Enhancement of Osteogenesis by Concanavalin A in Human Bone Marrow Mesenchymal Stem Cell Cultures

Running title: ConA enhances osteogenesis in MSC cultures

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ABSTRACT: *This study investigates concanavalin A (ConA) as a novel factor that may enhance osteogenesis of mesenchymal stem cells (MSCs) in vitro. Various factors have been studied as promoting factors for MSC osteogenesis in vivo and in vitro. However, their safety, effectiveness, and cost may not be ideal. So, human MSCs were cultured in osteogenic medium in the presence or absence of ConA. We used calcium assays to compare the effects of ConA and BMP-2 on MSC calcification. Also enzyme-linked immunosorbent assay (ELISA) and quantitative PCR were used to evaluate the expression levels of bone specific markers. ConA was observed to enhance the calcification and this effect was comparable to that of BMP-2. Combination of ConA and BMP-2 further enhanced the calcification slightly but significantly. ConA also increased osteocalcin and BMP-2 protein levels in the medium of MSC cultures. Furthermore, ConA increased osteocalcin, RUNX2, BMP-2, and BMP-4 mRNA expression levels. However, gene expression pattern of MSCs stimulated by ConA was different from that observed with BMP-2. These results, taken together, suggest that ConA and BMP-2 enhance MSC osteogenesis via different pathways. The ConA-induced bone formation in MSC cultures may be useful in regenerative medicine or tissue engineering in clinical studies and in*

basic research on bone formation.

KEY WORDS: Concanavalin A, Mesenchymal stem cells, Calcification, Osteogenesis

INTRODUCTION

Mesenchymal stem cells (MSCs) have multilineage developmental potential and can differentiate into osteoblasts, chondrocytes, or adipocytes *(1)*. MSCs can be easily obtained by bone marrow aspiration. Furthermore, MSCs can be expanded in large scale *in vitro* (2). The usage of MSCs with tissue-engineering techniques has been reported to promote regeneration of various tissues such as bone and cartilage (3-6).

After transplantation, MSCs that have differentiated into osteoblastic cells *in vitro* promote bone regeneration more prominently than do undifferentiated MSCs (7, 8). Until now, many cytokines, e.g. bone morphogenic protein-2 (BMP-2), transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF), and insulin-like growth factor (IGF-I) (9-11), along with prostaglandin E (PGE) (12) and statin (13), have been studied as promoting factors of MSC osteogenesis *in vivo* and *in vitro*. However, their safety, effectiveness, and cost may not be ideal.

In this study, we investigated concanavalin A (ConA) as a novel factor that may enhance osteogenesis of MSCs *in vitro*. ConA, a glycoprotein with suger-binding properties, activates lymphocytes (mitogen-activity) and

aggregates erythrocytes (14-16) and is useful in distinguishing cancer cells from normal cells (17). ConA also strengthens cell adhesion to various scaffolds (18). ConA may therefore have various applications in regenerative medicine. ConA promotes the formation of bone and cartilage-like tissues at the periosteum *in vivo (19, 20)* and in chondrocytes *in vivo (20, 21)*. However, the effects of ConA on MSC osteogenesis have not been fully understood. In this study, we used ConA to enhance bone formation so as to examine the efficacy of ConA for in vitro osteogenesis.

MATERIALS AND METHODS

Materials

ConA (SIGMA, St. Louis, MO) was diluted into phosphate-buffered saline (PBS) and the concentration was quantified by Lowry protein assay. BMP-2 was supplied from Yamanouchi Pharmaceutical Co., Ltd. (Tokyo, Japan). BMP-2 was adjusted at 300 μg/ml with LE6 buffer [pH 4.5, 5 mM L-glutamine (SIGMA), 5 mM NaCl (SIGMA), 2.5% glycine (SIGMA), 0.5% sucrose (SIGMA), and 0.01% Tween80 (SIGMA)]. BMP-2 was further diluted with PBS and added to the culture medium.

Cell culture

Human MSCs were purchased from Cambrex Bio Science (Walkersville, MD). These cells were identified as CD105-, CD166-, CD29-, and CD44-positive, and CD14-, CD34-, and CD45-negative by flow cytometric analysis. The multipotency of the cells was confirmed by the company. MSCs were maintained with Dulbecco's Modified Eagle's Medium (DMEM; SIGMA, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS; GIBCO BRL, Gaithersburg, MD) and antibiotic-antimycotic (100 units/ml penicillin G, 100 μg/ml streptomycin,

and 0.25 mg/ml amphotericin B, GIBCO BRL) (Medium A) at 37°C in 5% CO_2/95% air. MSCs obtained from the cultures at passages 3-5 were used for experiments. MSCs were seeded at 5 x10^3 cells/cm^2 in Medium A for each experiment. After MSCs reached to confluent, the medium was changed to osteogenic medium [Minimum Essential Alpha Medium (GIBCO BRL) containing the above antibiotic-antimycotic, 10^{-8} M dexamethasone (Dex; SIGMA), 10mM β-glycerophosphate (SIGMA), and 50 mg/ml ascorbic acid 2-phosphate (SIGMA)], then the effects of ConA or BMP-2 to MSC osteogenesis were analyzed as following.

