MSCs were cultured in maintenance medium and with bFGF at 3 d for MSC proliferation, and osteogenic medium was used from 9 d. WST: cell proliferation measured by WST-8 assay, ALP: cell differentiation measured by ALP activity, AR: the formation of bone nodule measured by Alizarin Red staining, PCR: cell differentiation measured by TaqMan real-time PCR. (B) MSCs culture for *in vivo* assays. (I), (II) MSCs were cultured with the same method as osteogenic (FGF+) (Fig. 1A(I)) and normal (Fig.1 A(III)), respectively, until 9 d. At 9 d, MSCs were seeded on OCP/Collagen disks or collagen disks. After 1 d culture, MSCs with OCP/Collagen disks or collagen disks were implanted in rat calvaria bone defect.

The cell number of normal MSCs, osteogenic (FGF+) MSCs, and osteogenic (FGF-) MSCs in the plates was determined at 3, 7 and 14 d (6, 10 and 17 d after MSC isolation, respectively) by counting living cells using a WST-8 assay (Cell Counting Kit-8, Dojindo, Kumamoto, Japan). ALP activity was measured using a commercially available labAssay™ ALP kit (Wako Pure Chemical Industries, Osaka, Japan). Briefly, on days 3, 7 and 14 of the culture period (6, 10 and 17 d after MSC isolation, respectively), normal MSCs, osteogenic (FGF+) MSCs, and osteogenic (FGF-) MSCs were lysed in 0.2 % Triton X-100 solution at room temperature. The lysates were subjected to ultrasonication and centrifugation (5000 g for 5 min). The supernatants were then collected and the ALP activity was determined by measuring the conversion of p-nitrophenol to p-nitrophenylate at pH 9.8 and 37 °C for 30 min. The ALP activity was normalised using the cell number that was determined by the WST-8 assay.

Alizarin red staining of MSCs
On days 3, 7 and 14 of the culture period (6, 10 and 17 d after MSC isolation, respectively), the monolayer of normal, osteogenic (FGF+), and osteogenic (FGF-) MSCs was washed twice with phosphate-buffered saline (PBS), rinsed with water, and stained with alizarin red S (Wako

Pure Chemical Industries) for 30 min at room temperature. After staining, the cells were rinsed with water and dehydrated in 70 to 100 % ethanol.

TaqMan quantitative real-time polymerase chain reaction
After the normal, osteogenic (FGF+), and osteogenic (FGF-) MSCs were incubated in 48-well plates for 7 d (10 d after MSC isolation), total RNA was extracted using a QIAshredder and RNeasy kit (Qiagen, Valencia, CA, USA). DNase treatment was performed using RQ1 RNase-free DNase (Promega). The first strand cDNA was synthesised from 1 µg of RNA using a SuperScript III Reverse Transcriptase (Invitrogen). The expression levels were determined for ALP, osteopontin (OPN), osteocalcin (OCN), osterix (Osx), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by real-time TaqMan polymerase chain reaction (PCR) analysis. Optimal oligonucleotide primers and TaqMan probes were designed with ProbeFinder (Roche Diagnostics, Basel, Switzerland) software using rat ALP, OPN, OCN, Osx, and GAPDH sequences from the GenBank database. The primer sets used for PCR were as follows: ALP, 5'-CCGAGTGGTGGTCACGAT-3' and 5'-ACGAGGTCACGTCCATCCT-3';

OPN, 5'-GAGTTTGGCAGCTCAGAGGA-3' and 5'-TCTGCTTCTGAGATGGGTCA-3'; OCN, 5'-ATAGACTCCGGCGCTACCTC-3' and 5'-CCAGGGGATCTGGGTAGG-3'; Osx, 5'-TGCCCCAACTGTCAGGAG-3' and 5'-GATGTGGCGGCTGTGAAT-3'; and GAPDH, 5'-TGGGAAGCTGGTCATCAAC-3' and 5'-GCATCACCCCATTTGATGTT-3'. TaqMan probes for the tested genes were labelled with carboxyfluorescein (FAM) fluorochrome with the following sequences: ALP, 5'-GGATGCTG-3' (Universal ProbeLibrary probe # 89); OPN, 5'-GGCTGAAG-3' (Universal ProbeLibrary probe # 41); OCN, 5'-CTTGGAGC-3' (Universal ProbeLibrary probe # 125); Osx, 5'-CTGGGCTG-3' (Universal ProbeLibrary probe # 26); and GAPDH, 5'-CATCACCA-3' (Universal ProbeLibrary probe # 9). TaqMan PCR was carried out in a 20 µL final volume containing 200 nM each of forward and reverse primers, 100 nM Universal ProbeLibrary probe, and a cDNA template. The cycling conditions were 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 1 s for 35 cycles. Real-time TaqMan PCR was performed using a LightCycler 1.5 (Roche Applied Science, Mannheim, Germany). All PCR reactions were performed in duplicate. The gene expression levels of ALP, OPN, OCN, and Osx were quantified as relative expression after normalization to the GAPDH expression level in the same reaction.

MSC seeding on OCP/Col or collagen disks

Osteogenic (FGF+) MSCs or normal MSCs were used for seeding on OCP/Col or collagen disks (Fig. 1BI and 1BII). The OCP/Col or collagen disks were placed in 24-well polystyrene plates, which inhibited cell attachment on the surface (Ultra Low Attachment Polystyrene; Corning Costar, Acton, MA, USA). After the third passage of the cells, which was almost 10 d after MSC isolation, normal MSCs or osteogenic (FGF+) MSCs were seeded onto OCP/Col or collagen disks at a cell density of 2×10^5 cells/disk/well in 500 µL of osteogenic differentiation medium. The 24-well plates containing the seeded disks were incubated on a rotary shaker (Multi Shaker MMA-210; Eyela, Tokyo, Japan) at a speed of 200 rpm for 6 h. The medium in each well was then changed to remove non-adherent cells. OCP/Col with normal MSCs (OCP/Col·nor-MSCs), OCP/Col, and collagen disks with osteogenic (FGF+) MSCs (OCP/Col·MSCs and Col·MSCs) were incubated at 37 °C in a 5 % CO_2 environment for 1 d. The preliminary study showed that the cell number one day after seeding 2.0×10^5 MSCs on OCP/Col and Col disks was approximately $78,055 \pm 5,701$ and $69,944 \pm 6,234$, respectively. There was no significant difference between them. Therefore, we concluded that the effects of MSC seeding on bone regeneration could be comparatively analysed between these groups.

