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**Osteogenic Differentiation is Synergistically Influenced by
Osteoinductive Treatment and Direct Cell–Cell Contact
Between Murine Osteoblasts and Mesenchymal Stem Cells**

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Osteogenic Differentiation is Synergistically Influenced by Osteoinductive Treatment and Direct Cell–Cell Contact Between Murine Osteoblasts and Mesenchymal Stem Cells

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Running Title: Synergistic influence of osteoblasts on MSC osteogenesis

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ABSTRACT

Mesenchymal stem cells (MSCs) exist in a highly specialized microenvironment involve a complex interplay of soluble signals and cell–cell contact with adjacent cells. However, very little is known about the effect of neighboring cells on MSC osteogenesis. Thus, the present study was designed to address whether osteoblasts play a synergistic role, promoting MSC osteogenesis in a direct cell–cell contact coculture model. Murine C3H10T1/2 and MC3T3-E1 cell lines were mixed at different ratios and seeded with an osteogenic-treated or an untreated medium. Weak osteogenesis was induced in MSCs cocultured in an untreated medium with different ratios of osteoblasts. An osteoblast-dependent increase in osteogenic gene expression of *Runx2*, type I collagen, and osteocalcin was observed over time. Moreover, both alkaline phosphatase (ALP) activity and calcium deposition were distinctly enhanced at levels that were proportional to the quantity of osteoblasts in the culture. The increases in mRNA expression and ALP activity were greater in cocultures treated with osteogenic supplements than in untreated cultures. However, the production of ALP activity followed by a distinct matrix mineralization was lower in osteogenic-treated cultures containing greater numbers of osteoblasts. It suggests that an appropriate number of osteoblasts may lead to weak osteogenesis of MSCs by direct cell–cell contact coculture in an untreated environment. Furthermore, additional osteogenic supplements may act synergistically with osteoblasts to accelerate matrix mineralization by reducing the process of osteogenic differentiation in osteogenic treated cocultures. These findings may improve the understanding of MSC osteogenesis and may provide benefits for regenerative medicine.

Keywords: Mesenchymal Stem Cell, Osteoblast, Osteogenesis, Coculture, Direct Cell–Cell Contact

INTRODUCTION

It has been demonstrated that adult mesenchymal stem cells (MSCs) are capable of giving rise to several different cell types, including myoblasts, adipocytes, fibroblasts, chondrocytes, and osteoblasts [2, 8, 16]. MSCs exist in a highly specialized microenvironment in which their maintenance, proliferation, migration, and differentiation involve a complex interplay of many local and systematic signals between stem cells and adjacent cells [4, 11, 14, 15, 18, 20, 21]. However, very little is known about the possible interaction between bone marrow MSCs and their neighboring cells during osteogenesis.

Osteoblasts are derived from MSCs and exist in close proximity to the bone marrow, which serves as a major source of MSCs and hematopoietic stem cells. They are located on the surface of bone, express multiple hormone receptors and various cell surface molecules, and secrete hormones and enzymes that maintain the balance between bone formation and resorption. Moreover, osteoblasts initiate the process of mineralization.

Osteoblasts were recently identified as offspring of the hematopoietic stem cells. However, it remains unclear as to whether and how osteoblasts affect the self-renewal and differentiation of another major stem cell population in bone marrow, MSCs. Few *in vivo* data are available on this, and *in vitro* studies using conditioned medium or coculture models have produced inconsistent results [3, 6, 7, 9, 13]. In experiments using conditioned medium, the real-time dynamic cell–cell interactions are missing and osteogenic inducers such as dexamethasone were used. Previous coculture studies used cells from different species and did not address the effect of direct cell–cell

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3 contact between osteoblasts and MSCs, which is of great interest given the close
4 proximity of these two cell types in vivo. In addition, in these coculture systems, the
5 state of differentiation of osteoblasts (which may have significant implications
6 regarding their effectiveness) has not been sufficiently considered. Furthermore, the
7 specific modules responsible for the stimulatory effect observed in osteoblasts in
8 some studies were not identified [13].
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20 Motivated by these issues, the present study was designed to address whether
21 osteoblasts play a synergistic role in the promotion of MSC osteogenesis in a direct
22 cell–cell contact coculture model. The murine MSC cell line C3H10T1/2, and murine
23 osteoblast cell line MC3T3-E1 (clone 14) were used to develop a direct cell–cell
24 contact coculture model, supplemented with/without osteogenic treatment. Cells were
25 seeded at different ratios of initial cell densities to observe the effect of the quantity of
26 osteoblasts on MSC osteogenesis in vitro. We found that the untreated cocultures were
27 able to induce weak osteogenesis in MSCs cocultured with various ratios of
28 osteoblasts. The osteogenic-treated cocultures exhibited marked expression of
29 osteogenic mRNA, such as *Runx2*, type I collagen, and osteocalcin. Moreover, both
30 alkaline phosphatase (ALP) activity and calcium deposition were distinctly enhanced
31 in these treated cocultures, at levels proportional to the quantity of osteoblasts.
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MATERIALS AND METHODS