Alizarin-Red Staining

MSCs were cultured in 48-well tissue culture plates (5-mm diameter) (BD Biosciences). After MSCs reached to confluent, the medium was changed to osteogenic medium with or without ConA and BMP-2. After 14 days, the culture medium was aspirated and the cell layer was washed twice with 10 mM Tris-Buffer containing 0.9% NaCl (pH7.6). The cell layers were fixed for 1 hour with 200 μl 95% ethanol. Fixed cell layers were stained by 200 μl 1% alizarin red S solution (Chroma-Gesellschaft Schmid GMBH&Co., Kongen, Gemany) for 5

min under shading at room temperature. After cell layers were washed twice with water, a photographic image was obtained.

Calcium Content

MSCs were cultured in 48-well tissue culture plates. After MSCs reached to confluent, the medium was changed to osteogenic medium with or without ConA or/and BMP-2. After 14 days, the medium was aspirated, cell layers were washed twice with 200 μl PBS, and 200 μl 10% trichloroacetic acid solution was then added to the cultures. The cells and extracellular matrix were homogenized for 10 seconds using Vibracell® (Sonics & Materials, Inc., Newtown, CT), and 11 μl 10 N NaOH was added for neutralization. The extracts were used for calcium quantification using a Ca C-test Wako kit (Wako Pure Industries, Ltd., Osaka, Japan) and a Multiwell spectrophotometer; Immno Mini NJ-2300 (Nalge Nunc International, Rochester, NY). Calcium ion concentration was determined at wavelength 570 nm.

Enzyme-linked immunosorbent assay (ELISA)

MSCs were cultured in 175 cm^2 flasks. After MSCs reached to confluent, the

medium was changed to osteogenic medium with or without ConA (8 μg/ml) or BMP-2 (50 ng/ml). Twenty-four hours before the end of culture, the medium was changed to phenol red-free DMEM supplemented with 1% antibiotic-antimycotic alone. Cultured medium was collected and ultrafiltrated by Amicon® Ultra-4 10,000 NMWL (Millipore Corporation, Billerica, MA). The concentrated fraction was then used for total protein quantification using the Protein Quantification Kit-Rapid (Dojindo Laboratories, Kumamoto, Japan), and for quantification of osteocalcin, BMP-2, and BMP-4 protein using the Gla-type Osteocalcin EIA kit (TaKaRa Bio Inc, Shiga, Japan), the Quantikine® Human BMP-2 Immunoassay, and the Quantikine® Human BMP-4 Immunoassay (R&D systems, Minneapolis, MN), respectively.

Real-Time PCR

MSCs were cultured in 90 mm diameter tissue culture plates (BD Biosciences). After MSCs reached to confluent, the medium was changed to osteogenic medium with or without ConA (8 μg/ml) or BMP-2 (50 ng/ml). After 3-21 days culture, the cell layers were washed twice with PBS. Total RNA was extracted using TRI®Reagent (SIGMA) and the RNeasy®Mini Kit (QIAGEN,

Tokyo, Japan). DNA was then degraded by DNase I (Invitrogen, Carlsbad, CA). Total RNA concentrations were measured by a ND-1000 spectrophotomer (NanoDrop Technologies, Wilmington, DE), and the total RNA content was then calculated. Total RNA was reverse transcribed with the Omniscript^{TM} RT kit (QIAGEN) and Oligo-dT Primer (Promega, Madison, WI) according to the manufacturer's protocol. Gene expression was analyzed with a TaqMan® probe (SIGMA), Absolute QPCR ROX Mix (AB Gene, Epsom, UK), and the 7900HT Fast Real-Time PCR system (Applied Biosystems, Foster City, CA). The following forward (F), reverse (R) and probe (P) pairs were employed: alkaline phosphatase (ALP) F: 5'-CCGTGGCAACTCTATCTTTGG-3', R: 5'-GCCATACAGGATGGCAGTGA-3', P: 5'-CCCCATGCTGAGTGACACAGACAAGAA-3'; osteocalcin (OC) F: 5'-GCCTCCTGAAAGCCGATGT-3', R: 5'-AAGAGACCCAGGCGCTACCT-3', P: 5'-CCAACTCGTCACAGTCCGGATTGAGCT-3'; runt-related transcription factor 2 (RUNX2) F: 5'-CTCCAACCCACGAATGCACTA-3', R: 5'-CGGACATACCGAGGGACATG-3', P:5'-CCACCTTTACTTACACCCCGCCAGTCAC-3'; bone morphogenetic protein 2 (BMP-2) F: 5'-TCAAGCCAAACACAAACAGC-3', R:

