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Effects of BMP-2 and vitamin D_3 on the osteogenic differentiation of adipose stem cells

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ABSTRACT

We studied the effect of bone morphogenetic protein-2 (BMP-2) and vitamin D_3 on the osteogenic differentiation of adipose stem cells (ASCs). ASCs were treated with 10, 50, and 100 ng/ml of BMP-2, and 10^{-8}, 10^{-7}, 10^{-6} M vitamin D_3. Then, to investigate the effects of combined treatment, ASCs were treated with BMP-2 and vitamin D_3 dose-dependently and time-dependently. The osteogenic differentiation was assessed by alkaline phosphatase (ALP) activities/staining and the mineralization was evaluated by Alizarin red S staining. ALP activity and mineralization dose-dependently increased in early stages (ALP on 7th day and mineralization on the 14th day) while all three doses of BMP-2 or vitamin D_3 showed comparable effects in late stages (ALP on the 14th day and mineralization on the 21st day) in ASCs. BMP-2 and vitamin D_3 had synergistic effect on the osteogenic differentiation of ASCs. While all three doses of BMP-2 acted similarly in reinforcing the effect of vitamin D_3, vitamin D_3 dose-dependently augmented the osteogenic effect of BMP-2. When BMP-2 was constantly treated, vitamin D_3 effect did not differ depending on the period of vitamin D_3 treatment. However, when vitamin D_3 was constantly treated, the BMP was more effective when treated for the last 7 days than when treated for the first 7 days. In conclusion, BMP-2 and vitamin D_3 promote osteogenic differentiation of ASCs, and can work synergistically. These results can be used to induce effective and economical osteogenic induction of ASCs for bone tissue engineering.

1. Introduction

Mesenchymal stem cells (MSCs) are an attractive cell source for applications in regenerative medicine owing to their good capacity for proliferation and differentiation. Although bone marrow-derived mesenchymal stem cells (BMSC) have been studied extensively, adipose stem cells (ASCs) have attracted recent attention because they can be acquired easily in large quantities with minimal morbidity, and they possess useful characteristics comparable to BMSC [1]. However, the osteogenic potential of ASCs was reported to be inferior to that of BMSCs when cultured in the same osteogenic medium [2]. Several methods including gene transfer have been used to enhance the osteogenic potential of ASCs [3,4]. However, simpler methods for inducing differentiation from ASCs are also needed for clinical applications. For this purpose, we focused on two substances, BMP-2 and vitamin D_3, which have been used to induce osteogenic differentiation from stem cells [5,6].

Vitamin D_3 has a range of physiological functions in the body, the most important being maintenance of the calcium and phosphorus balance [7]. In osteoblasts, vitamin D_3 normally inhibits cellular proliferation and induces differentiation [8]. Vitamin D_3 promotes osteogenic differentiation in human BMSCs and ASCs *in vitro* [9–11]. The low toxicity of $1,25(OH)_2D_3$ offers a huge advantage in clinical application [12]. Although a murine study reported that a treatment with vitamin D_3 promoted the early stages of osteoblastogenesis *in vitro* [9], other *in vitro* studies suggested that vitamin D_3 also induced the expression of both early and late stage markers of osteoblast differentiation, including ALP, osteopontin, bone sialoprotein, and osteocalcin in MSCs [5,6]. Vitamin D_3 enhances the dexamethasone – induced osteogenic differentiation of human MSCs, elevating the ALP activity and increases the level of matrix mineralization [5,6,13].

Bone morphogenetic proteins (BMPs) are characterized by their ability to induce ectopic ossification at extraskeletal sites [14,15]. BMPs induce osteoblast differentiation and matrix maturation *in vitro* in a wide range of rodent and human progenitor and stem cells [16,17]. BMPs signal via the heteromeric complexes of the types I and II serine threonine kinase receptors, which transmit downstream via the canonical Smad 1/5/8 pathway [18]. In

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addition to the canonical Smad pathway, BMPs can activate the mitogen-activated protein kinase pathway to control osteogenesis [19]. Of several BMPs, BMP-2 has been used to induce osteoblast differentiation *in vitro* in many human and animal models [20,21]. BMP-2 has been synthesized commercially using DNA recombination technology, and is currently being used clinically to enhance spine fusion [22].