Cell Cultures

The murine C3H10T1/2 cell line was a generous gift from Prof. Yeu Su (National Yang-Ming University, Taipei, Taiwan). The murine calvaria-derived MC3T3-E1 cell line at subclone 14, which has the osteoblastic phenotype, was purchased from ATCC. Each cell line was cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and 100 units/ml penicillin-streptomycin to enable basal cell growth. Cells were trypsinized and subcultured when cells reached 60–70% confluence for serial passages.

The Direct Cell–Cell Contact Coculture Model

MC3T3-E1 cells (clone 14) at passages 3 and 4, and C3H10T1/2 cells at passages 8–10 were plated onto 12-well culture plates and cocultured at various ratios of initial cell densities (Fig. 1): group B5 (pure MC3T3-E1 cells at 5,000 cells/cm²), group C1B4 (C3H10T1/2 at 1,000 cells/cm² and osteoblasts at 4,000 cells/cm²), group C2.5B2.5 (C3H10T1/2 at 2,500 cells/cm² and osteoblasts at 2,500 cells/cm²), group C4B1 (C3H10T1/2 at 4,000 cells/cm² and osteoblasts at 1,000 cells/cm²), and group C5 (pure C3H10T1/2 cells at 5,000 cells/cm²). Groups B5 and C5 were used as controls. To examine whether a synergistic interaction of direct cell–cell contact and soluble osteoinducible factors exists between C3H10T1/2 and MC3T3-E1 cells, all cocultures were divided into two groups: growth in culture medium treated with (OS) or without osteogenic (BS) supplements. For BS treatment, the culture medium used was the same as that described above in the presence of 2% FBS. For OS treatment, the medium used for BS was used, but supplemented with the following: 100 nm dexamethasone, 50 g/ml L-ascorbic acid, and 1 mM β-glycerophosphate. Half of the BS or OS medium (as appropriate) was replaced with fresh medium every 2–3 days.

ALP Activity Assay

ALP is one of the early markers of osteoblasts. To evaluate osteogenic differentiation, ALP activity was assessed by measuring the formation of p-nitrophenol (p-NP) from p-nitrophenyl phosphate, as per the manufacturer's instructions (Sigma). Cells were lysed in a buffer containing 1.5 M Tris, 1 mM ZnCl₂, and 1 mM MgCl₂ (pH 9.0), containing 2% Triton X-100, and reacted with a phosphate substrate reagent (2 mg/ml; Sigma) in a microplate that was protected from the light. The levels of p-NP were quantified according to a p-NP standard curve based on A405 and normalized to the respective DNA content of each coculture.

RNA Isolation and Reverse Transcription-Polymerase Chain Reaction

The mRNA expression of *Runx2* (a critical transcriptional factor that regulates skeletogenesis), type I collagen (a major organic component that exists in bone extracellular matrix), osteocalcin (a late and specific marker of bone formation), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; as an internal control for RNA loading) in BS- or OS-treated cocultures were determined at each time point by reverse transcriptase-polymerase chain reaction (RT-PCR) assay. Total RNA was extracted and collected by using TriZol reagent (Invitrogen) and the first-strand cDNA was synthesized by using the SuperScript First-Strand Synthesis System (Invitrogen) followed by the amplification of cDNA product using Platinum Taq DNA polymerase (Invitrogen). The sense and antisense sequences of the primers used for semiquantitative RT-PCR reactions are listed in Table 1. The reaction was performed under the following conditions: incubation at 94°C for 2 min; denaturation at 94°C for 45 s, annealing at 62°C (*Runx2*), 60°C (type I collagen and osteocalcin), or 52°C (GAPDH) for 30 s, and polymerization at 72°C for 60 s for 35 cycles; followed by a final extension at 72°C for 5 min. The expression of amplified products was evaluated using electrophoresis with 2% agarose gel and ethidium bromide staining. All