5’-ACGTCTGAACAATGGCATGA-3’, P: 5’-TCCTCTGGCTGATCATCTGAACTC-3’; bone morphogenetic protein 4 (BMP-4) F: 5’-GCCATTCCGTAGTGCCATCC-3’, R: 5’-TTCCCCGTCTCAGGTATCAA-3’, P: 5’-CTGGTAACCGAATGCTGATGGTCGT-3’, bone morphogenetic protein 6 (BMP-6) F: 5’-GCGACACCACAAAGAGTTCA-3’, R: 5’-CCCATACTACACGGGTGTCC-3’ P: 5’-AATTCCGCATCTACAAGGACTGTGT-3’; bone morphogenetic protein 7 (BMP-7) F: 5’-GAGTGTGCCTTCCCTCTGAACT-3’, R: 5’-AGAGGACGGAGATGGCATTG-3’, P: 5’-CTACATGAACGCCACCAACCACGCC-3’; bone morphogenetic protein 9 (BMP-9) F: 5’-ACCTGCCCTTCTTTGTTGTC-3’, R: 5’-ACTGCTCTCACCTGCCTCTG-3’, P: 5’-AGAGCGTGCTCAAGAAGCTGTCCAA-3’. The results were analyzed by a relative quantitative analysis method after correction with 18s ribosome RNA (TaqMan® Gene Expression Assays; Applied Biosystems) as an internal control.

Statistics

Data are presented as the mean of three independent experiments ± SD. Unless otherwise specified, paired t-tests were used when we compared test values with the control value. For comparisons among groups, one-way ANOVA with the Tukey post test was used.

RESULTS

Calcification

Cell layers in MSC cultures maintained in osteogenic medium were stained with Alizarin-Red to evaluate the degree of calcification. ConA enhanced the calcification by Day 14 (Fig. 1. A). Calcium contents of cell layers were determined to compare the effects of ConA and BMP-2 on MSC calcification. ConA increased calcium levels dose-dependently, and the effect reached a maximum at 8 μg/ml. The maximum effect of ConA was similar to that of BMP-2 at >50 ng/ml (Fig. 1. B, C). Combination effect of these two factors was determined in another independent culture. In the presence of ConA, BMP-2 slightly but significantly increased the calcium levels when administered at the same time (Fig. 2).

Osteocalcin

Bone-specific matrix protein, osteocalcin, was quantified by ELISA. The ostecalcin level did not increase until Day 14 regardless of the presence or absence of ConA, but ConA significantly increased the osteocalcin level in the medium of MSC cultures on Day 21 as compared to the basal level of control

cultures without ConA (Fig. 3).

Expression of bone-related genes

The mRNA levels of bone-related genes; BMP-2, 4, 6, 7, 9, alkaline phosphatase (ALP), osteocalcin, and runt-related transcription factor 2 (RUNX2) in the presence of 8 μg/ml ConA or 50 ng/ml BMP-2 were determined using real-time PCR analysis. BMP-2 increased ALP mRNA expression levels, whereas no appreciable ALP up-regulation was observed with ConA (Fig. 4. A). BMP-2 or ConA increased osteocalcin (Fig. 4. B) and RUNX2 (Fig. 4. C) mRNA levels on Day 21. In addition, BMP-2 increased BMP-2 and BMP-6 expression levels, but not BMP-4 expression levels, throughout the whole culture period (Fig. 4. D, E, F). In contrast, ConA increased BMP-2 and BMP-4 expression levels on Day 14, but not BMP-6 expression levels. BMP-7 and BMP-9 could not be detected throughout the whole culture period.

BMP protein levels

ConA increased BMP-2 protein levels in the culture medium on Day 14 (Fig. 5), whereas BMP-4 protein could not be detected throughout the whole culture

period.

Nodule formation and cell aggregation

ConA and BMP-2 induced cell aggregates and calcified nodules in MSC cultures. No difference in morphology was observed between MSC cultures exposed to ConA or BMP-2 (Fig. 6).