Although several studies have demonstrated that BMP-2 or vitamin D_3 enhances the osteogenesis of ASCs from a range of sources [3,23], there is a paucity of studies that investigated the dose–response relationship or combination treatments of these substances in human ASCs with particular focus on bone tissue engineering. Therefore, this study examined the optimal dose and timing for single or combined treatment of BMP-2 and vitamin D_3 for the osteogenic differentiation of human ASCs.

2. Materials and methods

2.1. Reagents

Human recombinant bone morphogenetic protein 2 (BMP-2) was provided by Korea Bone Bank (Seoul, Korea). 1α, 25-dihydroxyvitamin D_3 was obtained by Calbiochem (Merck, Darmstadt, Germany). Dexamethasone, L-ascorbic acid 2-phosphate, alkaline phosphatase yellow (pNPP) liquid substrate and cetylpyridinium chloride were supplied by Sigma (St. Louis, MO, USA). Alizarin red S was purchased from DC Chemical Co. Ltd. (Seoul, Korea). All other unspecified chemicals were acquired from Sigma.

2.2. ASCs isolation and cultivation

The ASCs were isolated from the lipoaspirates generated during an elective liposuction of three patients (mean age, 32 years; range, 31–33 years). Briefly, the lipoaspirates were washed 3 times with phosphate-buffered saline (PBS). The matrix was then digested with 1.5 mg/ml of collagenase, and filtered through a 100 μm nylon mesh. The erythrocytes were removed using an erythrocyte lysis buffer. The remaining cells were placed in culture flasks and cultured in Dulbecco's modified eagle medium (DMEM)/Ham F-12 (Invitrogen/GIBCO, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics. The cultures were maintained at 37 °C for 48 h in a 5% carbon dioxide (CO_2) humidified atmosphere. At confluence, the cells were detached from the culture dishes using 0.25% trypsin containing 1 mM ethylenediaminetetraacetic acid (EDTA; Gibco BRL), washed with PBS, counted and re-plated.

2.3. Induction of osteogenesis

All experiments were performed on the third passage cells after primary plating. For osteogenic induction culture, the cells were plated and cultured in the osteogenic media (OM; α-MEM media containing 1% antibiotics/antimycotics, 10% FBS, 100 nM dexamethasone, 50 μM L-ascorbate 2-phosphate, 10 mM β-glycerophosphate). The ASCs were cultured in 48-well plate at density of 3×10^4 cells per well. The medium was changed every third day. An alkaline phosphatase (ALP) assay was performed to detect the ostogenic differentiation of ASCs on days 7 and 14 (ALP), and Alizarin red S staining was performed to detect mineralized nodule formation on days 14 and 21. To determine the proper concentration of BMP-2 and vitamin D_3, the ASCs were cultured in BMP-2 (10, 50 or 100 ng/ml) and vitamin D_3 (10^{-8}, 10^{-7} or 10^{-6} M), and assayed at 7, 14 and 21 days for ALP and mineralization. To examine the dose-dependent effect of combined treatment, the ASCs were treated with a single fixed dose of BMP-2 (50 ng/ml) and three vitamin D_3 doses (10^{-8}, 10^{-7}, 10^{-6} M), and a fixed dose of vitamin D_3 (10^{-7} M) and three BMP-2 doses (10, 50, 100 ng/ml). In addition, to assess the time-dependent effect of combined treatment, the ASCs were treated continuously with 50 ng/ml of BMP-2 for 14 days plus 10^{-7} M vitamin D_3 added under three conditions: (1) treated from days 1 to 7; (2) treated from days 8 to 14; and (3) treated from days 1 to 14. In other experiments, the ASCs were treated continuously with 10^{-7} M Vitamin D_3 plus 50 ng/ml of BMP-2 added under three conditions: (1) treated from days 1 to 7; (2) treated from days 8 to 14; (3) treated from days 1 to 14.