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3 electrophoresis images were analyzed quantitatively using NIH Image J software, and
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5 normalized to their respective GAPDH values.
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10 **Alizarin Red S Staining for Matrix Mineralization**

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12 The matrix mineralization of cocultures cultured in BS or OS medium was detected by
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14 histochemical staining at day 28. Cells were fixed with 60% isopropanol with 2% alizarin red
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16 (Sigma) solution (pH 4.1–4.3) for 5 min at room temperature. Matrix mineralization was
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18 visualized by staining.
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25 **Statistical Analysis**

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27 All assays were repeated, with a minimum of $n=3$ per group. Data are expressed as mean
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29 and SD values. Statistical significance was determined using one-way ANOVA to compare
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31 means between groups, with a p value of less than 0.05 being considered significant.
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RESULTS

Osteogenic Gene Expression in BS- or OS-treated Cocultures at Day 3

In BS-treated cultures, there was no remarkable difference in *Runx2* mRNA expression between the groups at day 3 (Fig. 2). However, in OS-treated cultures, C4M1, C1M1, and C1M4 coculture groups exhibited a significant increase in *Runx2* mRNA expression in an osteoblast-cell-density-dependent manner. In these three cocultures, a greater number of osteoblasts showed higher *Runx2* mRNA expression. The expression of *Runx2* mRNA was also higher in group C5 than in group M5. Type I collagen is a major organic component contained in the bone extracellular matrix. In BS-treated cultures, all groups exhibited lower type I collagen mRNA expression than OS-treated cultures, with the exception of group M5 at day 3. However, all cultures exhibited much higher type I collagen mRNA expression with the osteogenic supplement compared to those in BS-treated cultures. The osteocalcin mRNA expression did not differ significantly between the groups at day 3, irrespective of whether culturing was in BS or OS. However, the expression of osteocalcin was greatly enhanced in the OS-treated groups compared to their BS-treated control.

Osteogenic Gene Expression in BS- or OS-Treated Cocultures at Day 28

In 28-day cultures, all cocultures exhibited a distinct decrease of *Runx2* mRNA expression at the end of culture (Fig. 2). With the exception of group M5, *Runx2* mRNA expression was higher following OS treatment than following BS treatment. Accumulation of type I collagen mRNA was observed in all groups, whether supplemented with or without osteogenic reagents. The expression of type I collagen was no more distinct in Group M5 (both with or without osteogenic medium) than in the other groups.

Osteocalcin is a specific bone marker at the late stage of bone formation. After a 28-day

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3 culture, osteocalcin mRNA expression was significantly increased in all groups, but especially
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5 in the OS-treated cultures. Osteocalcin levels in group C5 were greatly enhanced by OS
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7 treatment compared to BS treatment. Most cocultures exhibited an
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9 osteoblast-cell-density-dependent increase in the expression of *Runx2*, type I collagen and
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11 osteocalcin. Cocultures with more osteoblasts exhibited higher levels of osteogenic gene
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13 expression.
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20 **ALP Activity in BS-Treated Cocultures**

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22 In BS-medium culture, groups C5 and C4M1 did not exhibit a significant increase in ALP
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24 activity during the 28-day culture period (Fig. 3). However, the other three groups cocultured
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26 with osteoblasts at higher ratios exhibited a steady increase in ALP production over time.
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28 MSCs cocultured with fewer osteoblasts did not tend to go through the osteogenic
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30 differentiation in BS medium (i.e., without osteogenic supplements). Therefore, for MSCs, the
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32 lesser direct cell–cell contact with osteoblasts limited the contribution to the MSC osteogenic
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34 differentiation in vitro.
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41 **ALP Activity in OS-Treated Cocultures**

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43 The ALP activity from these OS-treated coculture groups yielded contrasting results from
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45 those in BS-treated culture (Fig. 4). In the osteogenically supplemented (OS) environment, all
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47 groups exhibited a time-dependent increase in ALP production during the culture period.
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49 Interestingly, the ALP activity at the end of the culture period was highest in group C5
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51 cultures. Group C4M1 also exhibited a remarkable time-dependent increase in ALP
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53 production compared to C5, except at day 28. In contrast, groups M5 and C1M4, which were
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55 cocultured at higher osteoblast ratios, exhibited slightly higher ALP activities at the end of the
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57 culture period than those cultured in BS medium.
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Matrix Mineralization and Calcium Deposition