DISCUSSION

In this study, we found that ConA enhanced calcification in MSC cultures in the presence of the osteogenesis induction medium, and its effect was partly similar to that of BMP-2. BMP-2, a member of the TGF-β super family (22), which induces ectopic bone formation *in vivo* (23). BMP-2 has also been shown to induce osteogenesis in MSC cultures in the presence of β-glycerophosphate and ascorbic acid 2-phosphate *in vitro* (9). In this study, the addition of BMP-2 increased calcium content in MSC cell layers, and ConA and BMP-2 additively enhanced the calcification (Fig. 2), suggesting that ConA and BMP-2 induce osteogenesis of MSCs by different mechanisms. BMP-2 activates smad1/5/8 by combining BMP receptors (BMPR-1 or BMPR-2), and the activated smad1/5/8 combines with smad4 and controls transcription of bone-related genes (24-27). On the other hand, ConA binds to various glycoproteins on the plasma membrane and aggregates cells. It has been reported that ConA has high affinity for osteogenin/BMP-3 (28). So, ConA could bind soluble morphogenetic proteins synthesized by MSCs and make them available at high concentrations to ConA-aggregated cells.

To characterize the osteogenic induction by ConA, we determined the protein

levels of osteocalcin and BMP, along with mRNA levels of bone-related genes. In MSC cultures, BMP-2 induced mRNA expression of ALP, RUNX2, and osteocalcin, as described previously (29). However, ConA elicited different gene-expression patterns: ConA increased RUNX2 and osteocalcin mRNA expression without an appreciable increase in ALP mRNA expression. BMP-2 increased BMP-2 and BMP-6 mRNA expression, whereas ConA increased BMP-2 and BMP-4 mRNA expression at high level and BMP-6 at low levels. It has been reported that BMP-6 mRNA expression is induced by Dex and that this induction is essential for osteogenesis of MSCs (30). ConA increased BMP-2 protein content in the medium of MSC cultures (Fig. 5). BMP-4 protein was absent in the supernatant in spite of the expression of BMP-4 mRNA, but this might be due to binding of ConA to BMP-4 in the cell layer. Thus, the osteogenesis induced by ConA may be accounted at least partly for autocrine syntheses of BMP-2 and BMP-4.

ConA induces hepatitis in rats and mice (31), however, it has been reported that biological properties of plant lectins are species dependent and cell dependent for their surrounding glycoproteins (32). Furthermore, ConA can be removed from culture cells by incubation with α-D-mannopyranoside as hapten

(33), and ConA bound to the bone graft produced by this method can be removed before transplantation (18). ConA or related glycoproteins may therefore be useful in producing bone grafts *in vitro*. For tissue-engineering, bone grafts are often formed with MSCs activated by growth factors such as BMP-2. However recombinant peptides are expensive. In contrast, ConA is inexpensive, with BMP-2 costing approximately a thousand times more than ConA for a medium change.

In conclusion, we have demonstrated that the addition of ConA to the osteogenic medium enhances osteogenesis of MSCs *in vitro*. ConA-induced bone formation in MSC cultures may be useful in regenerative medicine or tissue engineering in clinical studies and in basic research on bone formation.

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FIGURE LEGEND

Fig. 1 - Effects of ConA and BMP-2 on MSC calcification: (A) Cell layers were stained with Alizarin-Red on Day 14. MSCs were cultured with either non-osteogenic medium (a), or the osteogenic medium (b), and ConA (8μg/ml) added to osteogenic medium (c). Calcium content in MSC cultures in the osteogenic medium containing ConA (0 to 10μg/ml) or BMP (0 to 300 ng/ml) was quantified on Day 14. Data are presented as means ± standard deviation for three cultures (* P<0.05, ** P<0.01). Similar results were obtained in repeated studies.

Fig. 2 - The effects of a combination of ConA and BMP-2: MSCs were incubated in the osteogenic medium with 8 μg/ml ConA only or ConA with BMP-2 50, 100, or 300 ng/ml for 14 days. Data are presented as means ± standard deviation for three cultures. For comparison among groups, ANOVA with the Tukey post test was performed (***p<0.001, ** P<0.01 among each group). Similar results were obtained in repeated studies.