Implantation of OCP/Col·n-MSCs, OCP/Col·MSCs, Col·MSCs, OCP/Col, or collagen disks in a rat calvaria bone defect

Twelve-week-old male Wistar rats (SLC, Hamamatsu, Shizuoka, Japan) were used for this experiment. The principles of laboratory animal care as well as national laws were followed for the use of animals. All procedures were approved by the Animal Research Committee of Tohoku University.

The experimental rats were anaesthetised with an intraperitoneal (i.p.) injection of sodium pentobarbital (50 mg/kg) supplemented with ether inhalation. A calvaria bone defect with a 9 mm diameter was created as previously described (Kamakura *et al.*, 2006; Kawai *et al.*, 2009). The OCP/Col·nor-MSCs, OCP/Col·MSCs, and Col·MSCs disks that had been cultured for 1 d were implanted into the trephine defect. As a control, OCP/Col or collagen was implanted in the same manner. As a negative control, untreated animals were processed using the same method, but no disk was implanted in the bone defect. After the defects were treated, the ablated periosteum and the skin were repositioned and sutured, respectively. After 4 weeks, 5 rats in the OCP/Col·nor-MSCs group were euthanised and then fixed as described below. In addition, 5 rats each from the OCP/Col·MSCs, Col·MSCs, OCP/Col, collagen, and untreated groups were euthanised and fixed at 4 and 8 wks after implantation.

Radiographic analysis

The rats were anaesthetised with an i.p. injection of sodium pentobarbital (50 mg/kg) supplemented with ether inhalation. The rats were sacrificed and then the implants were resected together with the surrounding bones. The tissues were immersed in 4 % paraformaldehyde in 0.1 M PBS (pH 7.4) for the tissue fixation and kept overnight at 4 °C. The specimens were radiographed by means of a microradiography unit (Softex CMR Unit; Softex, Tokyo, Japan) with X-ray film (FR; Fuji Photo Film, Tokyo, Japan) under standardised conditions (20 kV, 5 mA, 1 min), in which OCP showed no radiopacity.

Analysis of bone microstructure

The calvaria bone was analysed using a Microfocus X-Ray CT System (Scan Xmate-E090; Comscantecno, Kanagawa, Japan). The bone specimens were scanned continuously at increments of 20 µm for 512 slices with a tube voltage of 90 kV and a tube current of 0.1 mA. The voxel size was $20 \times 20 \times 20$ µm³. After the scanning, the image data were transferred to a workstation, and the structural indices and the mineralisation of bone were calculated using a three-dimensional (3D) image analysis system (TRI/3D-Bon; Ratoc System Engineering, Tokyo, Japan). The TRI/3D-BON system builds 3D models from serial tomographic datasets for observation and morphometric analysis. The 3D images were segmented into voxels identified as bone and marrow. The gray-scale images were segmented using a strict filter to remove noise and a fixed threshold to extract the mineralised bone phase. The isolated small particles in the marrow space and the isolated small holes in the bone were removed using a cluster-labelling algorithm. The bone surface (BS) area was calculated using surface triangulation of the binary data based on the marching cubes method. The bone volume (BV) was calculated using tetrahedrons that corresponded to the enclosed volume of the triangulated surface. The total tissue volume (TV) was the entire volume of the analysis. The normalised index, bone surface-to-volume ratio (BS/BV), and bone

volume fraction (BV/TV) were then calculated from these values. In addition to bone structure, we also quantified the bone mineral density (BMD) at the level of calcified bone tissue. In advance, cylindrical phantoms containing an HA slurry with solids and various concentrations of the solution were imaged, and the linear relationship between the HA concentrations (0.3-0.7 g/cm^3) and absorption was verified. The BMD was calibrated using this relationship.

Tissue preparation
After the radiographic and micro-CT analyses, the samples were decalcified in 10 % ethylenediaminetetraacetic acid in 0.01 M phosphate buffer (pH 7.4) for 2-4 weeks at 4 °C. The samples were then dehydrated in a graded series of ethanol and embedded in paraffin. The centre of the defect was extracted and sectioned coronally at a thickness of 5 μm. The sections were stained with haematoxylin and eosin (H&E) and the photographs were taken with a photomicroscope (Leica DFC300 FX, Leica Microsystems Japan, Tokyo, Japan).

Quantitative micrograph analysis
Light micrographs of the sections stained with H&E were used for histomorphometric measurements. Photographs projecting the overall defect were taken for each specimen. The percentage of newly formed bone in the defect (new-Bone%) was calculated as the area of newly formed bone per area of the defect that was originally created by trephination × 100. Similarly, the percentage of remaining OCP in the defect (rem-Imp%) was calculated as the area of remaining OCP per area of the defect that was originally created by trephination × 100. The new-Bone% and rem-Imp % were quantified on a computer using Scion Image public domain software (Scion Corporation, Frederick, MD, USA).

Statistical analysis
Results are expressed as mean ± standard deviation (SD). All cellular experiments were performed at least three times and showed reliable reproducibility. Statistical analyses were performed for all of the cellular and histomorphometric experiments using commercial software (Ekuseru-Toukei 2006, Social Survey Research Information, Tokyo, Japan). One-way analysis of variance (ANOVA) was used to compare the means among groups. If the ANOVA was significant, then Tukey's multiple comparison analysis was used as a *post-hoc* test.