No remarkable calcium deposition was observed in group C5 after the 28-day culture period (Fig. 5) in BS-treated cocultures. However, clear accumulation of calcium was observed in all groups cultured in the OS condition. The accumulation of calcium deposition at week 4 in either the BS or OS condition was higher in Group M5 than in the other coculture groups. Groups C4M1, C2.5M2.5, and C1M4 expressed higher calcium deposition in the OS than in the BS condition. This suggests that both the higher number of osteoblasts and the presence of osteogenic supplements in these coculture groups enhanced the mineralization observed at the end of culture.

DISCUSSION

A monolayer coculture model was implemented in this study, with C3H10T1/2 and MC3T3-E1 cells at various ratios of cell density plated and cocultured in BS or OS medium, to determine whether direct cell–cell contact between osteoblasts and MSCs is critical for inducing or enhancing MSC osteogenesis. This study found a clear synergistic induction and enhancement of MSC osteogenesis in these coculture models. The results show that cocultures with a higher density of osteoblasts expressed greater mRNA expressions of *Runx2*, type I collagen, and osteocalcin, as well as ALP production in the BS-treated environment. Moreover, OS-treated cocultures exhibited much higher expressions of most target genes, but not of ALP activity or calcium deposition. This suggests that an appropriate number of osteoblasts may lead to weak osteogenesis of MSCs by direct cell–cell contact in an environment without additional osteogenic supplements. However, additional OS treatment may promote the activity of osteoblasts as synergistic modulators to accelerate matrix mineralization by impeding osteogenic differentiation in the cocultures.

Human bone-marrow-derived MSCs, by virtue of their capacity for self-renewal and multipotent differentiation, are considered promising candidate cells for regenerative medicine and tissue engineering applications. The multipotent differentiation capabilities of MSCs into various types of cell lineage, such as osteoblasts, chondrocytes, and adipocytes, have been characterized in numerous studies [2, 16]. Studies have used coculture systems to further investigate the possible induction of osteogenesis of MSCs by neighboring cells [5, 12, 17, 19, 22]. Osteoblasts, which are derived from bone-marrow MSCs, are the main cell type responsible for bone formation, and are one of the neighboring cell types close to the bone marrow. Direct cell–cell contact coculture models have produced further evidence that coculture of MSCs and osteoblasts [1, 13, 19] or osteoblast-derived soluble factors [9, 10, 19,

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3 23] leads to osteogenesis. It is believed that interaction between bone-marrow MSCs and their
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5 neighboring cells may lead to MSC osteogenesis via cell–cell contact or osteoblast-derived
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7 soluble factors. However, cells in cocultures derived from different species, such as humans,
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9 mice, and rabbits, may give rise to different results [13].
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15 It has been demonstrated that *Runx2* is as an essential transcription factor for the induction
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17 of early osteogenic differentiation. Studies of the expression of *Runx2* demonstrate that the
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19 early osteogenesis of cocultures is highly dependent on osteoblast numbers cocultured in an
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21 osteoinductive environment. Additional OS supplements were also critical to the overall
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23 enhancement of osteogenic gene expression compared to those in BS-treated cocultures. The
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25 gene expression at day 28 in BS-treated cocultures increased with the ratios of osteoblasts.
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27 Cocultures with MSCs and osteoblasts demonstrated that direct cell–cell contact was
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29 sufficient to induce osteogenic differentiation by enhancing the gene expressions of *Runx2*,
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31 type I collagen, and osteocalcin. More significant and distinct osteogenic differentiation was
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33 promoted by additional osteogenic supplements.
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41 Csaki et al. demonstrated that MSCs and osteoblasts actively search for cell–cell contact,
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43 leading to cell proliferation and osteogenic differentiation, only in monolayer cocultures with
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45 osteoinductive treatment. The quality of osteogenesis, as evidenced by protein expression, is
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47 proportional to the quantity of osteoblasts in the cocultures, a finding that is consistent with
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49 our results. A recent study has also suggested that primary bone-derived cells promote the
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51 osteogenesis of human embryonic stem cells in a coculture model by releasing bone
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53 morphogenetic proteins 2 and 4 [1]. These findings demonstrate that the osteogenesis of stem
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55 cells may be induced or promoted in a coculture system through either direct cell–cell contact
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57 or secreted soluble factors derived from osteoblasts.
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6 Our findings show that direct cell–cell contact between MSCs and osteoblasts contributed
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8 little to MSC osteogenic differentiation in the BS condition. In addition, the cocultures
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10 required at least one osteoinductive factor to induce or enhance the osteogenic differentiation
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12 in MSCs: either an appropriate number of osteoblasts or treatment with osteogenic
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14 supplements. Culture conditions that included both a large quantity of osteoblasts and
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16 osteogenic supplements resulted in synergistic inhibition during osteogenesis by shrinking the
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18 process of osteogenic differentiation.
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25 The untreated cocultures with murine MSCs and osteoblasts were sufficient to induce a
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27 weak osteogenesis when cultured in BS medium without any osteogenic supplement.
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29 Furthermore, the overall level of osteogenesis in the untreated cocultures remained lower than
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31 in the OS-treated cocultures. This information indicates that direct cell–cell contact and
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33 soluble osteogenic factors might act as synergistic modulators to promote or inhibit
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35 osteogenic differentiation in MSCs. Understanding the possible synergistic interactions
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37 between MSCs and osteoblasts might be an alternative approach to manipulating the fates of
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39 stem cells and for differentiating lineages in cell-therapeutic strategies and regenerative
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For Peer Review