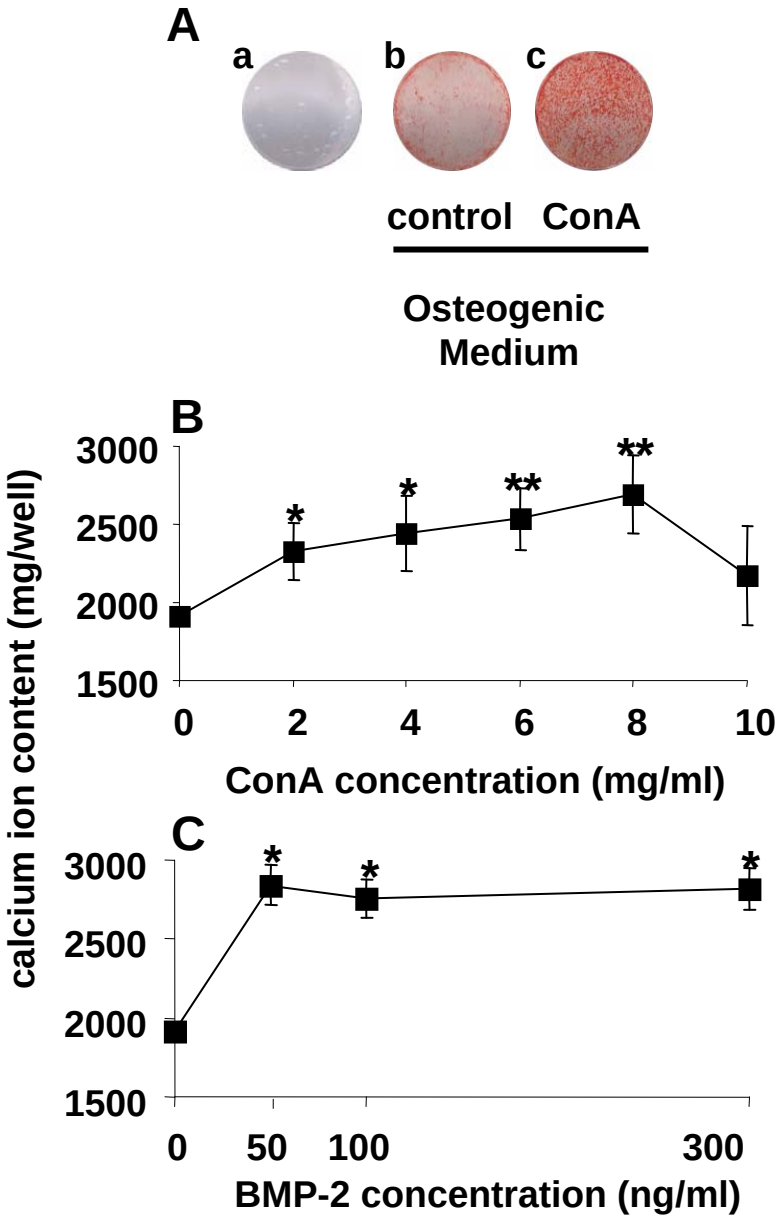
Fig. 3 - Osteocalcin levels in the medium of MSC cultures in the presence or

absence of ConA: MSCs were incubated in the osteogenic medium with 8 μg/ml ConA for 3-21 days. The osteocalcin protein levels in the medium were quantified by ELISA. Data are presented as means ± standard deviation for three cultures (***p<0.001). Similar results were obtained in repeated studies.

Fig. 4 - Effects of ConA (8μg/ml) and BMP-2 (50 ng/ml) on the expression of bone-related genes in MSC cultures: The messenger RNA levels of osteogenic marker genes, including (A) ALP, (B) osteocalcin, (C) RUNX2, (D) BMP-2, (E) BMP-4, and (F) BMP-6, were determined by real-time PCR. Data are presented as means ± standard deviation for four replicate wells. (* P<0.05, ** P<0.01, ***p<0.001). Similar results were obtained in repeated studies.

Fig. 5 - Effects of ConA on BMP-2 protein levels in the culture medium: MSCs were incubated in the osteogenic medium in the presence of 8μg/ml ConA for 3-21 days. BMP-2 protein levels in the culture medium were quantified by ELISA. Data are presented as means ± S.D. Data are presented as means ± standard deviation for three replicate cultures (***p<0.001). Similar results were obtained in repeated studies.