2.4. ALP assay and Alizarin red S staining

To quantify the ALP enzymatic activity, ASCs (3×10^4/well) were seeded in 48-well tissue culture plates and cultured for 7 and 14 days. The cells on the plates were then washed twice with PBS and lyzed with a lysis buffer [0.5 M Tris (pH9.0), 0.9% NaCl (150 mM), 1% Triton X-100 1 mM EDTA]. After 16 h, their enzymatic activities were evaluated using a spectrophotometer at 405 nm. For ALP staining, the induced ASCs were fixed in 10% formalin for 10 min. The cells were permeabilized for 30 min with 0.1% Triton-100 in PBS, and treated with nitro blue tetrazolium and 5-bromo-4-chroro-3-indolyl phosphate for 10–30 min. To measure the level of calcium deposition in the extracellular matrix, the ASCs were seeded in 48-well tissue culture plates and cultured for 14 or 21 days under osteogenic media (OM). The cultured cells and matrix were washed twice with PBS and fixed in 70% ethyl alcohol for 1 h. After three washes with PBS, they were then stained with 2% alizarin red solution (pH 4.2) (Junsei Chemical, Tokyo, Japan) for 10 min to stain the calcium deposits. The cells and matrix were then washed five times with distilled water, followed by PBS for 15 min to remove the non-specific stained cells. To quantify the degree of mineralization, they were extracted using 10% (w/v) cetylpyridinium chloride in 10 mM sodium phosphate (pH 7.0) for quantification. The concentration was evaluated by measuring the absorbance at 562 nm on a multiplate reader using an Alizarin red S standard curve in the same solution. All values are expressed as the fold changes over the control, which were ASCs treated with standard OM.

2.5. Statistical analysis

Statistical analysis was performed using two-way ANOVA test with Bonferroni corrections for multiple comparison on Prism 4 software (Microsoft, Seattle., WA, USA). All quantitative data is expressed as the group means and standard deviations. A p value <0.05 was considered significant.

3. Results

3.1. Effects of several doses of BMP-2 and vitamin D_3 on the osteogenic differentiation of ASCs

BMP-2 over 10 ng/ml increased the ALP activity and mineralization significantly compared to the control on all tested days. The difference from the concentration gradient was not detectable except on the 14th day in the mineralization (Fig. 1A and B). Vitamin D_3 from 10^{-8} M produced a significant and dose-dependent increase in ALP activity on the 7th day and in mineralization on the 14th day in ASCs, respectively. While this difference with concentration gradient diminished for ALP activity on 14th day, it persisted for the mineralization on 21st day in ASCs (Fig. 1C and D).