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TABLE LEGENDS

Table 1. Primer sequences for RT-PCR.

FIGURE LEGENDS

Figure 1. Schema of the direct cell–cell coculture model. C3H10T1/2 cells were cocultured with MC3T3-E1 cells at different ratios: 5:0, 4:1, 1:1, 1:4, and 0:5. Groups C5 and M5 were used as controls. Cells were uniformly mixed before seeding onto 12-well culture plates.

Figure 2. The expressions of *Runx2*, type I collagen, and osteocalcin were analyzed as osteogenic markers by semiquantitative RT-PCR at days 3 and 28. C3H10T1/2 cells were cocultured with MC3T3-E1 cells at various ratios in untreated (BS) or osteogenic-treated (OS) medium. (* $p < 0.05$, $n = 3$ for each group; data are mean and SD values).

Figure 3. ALP activity in cocultures with C3H10T1/2 and MC3T3-E1 cells maintained in BS treatment were assayed and determined spectrophotometrically during the 28-day culture period. Few alterations in ALP production were observed in groups C5 and C4M1 over time. However, groups C2.5M2.5 and C1M4 exhibited an almost linear increase in ALP production during the culture period (* $p < 0.05$, $n = 3$ for each group; data are mean and SD values).

Figure 4. ALP activity in cocultures with C3H10T1/2 and MC3T3-E1 cells maintained in OS medium were assayed and determined spectrophotometrically during the 28-day culture period. ALP activity in group C5 expressed a surprising increase, reaching its highest point at the end of the culture period. All OS-treated groups exhibited a steady increase in ALP production over time. ALP activity was overall relatively higher in the OS- than in the BS-treated cocultures (* $p < 0.05$, $n = 3$ for each group; data are mean and SD values).

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6 Figure 5. The matrix mineralization of all OS-treated cocultures during osteogenesis was
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8 estimated by alizarin red S staining at week 4. Calcium deposition accumulated over time,
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10 reaching its highest level at the end of the culture period. Both BS- and OS-treated M5 groups
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12 exhibited the highest levels of matrix mineralization. All groups except the BS-treated group
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14 C5 coculture exhibited an osteoblast-dependent accumulation of calcium deposition over time.
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17 Magnification, $\times 50$.
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For Peer Review

Table 1. Primer sequences for RT-PCR.

Target genes	Annealing Temp. (°C)	Primer sequences	Product Size
<i>Runx2</i>	62	Forward: GAC AGA AGC TTG ATG ACT CTA AAC C Reverse: TCT GTA ATC TGA CTC TGT CCT TGT	171
<i>Collagen Type I</i>	60	Forward: GCA TGG CCA AGA AGA CAT CC Reverse: CCT CGG GTT TCC ACG TCT C	83
<i>Osteocalcin</i>	60	Forward: ATG AGG ACC CTC TCT CTG CTC Reverse: ATG AGG ACC CTC TCT CTG CTC	270
<i>GAPDH</i>	52	Forward: AAC TAC ATG GCT ACA TGT TCC A Reverse: CCA TTC TCG GCC TTG ACT GT	73

Group	C5	C4M1	C2.5M2.5	C1M4	M5
<i>Cell density: cells/cm²</i>					
C3H10T1/2	5000	4000	2500	1000	0
MC3T3-E1	0	1000	2500	4000	5000











BS					
OS					

Figure 1. Schema of the direct cell–cell coculture model. C3H10T1/2 cells were cocultured with MC3T3-E1 cells at different ratios: 5:0, 4:1, 1:1, 1:4, and 0:5. Groups C5 and M5 were used as controls. Cells were uniformly mixed before seeding onto 12-well culture plates.