Fig. 6 - The appearance of MSCs exposed to ConA or BMP-2: MSCs were incubated in the osteogenic medium in the absence of these peptides (A; control), in the presence of 50 ng/ml BMP-2 (B) or 8 μg/ml ConA (C). White and black arrows indicate the calcified nodules induced by BMP-2 and ConA, respectively. Bar=200 μm.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b
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Osteogenic Medium
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ConA concentration (mg/ml)
C
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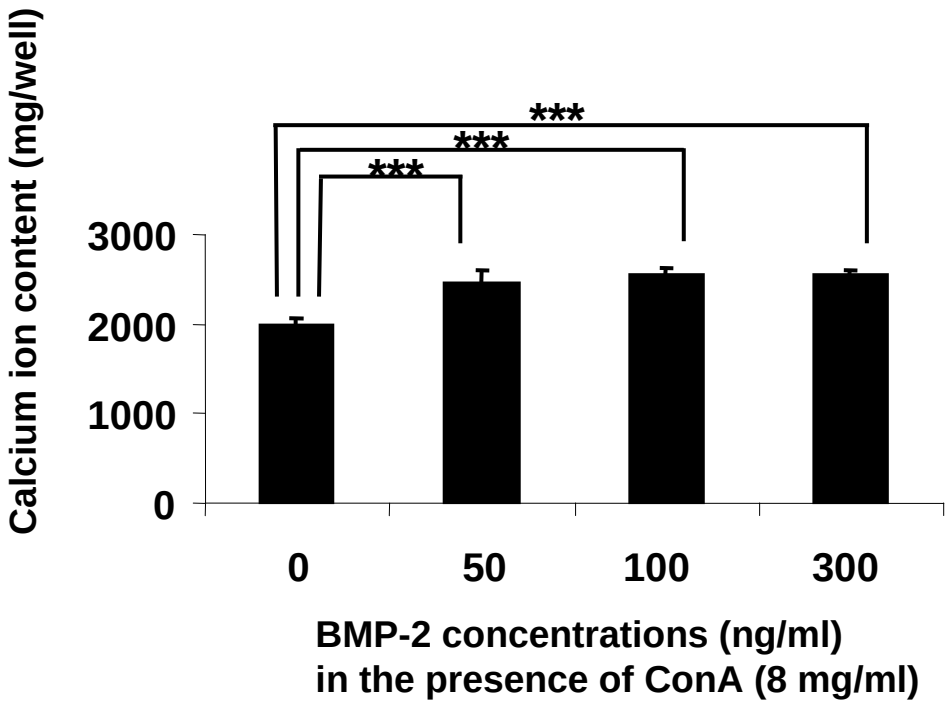
BMP-2 concentration (ng/ml)

Fig.1


Calcium ion content (mg/well)
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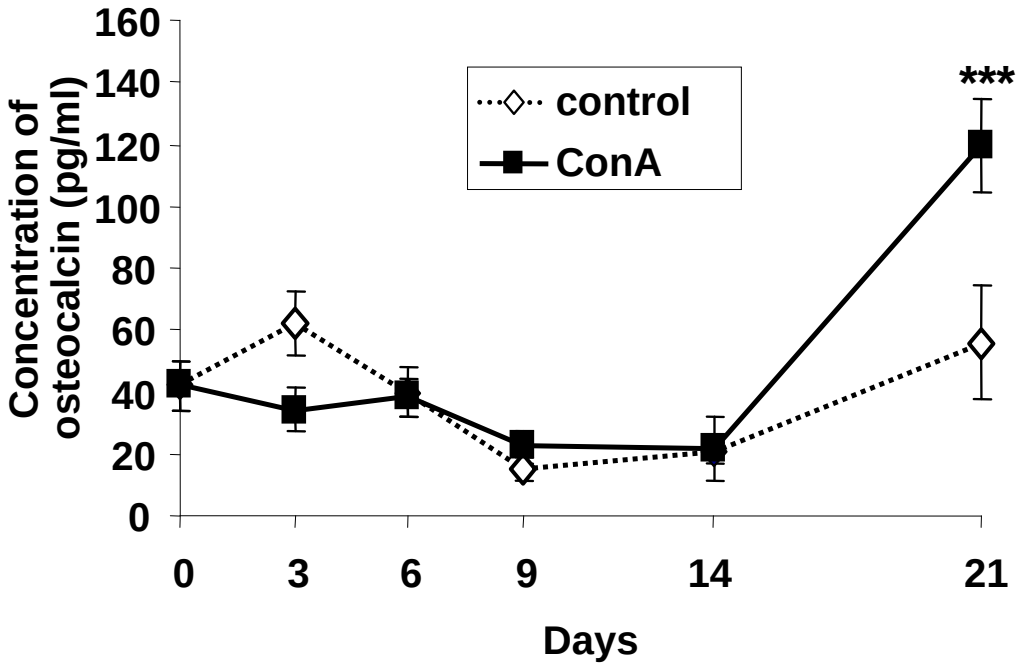
BMP-2 concentrations (ng/ml)
in the presence of ConA (8 mg/ml)