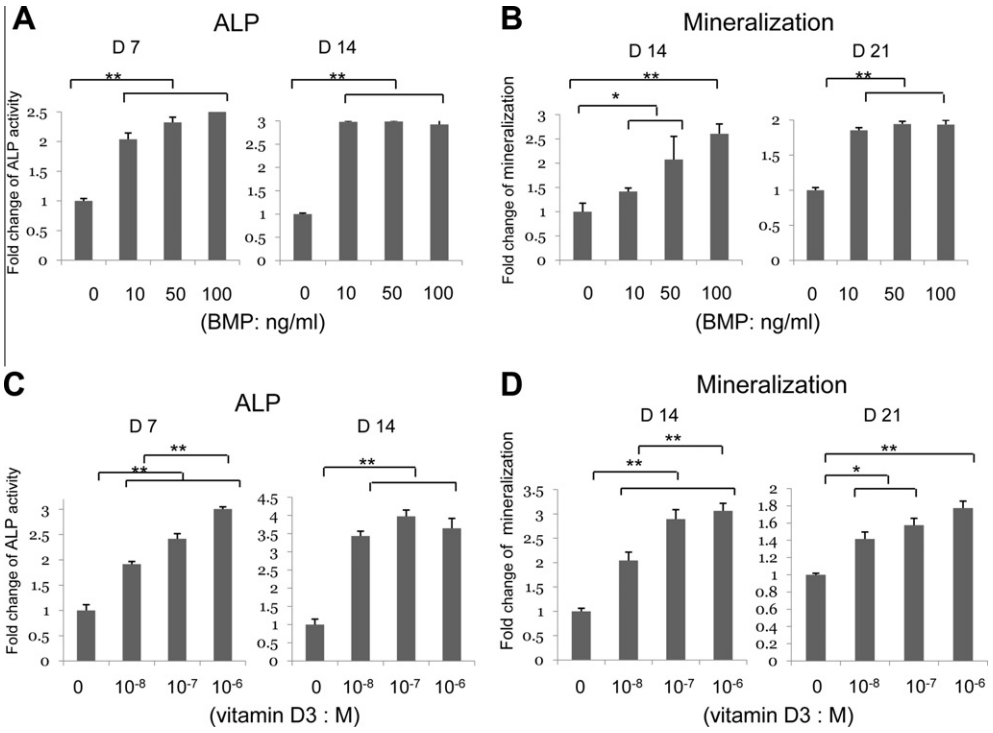

Fig. 1. The result of BMP-2 (A) and (B) and vitamin D_3 treatment (C) and (D) on the osteogenic culture of ASCs: the ALP activity (A) and (C) and mineralization from Alizarin red S staining (B) and (D) are shown. The bars represent the mean ± SD ($N = 3$; $^{**}p < 0.01$; $^{*}p < 0.05$). NS: not significant), D: day.