Results

MSC proliferation and differentiation
Fig. 2a shows the proliferation of normal, osteogenic (FGF+), and osteogenic (FGF-) MSCs. Six days after MSC isolation, the number of MSCs in each culture condition was almost the same. However, at 10 d after MSC isolation, the number of osteogenic (FGF+) MSCs was significantly higher than the normal and osteogenic (FGF-) MSCs. The number of osteogenic (FGF-) MSCs was lower than normal MSCs throughout the culture period. There was a significant difference in MSC proliferation between FGF(+) and FGF(-) cultures and FGF(+) and normal cultures on days 10 and 17 of the culture period ($p < 0.01$). The use of osteogenic medium increased the ALP activity of MSCs during the entire observation period regardless of the presence or absence of bFGF (Fig. 2b). Ten days after MSC isolation, osteogenic (FGF+) MSCs exhibited the highest level of ALP activity, which was 1.9 and 64.3 times greater than the osteogenic (FGF-) and normal MSCs, respectively. There was a significant difference in ALP activity of the MSCs between each condition at

Table 1. Results of bone mineral density (BMD) and new-Bone% in OCP/Col with osteogenic (FGF+) MSCs (OCP/Col·MSCs) or normal MSCs (OCP/Col·nor-MSCs) ay 4 weeks after implantation in rat calvaria defect. *$p < 0.05$.

	OCP/Col•MSCs	OCP/Col•nor-MSCs
BMD (mg/cm³)	693.0 (± 50.1) *	560.4 (± 81.9)
new-Bone (%)	44.08 (± 1.74) *	30.29 (± 2.70)

Table 2. Quantification of the bone microstructure in OCP/Col•MSCs and OCP/Col at 8 weeks after implantation. *$p < 0.05$. TV, total tissue volume; BV, bone volume; BS, bone surface; BS/BV, bone surface to volume ratio; BV/TV, bone volume fraction; BMD, bone mineral density.

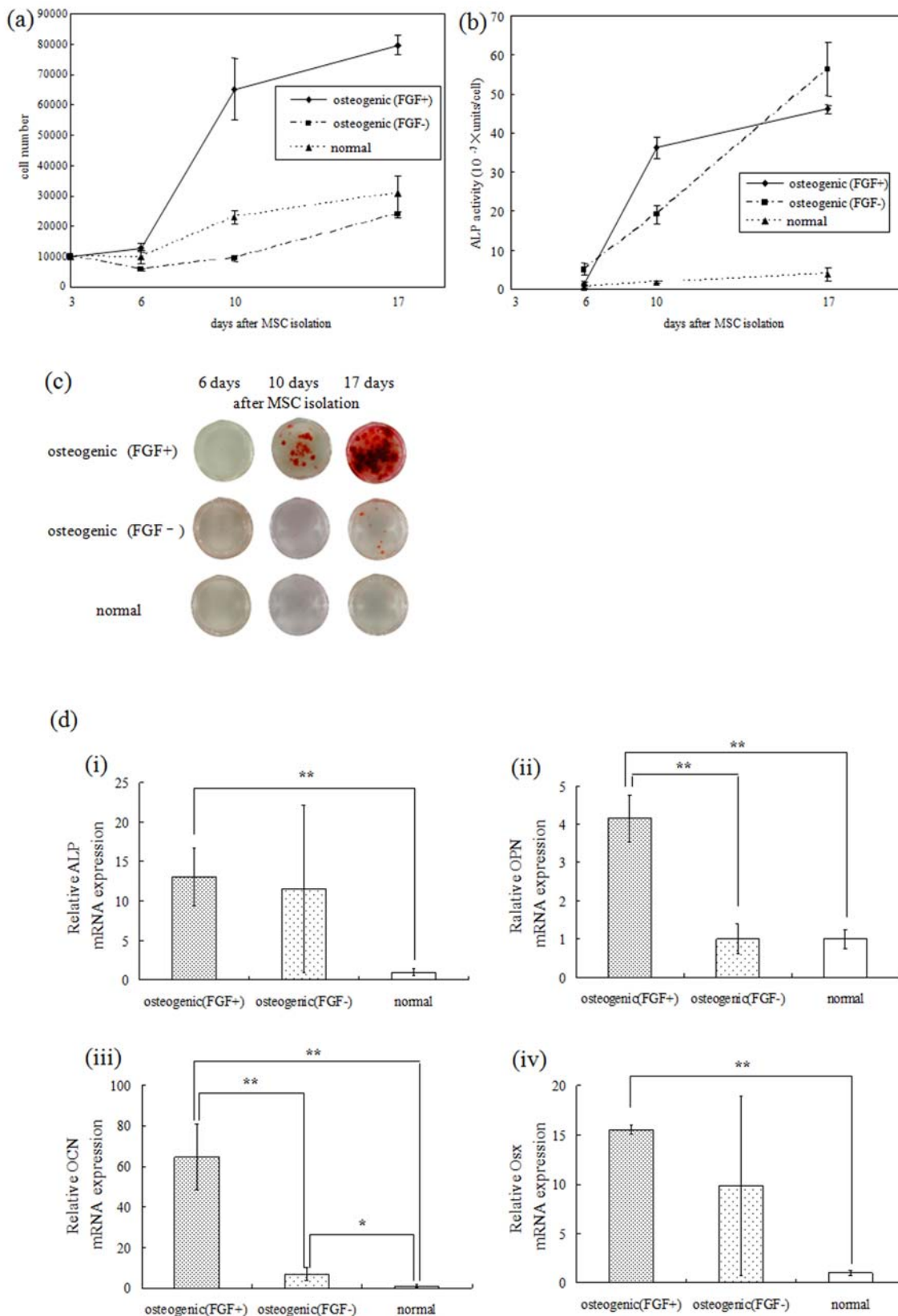|	OCP/Col•MSCs	OCP/Col
TV (mm³)	45.76±10.77	39.85±8.09
BV (mm³)	26.80±11.82	23.42±6.58
BS (mm²)	309.09±58.22	259.34±40.17
BS/BV (1/mm)	12.41±2.99	11.33±1.48
BV/TV (%)	56.70±11.83	58.26±5.41
BMD (mg/cm³)	657.33±18.16*	632.70±3.89

Fig. 2. (a) Results of MSC proliferation after isolation in each culture medium. (b) Results of ALP activity of MSCs after isolation in each culture medium. (c) Alizarin red staining of MSCs cultured in each medium at 6, 10 and 17 d after isolation. (d) Normalised (i) ALP, (ii) OPN, (iii) OCN, and (iv) Osx mRNA expression (to GAPDH) at 10 d after MSC isolation by TaqMan real-time PCR. *$p < 0.05$, **$p < 0.01$.