Fig.2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Days

Fig.3

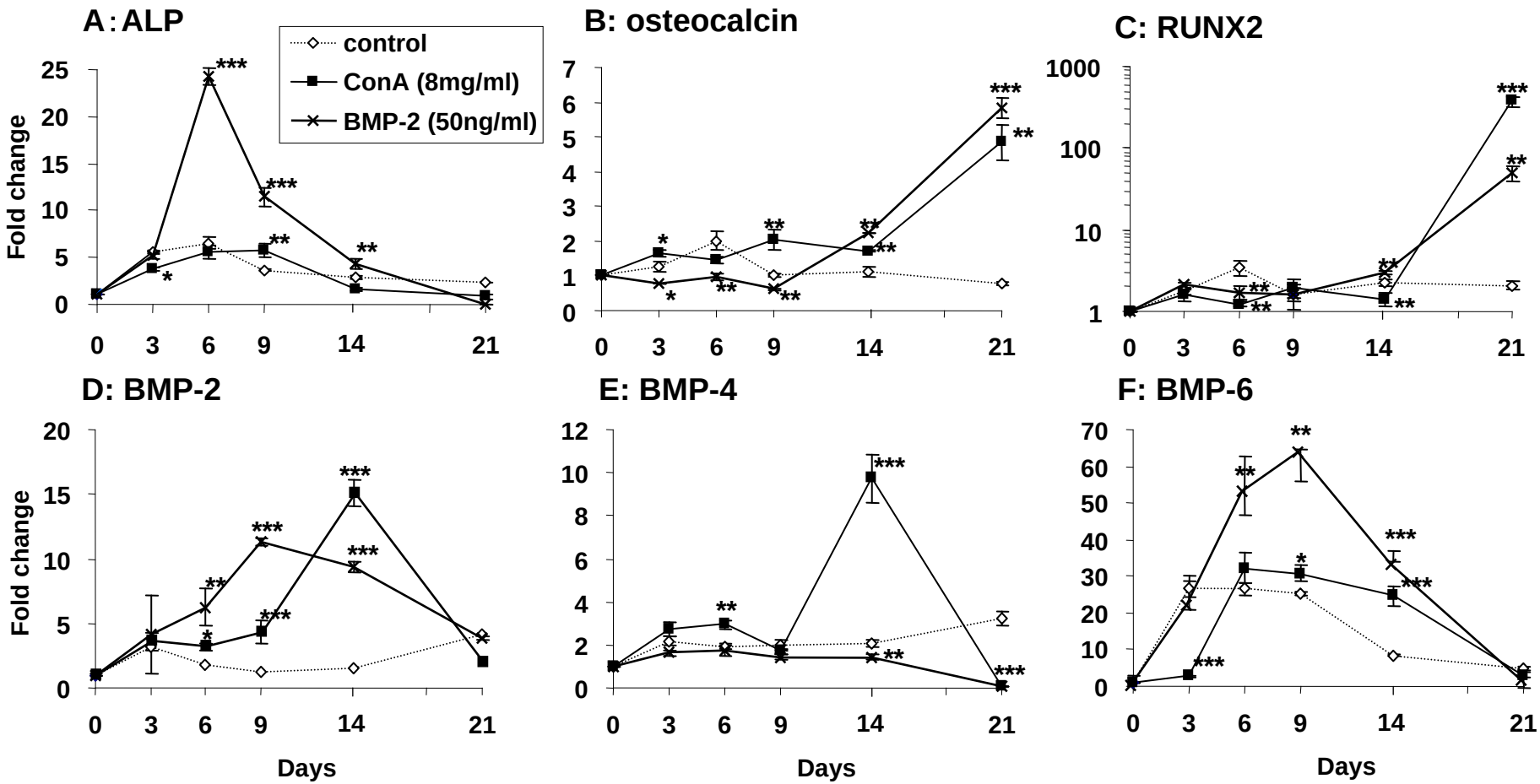
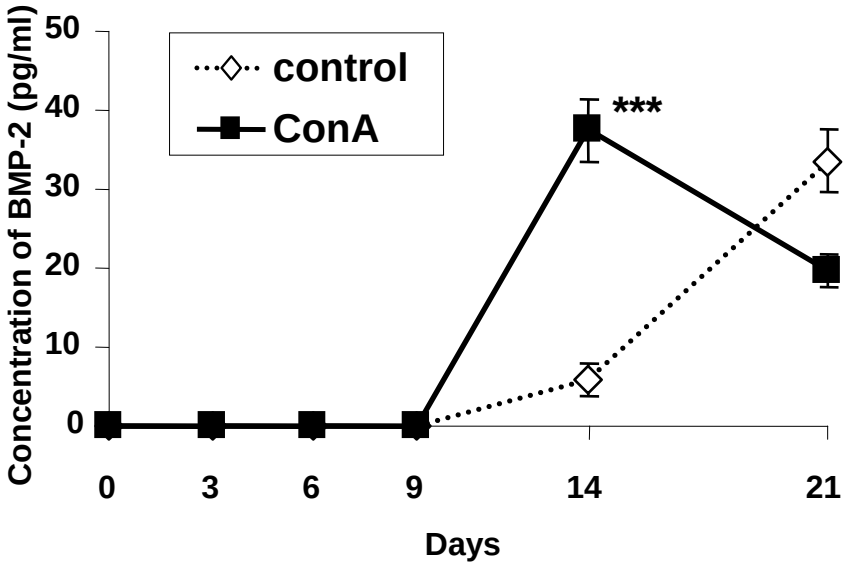