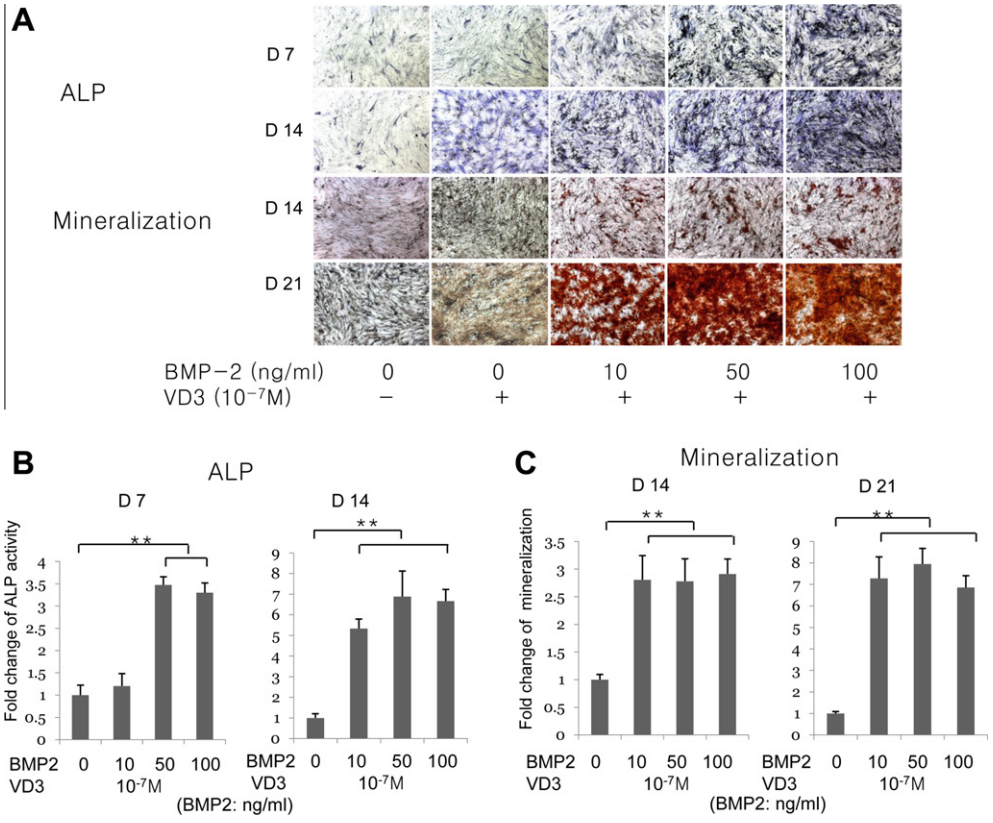

Fig. 2. The effects of several doses of BMP-2 with a fixed-dose of vitamin D_3 on the osteogenic differentiation of ASCs are shown: the findings from ALP staining and Alizarin red S staining (A), the quantification of the ALP activity (B) and mineralization (C). The bars represent the mean ± SD ($N = 3$; $^{**}p < 0.01$; $^{*}p < 0.05$). NS: not significant, D: day, VD_3: vitamin D_3.

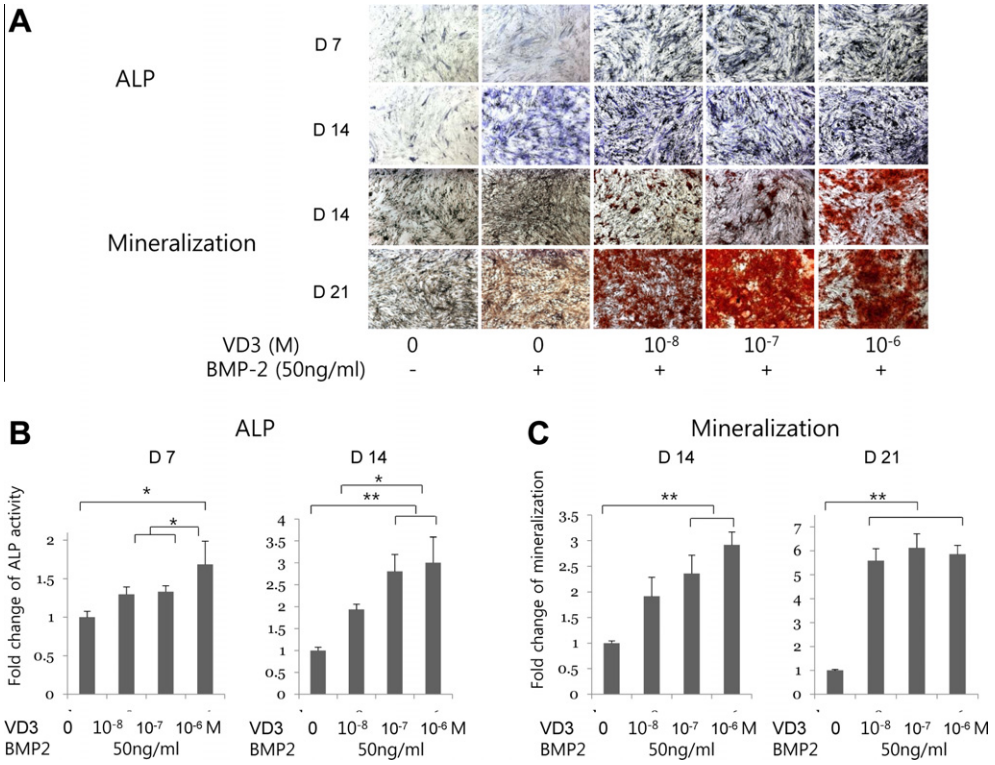

Fig. 3. The effects of several doses of vitamin D_3 with a fixed-dose of BMP-2 on the osteogenic differentiation of ASCs are shown: the findings from ALP staining and Alizarin red S staining (A), the quantification of the ALP activity (B) and mineralization (C). The bars represent the mean ± SD ($N = 3$; $^{**}p < 0.01$; $^{*}p < 0.05$). NS: not significant, D: day, VD_3: vitamin D_3.