10 d ($p < 0.01$). The ALP activity of FGF(-) MSCs was higher than the FGF(+) MSCs on day 17. In addition, there was a significant difference in ALP activity of the MSCs between the FGF(+) and FGF(-) groups ($p < 0.05$) and between the FGF(+) and normal culture groups on day 17 ($p < 0.01$). The effect of osteogenic (FGF+) medium on the development of mineralised structures in MSC cultures is shown in Fig. 2c. Alizarin red S staining was used as an indicator of calcification. The addition of bFGF to the osteogenic medium markedly enhanced the amount of mineralised bone-like tissue formation. No calcareous deposit was observed in normal MSCs through 17 d of the culture period. Fig. 2d shows the expression of osteogenic genes quantified by real-time TaqMan PCR of osteogenic (FGF+), osteogenic (FGF-), and normal MSCs 10 d after MSC isolation. The expression levels of ALP, OPN, OCN, and Osx were approximately 13.1, 4.1, 64.5 and 15.5-fold higher, respectively, than those of normal MSCs when the cells were grown in osteogenic (FGF+) medium. The expression levels of OPN and OCN in osteogenic (FGF-) MSCs were significantly lower than those of osteogenic (FGF+) MSCs.

In vivo bone-formation ability of osteogenic (FGF+) MSCs and normal MSCs

We next examined the process of bone repair by micro-CT in critical-sized defects produced in rat calvaria. Table 1 shows the BMD estimated by micro-CT analysis and the new-Bone% estimated by histomorphometric measurements of the OCP/Col·MSCs or OCP/Col·nor-MSCs 4 weeks after implantation. Both the BMD and new-Bone% of OCP/Col·MSCs were significantly higher than those of OCP/Col·nor-MSCs.

Bone regeneration by the OCP/Col scaffold seeded with osteogenic (FGF+) MSCs

The results of the *in vitro* and *in vivo* studies described above demonstrate the significant superiority of the osteogenic (FGF+) condition over the other culture conditions. Therefore, osteogenic (FGF+) MSCs were used to analyse bone regeneration in combination with the OCP/Col scaffold in a rat calvaria defect in order to evaluate whether the crystals of OCP in a collagen matrix can stimulate the introduced MSCs. The results of the radiographic examination are shown in Fig. 3. It has been previously shown that soft x-ray conditions used for measurement do not exhibit any radiopacity in OCP/Col disks before implantation (Kamakura *et al.*, 2006). OCP/Col·MSCs and OCP/Col disks implanted in a rat calvaria defect showed radiopacity at 4 and 8 weeks post-implantation. Col·MSCs or collagen disks implanted in the defect also showed isolated, round radiopacities scattered throughout the defect. There was slight radiopacity along the defect margin in the untreated group. Table 2 shows the quantification of the bone microstructure in the OCP/Col·MSCs and OCP/Col disks 8 weeks post-implantation. There were no significant differences in radiomorphometric parameters (BS/BV and BV/TV) between the OCP/Col·MSCs and OCP/Col groups. However, the BS of the OCP/Col·MSCs group was greater than the OCP/Col group, and the BMD of the OCP/Col·MSCs group was
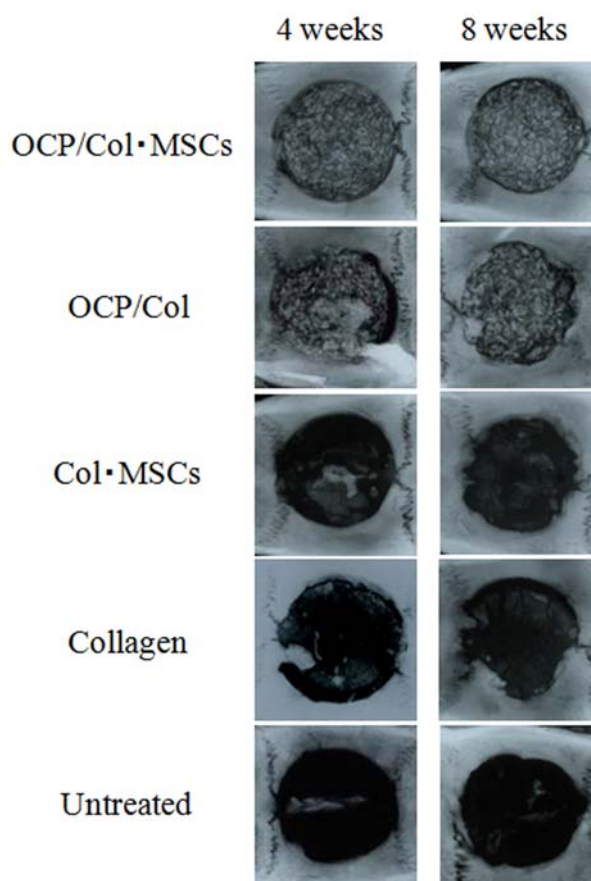

Fig. 3. Radiographs of rat calvaria defect treated with OCP/Col·MSCs, OCP/Col, Col·MSCs, or collagen at 4 and 8 weeks. Defect size was 9 mm in diameter.

significantly higher than the OCP/Col group. Figs. 4 and 5 show the histological sections of each material in the presence or absence of cultured MSCs implanted in a rat calvaria defect. At 4 weeks, newly formed bone was clearly observed in the connective tissue of the defect implanted with a OCP/Col·MSCs disk. In contrast, newly formed bone was limited to the area within the defect for rats implanted with an OCP/Col disk. Newly formed bone was minimal in the defect implanted with Col·MSCs or collagen disks as well as the group left untreated. At 8 weeks, a greater expansion of newly formed bone was observed in the calvaria defect implanted with OCP/Col·MSCs or OCP/Col disks. Some OCP granules within the OCP/Col disk were surrounded by multinucleated giant cells (MNGCs), but inflammatory cell infiltration was not observed at the implantation site. In the Col·MSCs and collagen groups, bone formation was observed sporadically in the collagen disks. In addition, bone regeneration in the untreated control was restricted to the defect margin.