A : ALP
control
ConA (8mg/ml)
BMP-2 (50ng/ml)
Fold change
B: osteocalcin
C: RUNX2
D: BMP-2
E: BMP-4
F: BMP-6
Days

Fig.4


Concentration of BMP-2 (pg/ml)
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Days

Fig.5

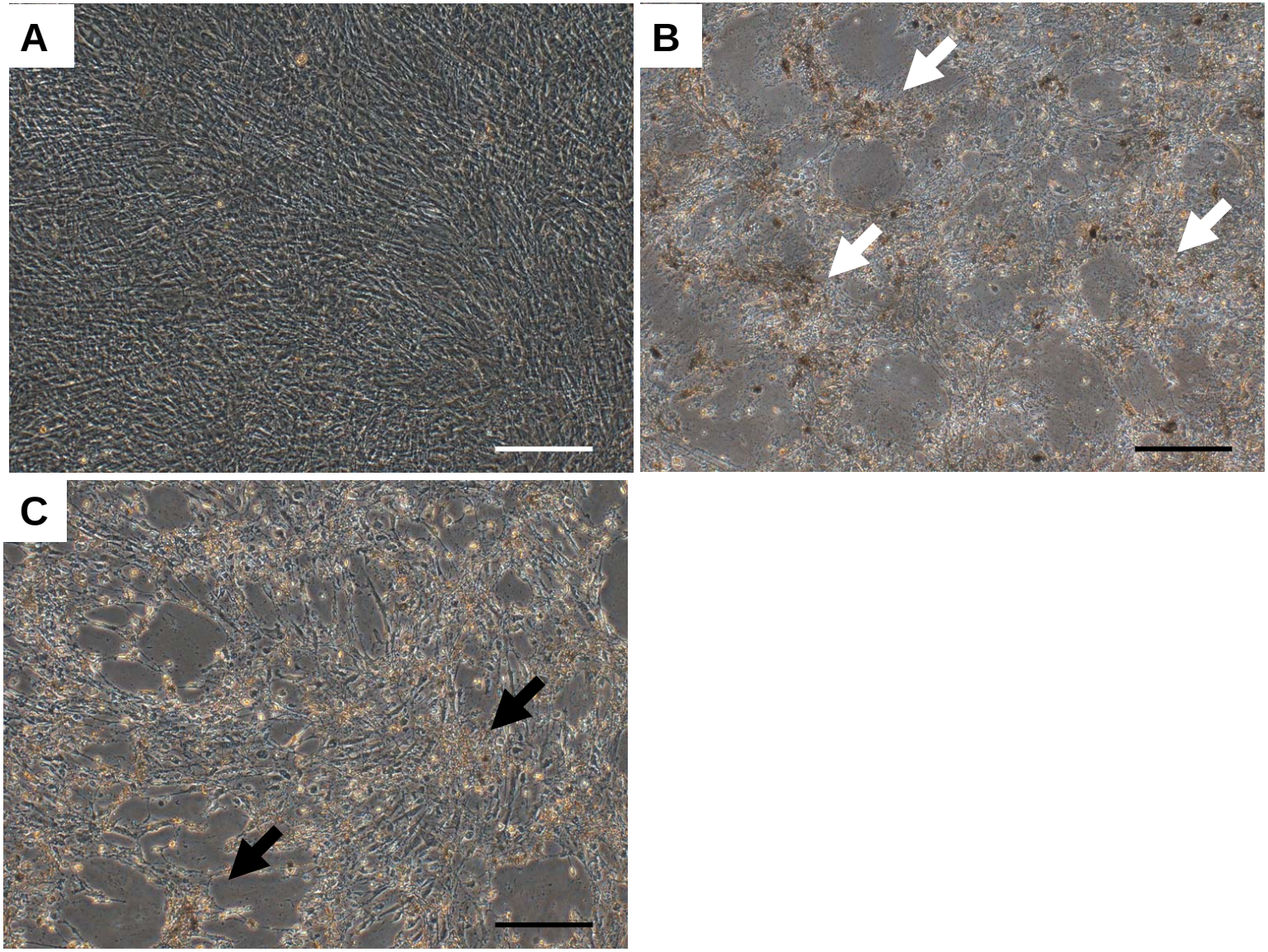

Fig.6