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Osteocytes up-regulate the terminal differentiation of pre-osteoblasts via gap junctions

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ABSTRACT

We examined cell-to-cell interaction between pre-osteoblasts and osteocytes using MC3T3-E1 and MLO-Y4, respectively. First, GFP expressing MC3T3-E1 (E1-GFP) cells were generated to isolate the cells from co-culture with MLO-Y4. No changes were observed in the expression of osteogenic transcription factors Runx2, Osterix, Dlx5 and Msx2, but expression of alkaline phosphatase (ALP) and bone sialoprotein (BSP) in E1-GFP co-cultured with MLO-Y4 was 300–400-fold greater than that in mono-cultured E1-GFP. In addition, mineralized nodule formation was drastically increased in co-cultured E1-GFP cells compared to mono-cultured cells. Patch clamp assay showed the presence of gap junctions between E1-GFP and MLO-Y4. Furthermore, when the gap junction inhibitor carbenoxolone (CBX) was added to the culture, increased expression of ALP and BSP in E1-GFP co-cultured with MLO-Y4 was suppressed. These results suggest that gap junction detected between pre-osteoblasts and osteocytes plays an important role on the terminal differentiation of pre-osteoblasts.

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1. Introduction

Gap junctions, which mediate direct cell-to-cell-communication, play important roles in the maintenance of complex cellular functions. Disruption of these intercellular junctions leads to tissue dysfunction and a variety of physiological disorders [1]. Gap junctions are formed by the juxtaposition of two hemichannels composed of transmembrane proteins called connexins (Cx), and are localized to the plasma membrane where they can contact neighboring cells, thereby facilitating the rapid diffusion of small molecules and ions less than 1 kDa in a process known as gap junctional communication [2].

Bone is a dynamic tissue that undergoes constant remodeling in response to physical changes, including fluctuations in hormone levels, growth factors, and mechanical load [3]. Bone remodeling is constantly controlled by bone-resorbing osteoclasts and bone-forming osteoblasts, as well as osteocytes, which are embedded in the mineralized bone matrix [4]. In addition to endocrine, paracrine, and autocrine factors, direct cell-to-cell communication through gap junctions plays an important role in coordinating the activities of bone cells [5]. Recently, it was reported that osteocytes communicate with osteoblasts as well as other osteocytes through abundant gap junctions composed of Cx43. Gap junctions are permeable to molecules of up to 1.2 kDa, making them cable of propagating signals produced by ions, metabolites, and second messengers such as cAMP, cADP-ribose, and inositol derivatives [2]. Interestingly, disruption of the Cx43 gene in mice results in a delay in endochondral and intramembranous ossification. Calvarial osteoblasts isolated from Cx43 knockout mice demonstrate delayed expression of many osteoblast-specific markers, including osteocalcin (OCN), bone sialoprotein (BSP), and type I collagen [6]. Furthermore, manipulation of gap junctional communication by overexpression of Cx43 in the cultured osteoblastic cell lines alters basal expression of OCN, BSP, and alkaline phosphatase (ALP) in a reciprocal manner [7]. These findings strongly suggest that gap junctions are a fundamental part of the regulatory mechanism that controls the gene expression of osteoblast-specific markers during osteoblast differentiation and bone remodeling [2]. However, it has not been demonstrated whether osteocytes regulate osteogenic gene transcription and terminal differentiation of osteoblasts via gap junctions.

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Therefore, to understand the role of osteocytes as a micro-environmental factor in the terminal differentiation of pre-osteoblasts, we examined direct cell-to-cell interaction between MC3T3-E1 and MLO-Y4 cells in a unique co-culture system. The preosteoblast-like cell line MC3T3-E1 was derived from normal mouse bone tissue. MLO-Y4 cells are a line isolated from osteocalcin promoter-driven T-antigen transgenic mice and exhibit a stable osteocytic phenotype. When MLO-Y4 and MC3T3-E1 cells were co-cultured, the mRNA expression levels of ALP and BSP were strongly increased in MC3T3-E1 cells through gap junctions with MLO-Y4, and this was followed by enhanced mineralization in culture. Interestingly, increased ALP and BSP expression was not accompanied by an increase in the expression of osteogenic transcription factors, such as Runx2 and Osterix. These results suggest that cell-to-cell communication between pre-osteoblasts and osteocytes is sufficient for increased expression of ALP and BSP, and for subsequent terminal differentiation of pre-osteoblasts.