Histomorphometric findings for the new-Bone% and rem-Imp% are shown in Figs. 6 and 7, respectively. The values for new-Bone% ± SD in the OCP/Col·MSC, OCP/Col, Col·MSC, collagen, and untreated groups were 44.1 ±1.7, 22.7 ±1.3, 16.6 ±0.9, 10.8 ±1.5 and 4.1 ±0.8 at 4 weeks, and 52.6 ±1.3, 39.7 ±1.6, 21.2 ±2.8, 16.6 ±1.2 and 4.2 ±3.7 at 8 weeks, respectively. The values for rem-
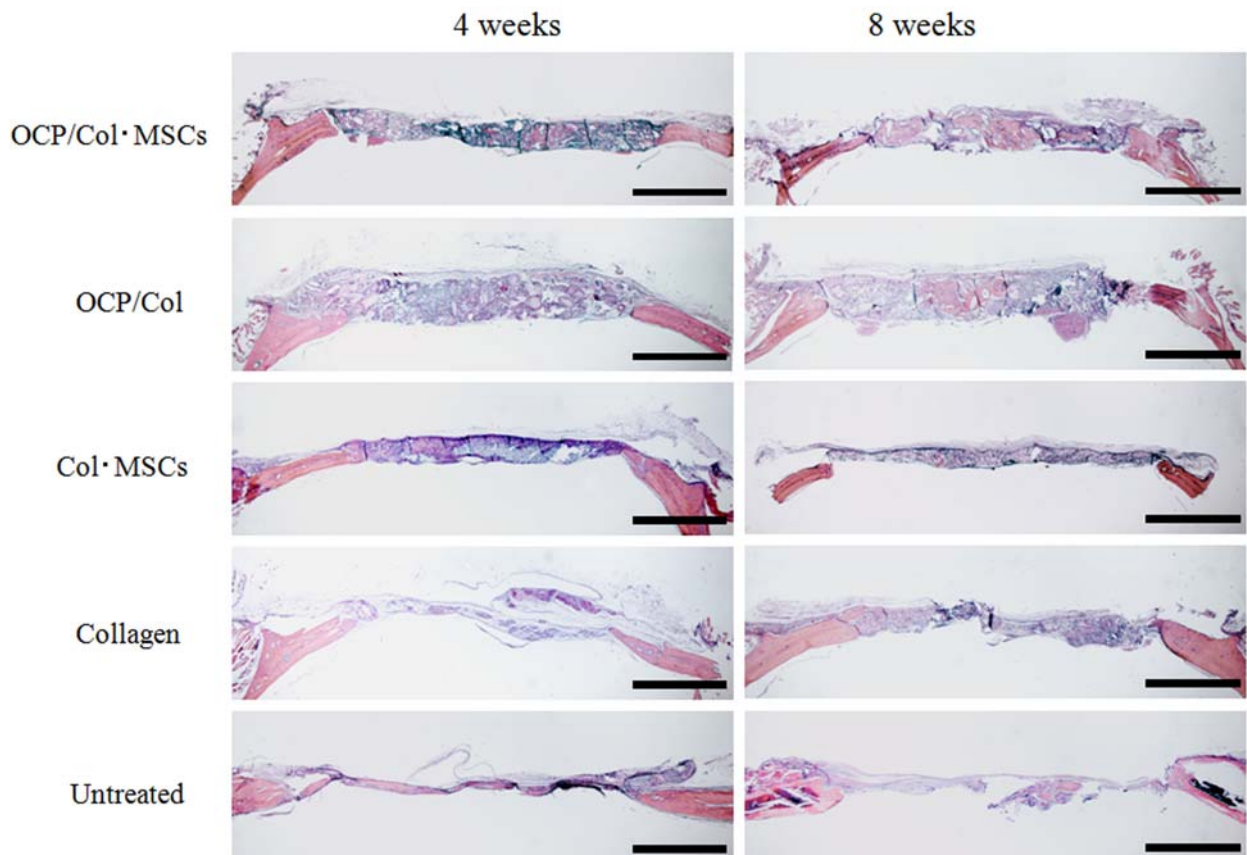
Fig. 4. Overview of histological sections with haematoxylin-eosin staining on calvaria defect treated with OCP/Col·MSCs, OCP/Col, Col·MSCs, or collagen at 4 and 8 weeks. Bars = 2 mm.
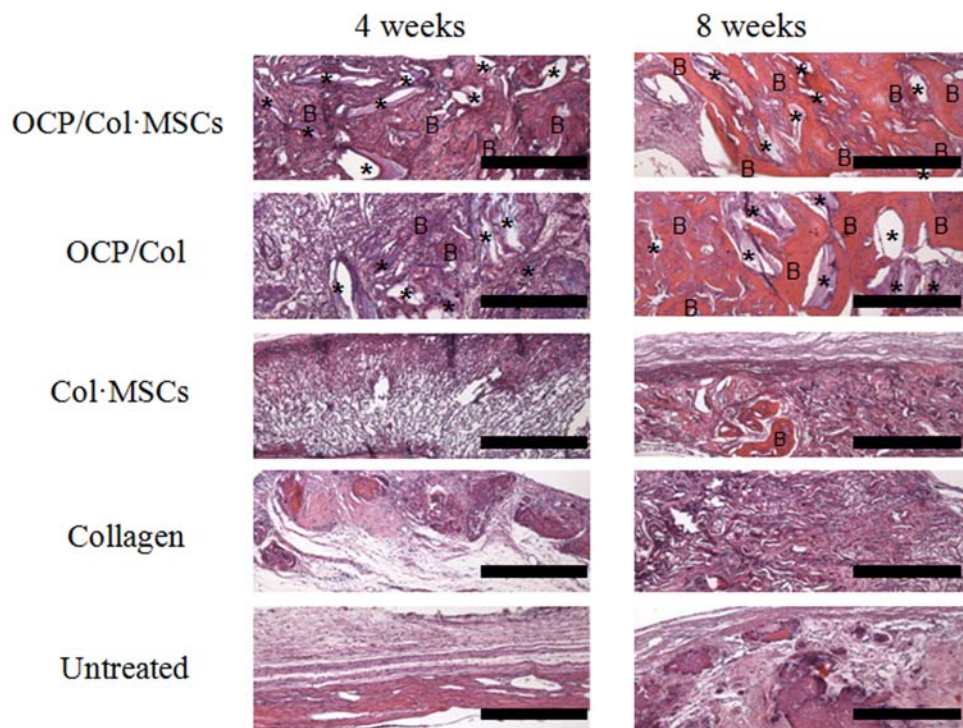