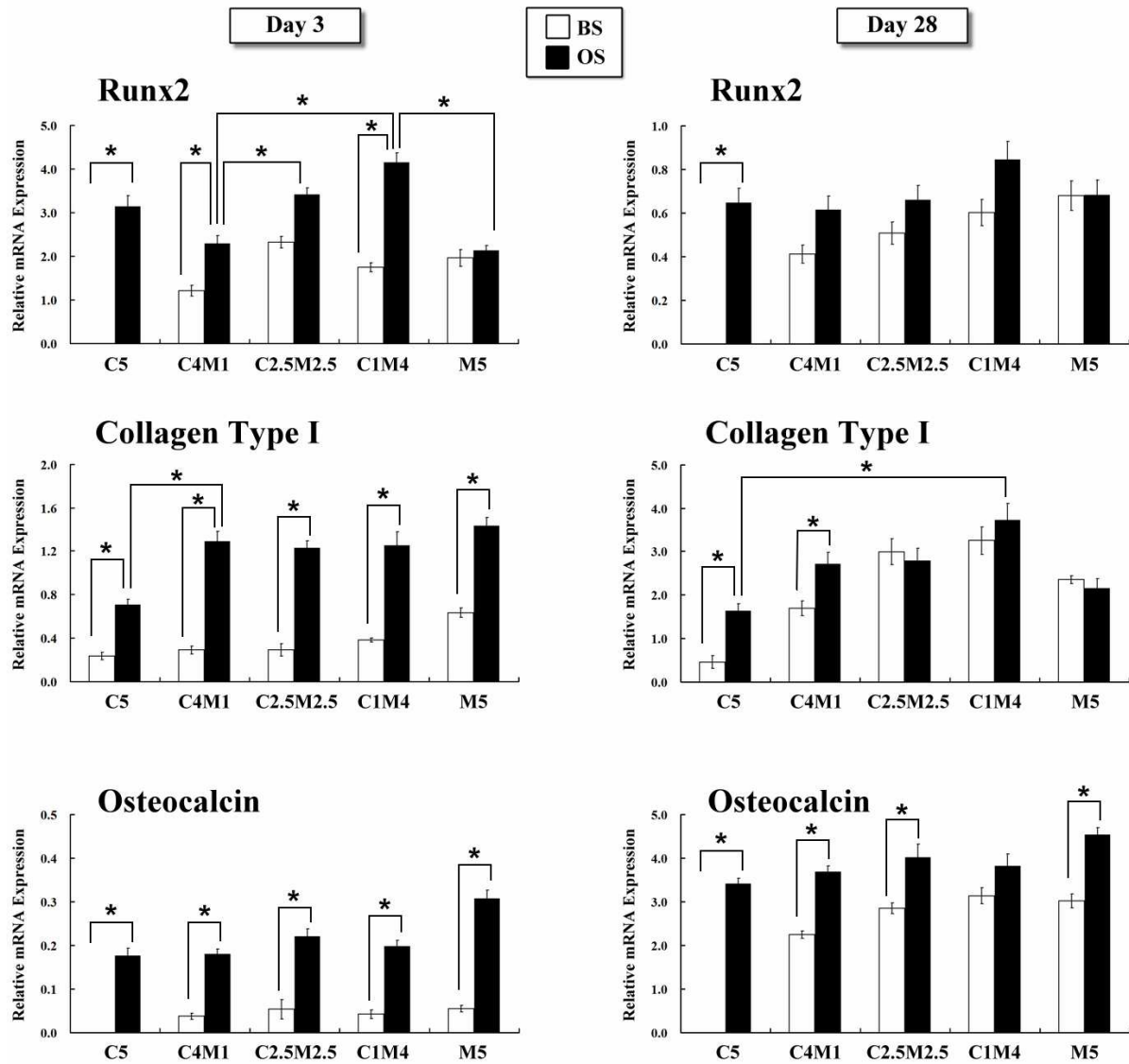


Figure 2. The expressions of *Runx2*, type I collagen, and osteocalcin were analyzed as osteogenic markers by semiquantitative RT-PCR at days 3 and 28. C3H10T1/2 cells were cocultured with MC3T3-E1 cells at various ratios in untreated (BS) or osteogenic-treated (OS) medium. (* $p < 0.05$, $n = 3$ for each group; data are mean and SD values).