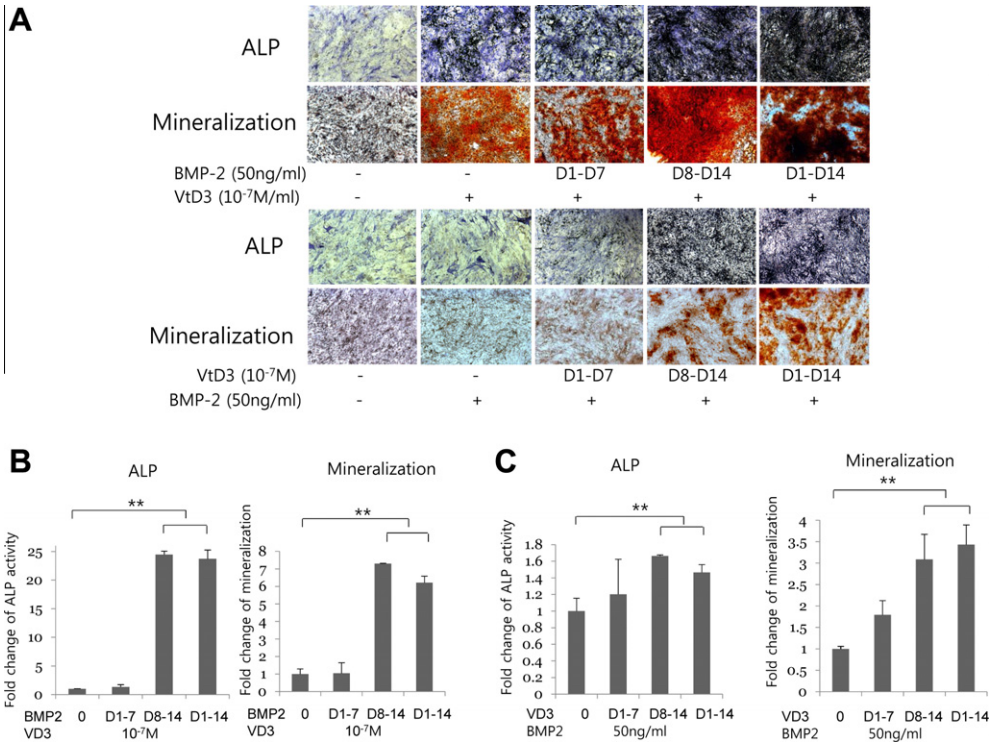

Fig. 4. The time-dependent effect of BMP-2 and vitamin D_3 combination on each other: the findings from ALP staining and Alizarin red S staining (A), and the quantification of ALP activity and mineralization are shown (B) and (C). Vitamin D_3 at 10^{-7} M was treated continuously for 14 days and BMP-2 at 50 ng/ml was added for the first 7 days, last 7 days or for the entire 14 days (A-upper panels, B). BMP-2 at 50 ng/ml was treated continuously for 14 days and vitamin D_3 at 10^{-7} M was added on the first 7 days, last 7 days or for the entire 14 days (A-lower panels, C). The bars represent the mean ± SD (N = 3; $^{**}p < 0.01$; $^{*}p < 0.05$). NS: not significant, D: day, VD_3: vitamin D_3.

3.2. Combination effects of BMP-2 and vitamin D_3 on the osteogenic differentiation of ASCs when treated together

We tested the combination effects of BMP-2 and vitamin D_3 in ASCs. When different doses of BMP-2 (10, 50 or 100 ng/ml) were treated along with fixed dose of vitamin D_3 (10^{-7} M), BMP-2 over the concentration of 50 ng/ml significantly elevated ALP activity by 3-fold compared with the control (treatment vitamin D_3 only) on the 7th day (Fig. 2A and B). All three doses of BMP-2 significantly increased ALP activity on the 14th day, (Fig. 2A and B) and mineralization on the 14th day and the 21st day by several fold compared with the vitamin D_3 only treatment (Fig. 2A and C). Synergistic effects of BMP-2 and vitamin D_3 on the osteogenic differentiation was clearly demonstrated on this setting.