Fig. 5. Histological examination of calvarial defects with implantation of OCP/Col·MSCs, OCP/Col, Col·MSCs, or collagen. Bars = 500 μm. * = remaining OCP. B = newly formed bone.
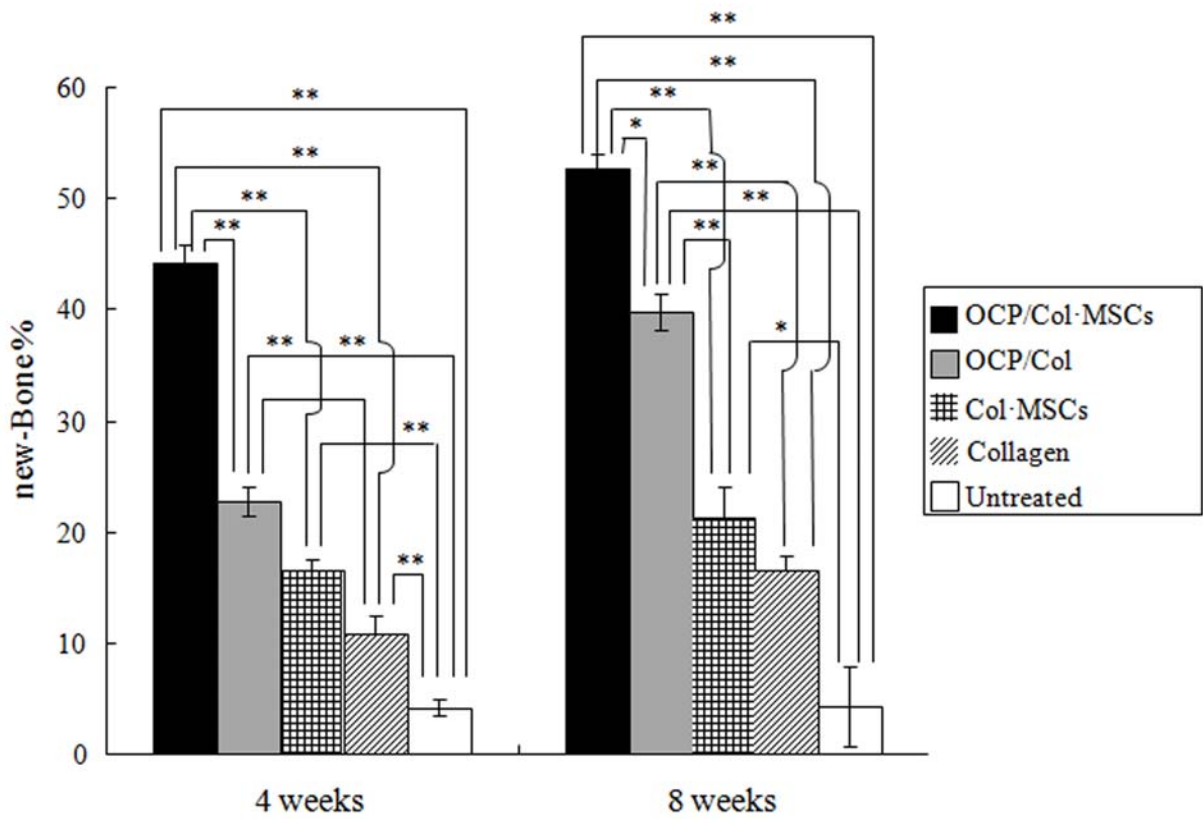
Fig. 6. Histomorphometric findings regarding the area of newly formed bone (new-Bone%) in the defects. $*p < 0.05$, $**p < 0.01$.
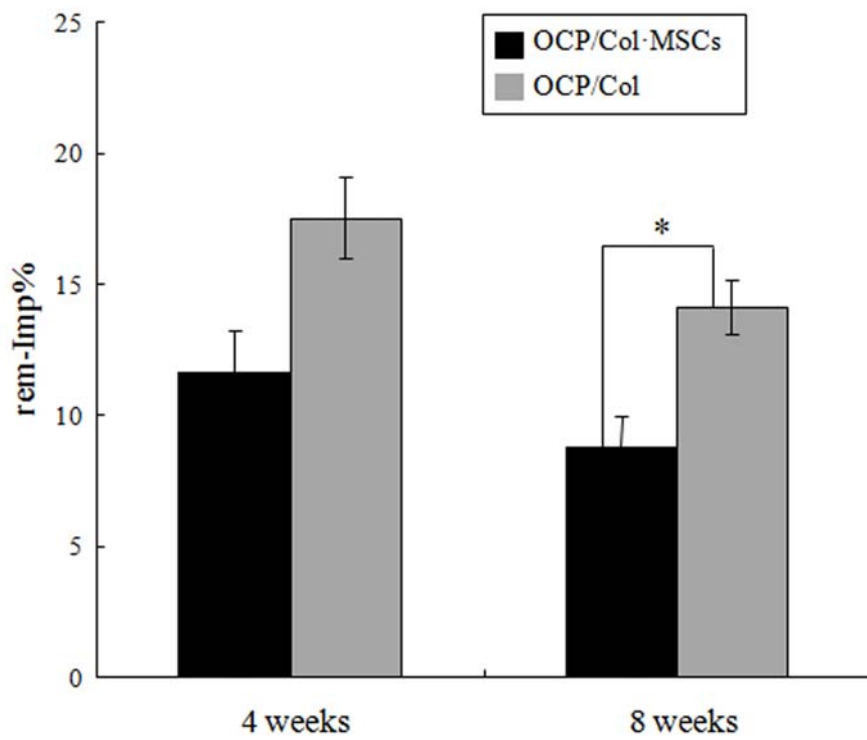