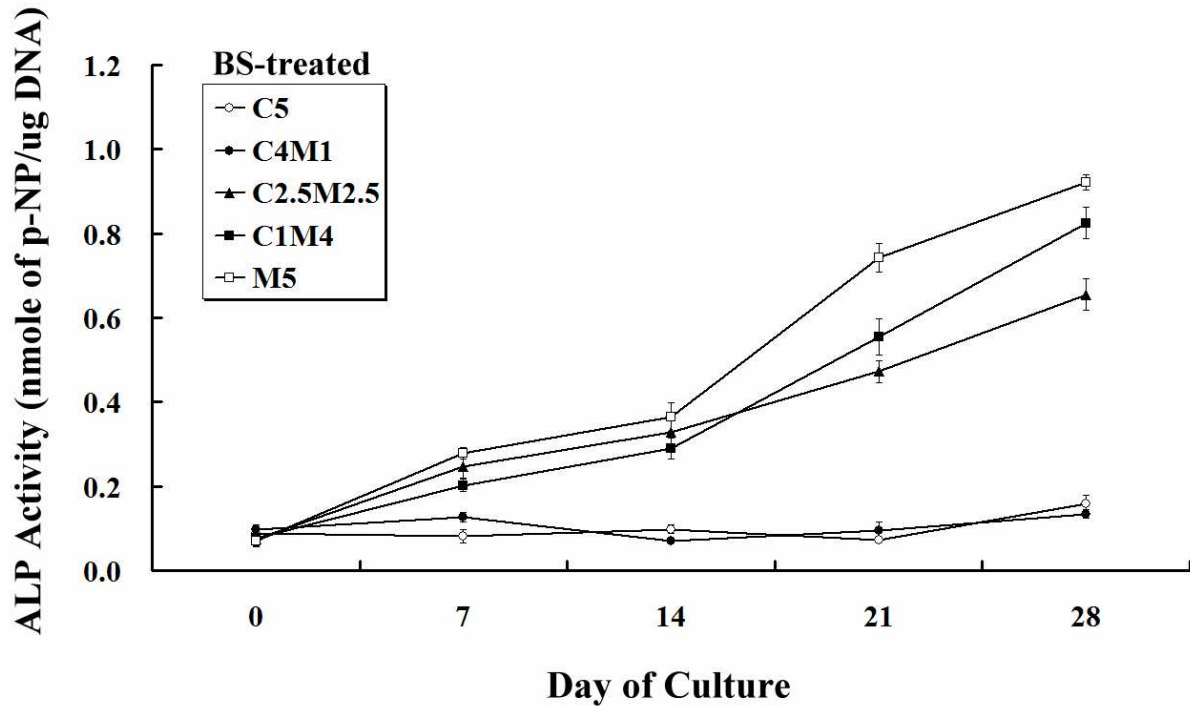


Figure 3. ALP activity in cocultures with C3H10T1/2 and MC3T3-E1 cells maintained in BS treatment were assayed and determined spectrophotometrically during the 28-day culture period. Few alterations in ALP production were observed in groups C5 and C4M1 over time. However, groups C2.5M2.5 and C1M4 exhibited an almost linear increase in ALP production during the culture period ($*p < 0.05$, $n = 3$ for each group; data are mean and SD values).