Conversely, when different doses of vitamin D_3 (10^{-8}, 10^{-7}, and 10^{-6} M) were treated along with fixed dose of BMP-2 (50 ng/ml), vitamin D_3 at the concentration of 10^{-6} M significantly increased ALP activity compared with the control (treatment with BMP-2 only) on the 7th day. On the 14th day, vitamin D_3 over 10^{-7} M was effective (Fig. 3A and B). The mineralization significantly increased over the concentration of 10^{-7} M on the 14th day, and over 10^{-8} M on the 21st day (Fig. 3A and C). While all three doses of BMP-2 acted similarly in reinforcing the effect of vitamin D_3, vitamin D_3 dose-dependently augmented the osteogenic effect of BMP-2.

3.3. Time-dependent effects of BMP-2 and vitamin D_3 combination on the osteogenic differentiation of ASCs

To test the optimal timing to enhance the osteogenesis of ASCs by time-dependent treatment of vitamin D_3 on BMP-2, ASCs were treated with BMP-2 at 50 ng/ml for 14 days. During the interval, ASCs were added with vitamin D_3 at 10^{-7} M in three conditions: (1) treated for the first 7 day; (2) treated for the last 7 days; (3) treated for whole 14 days. Conversely, the effect of sequential treatment of BMP-2 on vitamin D_3 was tested. ASCs were treated with vitamin D_3 for 14 days and added with 50 ng/ml BMP-2 in three conditions: (1) treated for the first 7 day; (2) treated for the last 7 days; (3) treated for whole 14 days. The result of ALP assay and Alizarin red S staining demonstrated that when vitamin D_3 was constantly treated, the BMP was more effective when treated for the last 7 days than when treated for the first 7 days, showing obvious synergistic effects (Fig. 4A and B). When BMP-2 was constantly treated, vitamin D_3 was also more effective when treated for last 7 days although the difference was not as great as seen with the addition of BMP-2 on vitamin D_3 treated ASCs (Fig. 4A and C).

4. Discussion

In this study, we aimed to find the best condition to induce osteogenic differentiation from ASCs using two available substances, BMP-2 and vitamin D_3 , that have been known to induce bone formation. The results confirmed that the osteogenic differentiation of ASCs can be greatly promoted using BMP-2 and vitamin D_3. It was demonstrated that relatively low concentrations of either substance were sufficient to enhance the osteogenic differentiation of ASCs. The prime meaning of this study lies in investigating simple ways of facilitating the osteogenic differentiation of ASCs without resorting to more complicated means. Although the BMSCs underwent osteogenic differentiation with dexamethasone, ascorbate and β-glycerophosphate [6], ASCs appear to require more factors to achieve osteogenic differentiation comparable to BMSCs. A combination of BMP-2 and vitamin D_3 had a synergistic effect in certain settings, with lower concentrations of each factor being enough to show the synergy. These findings suggest that osteogenic differentiation can be induced from ASCs at a lower cost when a clinical application is considered.

The optimal timing to enhance the osteogenesis of ASCs was examined by a time-dependent treatment of BMP-2 on a constant treatment of vitamin D_3 or vice versa. Although it was suggested that BMP-2 induced the early osteogenic differentiation of MSCs and vitamin D_3 worked in the later period [6], our results suggest that BMP-2 potentiates the osteogenic effect of vitamin D_3 when treated in later period. When the vitamin D_3 treatment was added to the ASCs treated constantly with BMP-2, the later treatment was more effective than the early treatment but the difference was not as great. Therefore, according to these results, vitamin D_3 treatment throughout the culture period with BMP-2 added in the later period is an effective and economical way of inducing the osteogenic differentiation of ASCs. In conclusion, both BMP-2 and vitamin D_3 promote the osteogenic differentiation of adipose stem cells, and can work synergistically. The results can be used to develop methods for the effective and economical osteogenic induction of ASCs in bone tissue engineering.

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