Fig. 7. Quantitative analysis of remaining implant OCP granules (rem-Imp%) in the defect. $*p < 0.05$.

Imp%±SD in the OCP/Col·MSC and OCP/Col groups were 11.6 ±1.6 and 17.5 ±1.6 at 4 weeks, and 8.9 ±1.1 and 14.1 ±0.0 at 8 weeks, respectively. The new-Bone% of the OCP/Col·MSC group was significantly higher than the OCP/Col group. Moreover, the new-Bone% of the OCP/Col·MSC and OCP/Col groups significantly increased at 8 weeks. The analysis revealed that the OCP in the OCP/Col·MSC group was resorbed significantly more than that of the OCP/Col group. In the OCP/Col·MSC and Col·MSC groups, there was a significant difference in new-Bone% throughout the implantation period. In addition, the value for the new-Bone% of the Col·MSC group was slightly higher than the collagen alone group throughout the implantation period.

Discussion

MSCs have been considered to be a suitable autologous cell source for the generation of tissue-engineered bone. Defining the culture conditions for the *in vitro* proliferation and differentiation of MSCs is important for tissue engineering therapy. Several reports have shown that bFGF markedly increases the growth rate and lifespan of MSCs. Furthermore, the incubation of MSCs with bFGF retains their differentiation potential throughout numerous mitotic divisions (Tutsumi *et al.*, 2001; Bianchi *et al.*, 2003). In these reports, bFGF was added at the first passage and continued until the cells were confluent. When the cells reached confluence, differentiation was induced by osteogenic medium containing dexamethasone, ascorbic acid and β-glycerophosphate without bFGF. In our present study, we used this osteogenic culture condition as a "normal" condition (Fig. 1). We showed that osteogenic medium containing bFGF (osteogenic (FGF+)) enhanced the proliferation of MSCs when added early in the culture period (Fig. 1), but also increased the activity of ALP, the expression level of osteogenic differentiation related genes, and the amount of mineralised nodule formation (Fig. 2). Pitaru *et al.* previously showed that bFGF has the capacity to stimulate both the growth and differentiation of rat MSCs (Kotev-Emeth *et al.*, 2002; Pitaru *et al.*, 1993). Scutt *et al.* also reported that bFGF could stimulate the osteogenic differentiation of rat MSCs, and that continued exposure to bFGF increased the formation of bone-like tissue *in vitro* (Scutt and Bertram, 1999). Our results are consistent with these reports. In this study, we observed very weak alizarin red staining in osteogenic (FGF-) MSCs with increasing ALP activity by day 17 of the culture period (Figs. 2b and 2c). There are several possible explanations for these findings, such as (1) the cell proliferation in the absence of FGF was slow, whereas that in the presence of FGF was markedly enhanced; (2) ALP activity was expressed as a value per unit cell; or (3) the lower cellular density in the absence of FGF resulted in the detection of very few mineral deposits. Similar findings have also been reported in a previous study (Maegawa *et al.*, 2007).

We further examined the *in vivo* bone-forming efficiency of MSCs cultured in osteogenic (bFGF+) medium or normal medium using OCP/Col scaffolds. Enhanced new bone formation and a higher bone mineral density were observed with the transplantation of MSCs that had been cultured in osteogenic (FGF+) medium (Table 1). Yoshikawa *et al.* previously reported that *in vitro* ALP activity and bone Gla protein content correlated well with the amount of newly formed bone *in vivo* (Yoshikawa *et al.*, 1992). In fact, the present procedure using osteogenic medium containing bFGF increased ALP activity compared to normal culture conditions, which resulted in enhanced formation of new bone.

It has been reported that several weeks are required to prepare and differentiate MSCs to osteoblastic cells for transplantation (i.e., primary culture in standard medium for 10 d followed by a subculture in differentiation medium for 2 weeks (Yoshikawa *et al.*, 1996; Okamoto *et al.*, 2006)). In contrast, the present procedure shortened the time required for transplantation to approximately 10 d. These results show the usefulness of our *in vitro* cell culture expansion-differentiation technique using an osteogenic (FGF+) condition, which was confirmed after *in vivo* implantation. Since shortening the culture period for osteogenic differentiation reduces the time required for the bone defect to heal, the present procedure used to accelerate both the growth and biochemical functions of MSCs could be therapeutically useful. However, it remains to be shown that the present material would have a similar effect on human MSCs.