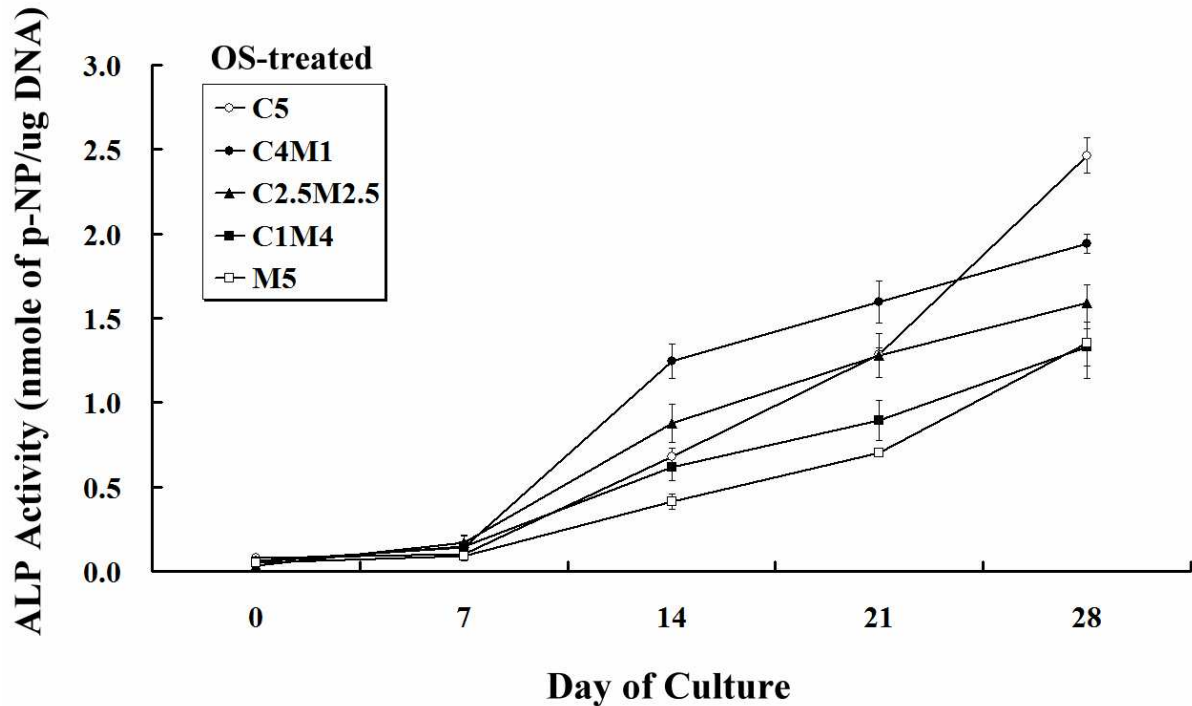


Figure 4. ALP activity in cocultures with C3H10T1/2 and MC3T3-E1 cells maintained in OS medium were assayed and determined spectrophotometrically during the 28-day culture period. ALP activity in group C5 expressed a surprising increase, reaching its highest point at the end of the culture period. All OS-treated groups exhibited a steady increase in ALP production over time. ALP activity was overall relatively higher in the OS- than in the BS-treated cocultures ($*p < 0.05$, $n = 3$ for each group; data are mean and SD values).

Alizarin Red S Staining at Week 4

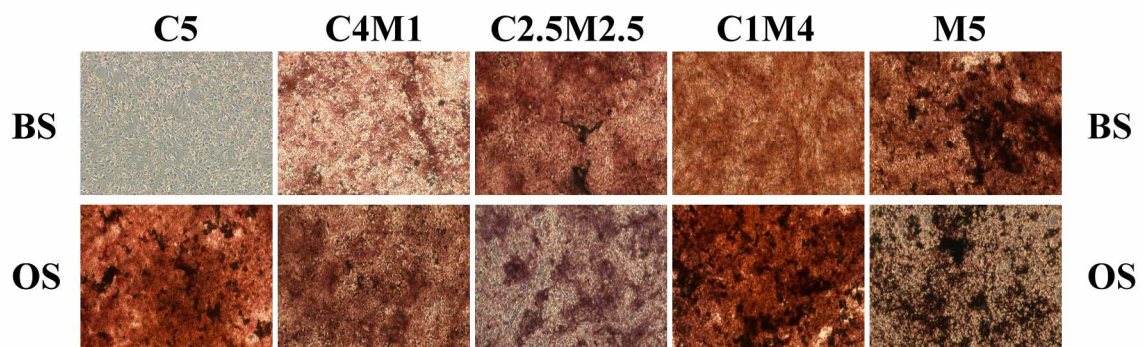


Figure 5. The matrix mineralization of all OS-treated cocultures during osteogenesis was estimated by alizarin red S staining at week 4. Calcium deposition accumulated over time, reaching its highest level at the end of the culture period. Both BS- and OS-treated M5 groups exhibited the highest levels of matrix mineralization. All groups except the BS-treated group C5 coculture exhibited an osteoblast-dependent accumulation of calcium deposition over time. Magnification, $\times 50$.