Several studies have suggested that inorganic ceramic materials not only act as simple scaffold materials, but also affect osteoblast functions on the cell surface through direct interactions (Kokubo *et al.*, 2009). A recent study showed that β-TCP can affect the signal transduction of human primary osteoblasts (Lu and Zreiqat, 2010). Dissolution of the material elements, such as calcium ions, and the adsorption of biomolecules on their surfaces explain the bioactivity of these ceramics (Kokubo, 1991; Ducheyne and Qui, 1999). β-TCP has also been shown to affect MSC functions, including protein synthesis (Kasten *et al.*, 2008), bone induction within a gelatin sponge matrix in relation to its dose (Takahashi *et al.*, 2005), and the osteogenic ability after chondrogenic differentiation (Janicki *et al.*, 2010). BCP and Ca-deficient HA reduced MSC differentiation and the calcification ability when added directly to the cell culture media (Saldaña *et al.*, 2009). Ca-deficient HA was also found to induce a stronger inhibition of MSC differentiation than BCP when the cells were indirectly co-cultured with these materials, which was explained by calcium uptake from the medium due to the crystal imperfection of Ca-deficient HA (Saldaña *et al.*, 2009). Therefore, calcium phosphate ceramics may affect MSC differentiation.

We previously showed that OCP/Col disks induce bone regeneration better than collagen disks (Kamakura *et al.*, 2006; Kawai *et al.*, 2009). Interestingly, in the present study we found that OCP/Col disks implanted without MSCs into bone defects enhanced bone regeneration better than collagen disks with MSCs (Figs. 3-6). However, micro-CT quantification demonstrated significant differences in bone mineral density between the OCP/Col composites implanted with and without MSCs, suggesting that MSCs inoculated on the OCP/Col composite were effective at inducing bone maturation (Table 2). In fact, the amount

of newly formed bone was higher in the OCP/Col·MSCs group than the Col·MSCs group. These results suggest that the inclusion of OCP crystals in this composite material supports both host cells and transplanted MSCs for the induction of new bone formation. Recent studies have demonstrated that OCP is capable of inducing the differentiation of stromal cells into osteoblastic cells *in vitro* (Shelton *et al.*, 2006; Liu *et al.*, 2007; Anada *et al.*, 2008). It has been suggested that the stimulatory capacity of OCP to raise osteoconductive characteristics stems from a physicochemical change caused by the conversion of OCP to HA (Suzuki *et al.*, 2006a; Suzuki *et al.*, 2006b). Even with the OCP/Col composite, OCP crystals converted to apatite under physiological conditions (Suzuki *et al.*, 2009). The OCP-HA conversion of OCP crystals is accompanied by calcium ion consumption as well as phosphate ion release into the surrounding solution (Suzuki *et al.*, 2006a), and it has been hypothesised that these ions can modify osteoblast functions (Bellows *et al.*, 1992; Dvorak *et al.*, 2004). In fact, the corresponding changes in ion concentration have also been observed in culture medium incubated with OCP (Anada *et al.*, 2008; Takami *et al.*, 2009), suggesting that the solution-mediated stimulation of osteoblasts occurs not only under *in vitro* physiological conditions, but also after *in vivo* implantation.

The amount of OCP remaining 8 weeks after implantation was significantly different between the groups with and without MSCs (Fig. 7). Previous *in vivo* studies have shown that OCP increases the number of osteoclast-like cells that are positive for tartrate-resistant acid phosphatase activity (Imaizumi *et al.*, 2006; Kikawa *et al.*, 2009; Murakami *et al.*, 2010). These results suggest that OCP itself enhances bone formation, which is accompanied by biodegradation and a concomitant increase in osteoclastic cellular resorption of OCP (Imaizumi *et al.*, 2006; Kikawa *et al.*, 2009; Murakami *et al.*, 2010). We previously reported (Takami *et al.*, 2009) that OCP can induce the formation of osteoclasts from precursors in bone marrow cells if co-cultured with osteoblasts, even in the absence of vitamin D_3, which is a factor that increases the osteoclast-inducing molecule receptor activator of NF-κB ligand (RANKL) in osteoblasts. Therefore, it should be determined whether OCP has a similar effect on MSCs for inducing osteoclast differentiation.

Conclusions

The results of the present study demonstrate that OCP crystals in a collagen matrix are able to support exogenous MSC-mediated bone regeneration in an OCP/Col scaffold implanted in rats with critical-sized defects. These results also suggest that OCP crystals have a similar effect on host osteoblasts that invade the OCP/Col scaffold within the implantation site. The MSCs grown using the present cell culture procedure with osteogenic (FGF+) medium accelerated the growth and differentiation of the cells more efficiently, and may provide a useful cellular source for bone regeneration if combined with the OCP/Col scaffold. Taken together, our results show that OCP/Col·MSCs disks have osteogenic capabilities that are supported

by the physicochemical properties of the OCP structure and are therefore potential candidates for autologous transplantation.

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Discussion with Reviewers

Reviewer I: As mentioned in the Introduction section, OCP tends to convert to HA in a proper aqueous environment. Did this conversion occur during the *in vitro* culture of MSCs and biomaterials? Did this conversion occur in the *in vivo* experiments? If the answer is yes to either question, is it possible that the better bone generation on OCP/Col could be attributed to this conversion (i.e., attributed to HA, not to OCP)?

Authors: We did not analyse the OCP-HA conversion *in vitro* and *in vivo* in the present study. However, the previous studies confirmed that OCP without any matrix materials and OCP in Col matrix or in Alginate matrix tend to convert to HA phases in the cell culture media with or without osteoblastic cells and also *in vivo* if implanted in rat calvaria defects (Suzuki *et al.*, 2006b, text reference) and in mouse (Suzuki *et al.*, 1991, text reference) and rat (Suzuki *et al.*, 2009, text reference) subperiosteal region on calvaria and in mouse abdomen subcutaneous tissue near facia (Suzuki *et al.*, 1993). The previous report (Suzuki *et al.*, 2006b) showed that bone regeneration determined histomorphometrically and osteoblastic differentiation at mRNA level in semi-quantitative (Suzuki *et al.*, 2006b) and quantitative (Anada *et al.*, 2008, text reference) analyses are enhanced by OCP which is hydrolysing into HA *in situ* whereas they are not enhanced by OCP hydrolysate (Ca-deficient HA material obtained *via* hydrolysis experiment of OCP). From these results, we concluded that a process from OCP to HA itself, not OCP crystals or HA crystals, should exhibit a stimulatory effect on osteoblastic differentiation and bone regeneration.