2. Materials and methods

2.1. Cells and reagents

All cells were maintained in growth medium composed of α-MEM (Wako, Osaka, Japan), 10% fetal bovine serum (FBS; Japan Bioserum Tokyo, Japan), and 1% penicillin–streptomycin (Wako, Osaka, Japan) at 37 °C in an atmosphere containing 5% CO_2. Carbenoxolone (CBX) was purchased from Sigma (St. Louis, MO).

2.2. Generation of GFP-expressing cells

MC3T3-E1 cells were seeded in a 6-well plate at a density of 1×10^5/well, and cultured for 18 h. Each well was then incubated with 2 ml of α-MEM containing 6.25 μl of Lipofectamine LTX (Invitrogen, Carlsbad, CA) with 2 μg of GFP-expression vector (pEGFP-N1, Takara Bio, Shiga, Japan) for 24 h. GFP-expressing cells were selected by culturing with G418 (2 mg/ml, Sigma, St. Louis, Mo), and their purity was confirmed by flow cytometry (BD FACS Aria; BD Biosciences, San Jose, CA).

2.3. Co-culture of E1-GFP and MLO-Y4 cells

An aliquot of 2×10^6 MLO-Y4 cells were gently mixed with 2×10^6 of E1-GFP cells in a 15 ml tube. The mixed cells were seeded into 10 cm dishes with growth medium and cultured for 1–72 h. For the ratio-dependent assay, cell numbers of each type cell were seeded for co-culture as shown in Supplemental Table 1. GFP-expressing cells were isolated using a FACS Aria (BD Biosciences). E1-GFP cells at 4×10^6 cells per well were used as a control. For separated culture experiments, E1-GFP cells were cultured at a density of 1×10^6 in a well covered by a filter chamber (1 μm pore size; Becton and Dickinson, Franklin Lakes, NJ) placed on 1×10^6 of MLO-Y4 cells for 24 h.

2.4. Whole-cell patch-clamp recording

Cultured cells were transferred to a recording chamber that was continuously perfused with a solution containing 126 mM NaCl, 3 mM KCl, 2 mM $MgSO_4$, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 2.0 mM $CaCl_2$, and 10 mM D-glucose. Electrical signals were recorded by amplifiers (Axoclamp 700B, Axon Instruments, Foster City, CA), digitized (Digidata 1422A, Axon Instruments), observed on-line and stored on a computer hard disk using Clampex (pClamp 10, Axon Instruments). The composition of the pipette solution for recordings was as follows: 70 mM of potassium gluconate, 70 mM of KCl, 10 *N*-(2-hydroxyethyl) piperazine-*N*′-2-ethanesulfonic acid (HEPES), 15 mM of biocytin, 0.5 mM of EGTA, 2 mM of $MgCl_2$, 2 mM of magnesium adenosine triphosphate (ATP), and 0.3 mM of sodium guanosine triphosphate (GTP). Thin-wall borosilicate patch electrodes (2–5 MΩ) were pulled on a Flaming-Brown micropipette puller (P-97, Sutter Instruments, Novato, CA). Recordings were obtained at 30–31 °C. Membrane currents and potentials were low-pass filtered at 5–10 kHz and digitized at 20 kHz.

2.5. Analysis of mRNA expression

Total RNA was isolated using RNAiso Plus (Takara Bio) according to the manufacturer's instructions. First-strand cDNA was synthesized as previously reported [8]. cDNA was then diluted five-fold in sterile distilled water and 2 μl of cDNA was subjected to real-time RT-PCR using SYBR Premix Ex Taq™ II (Takara Bio) on a CFX96 Real-Time System (Bio-Rad Laboratories, Hercules, CA). The primer sets used are described in Supplemental Table 2. Each reaction was performed three times using cDNAs from different sample RNAs. mRNA expression levels were normalized to that of β-actin.

2.6. Noggin and CBX treatment

E1-GFP cells were co-cultured with MLO-Y4 cells in the presence or absence of recombinant mouse noggin (1 mg/ml) and cultured for 12 h. After incubation, the E1-GFP cells were isolated and subjected to real-time RT-PCR analysis. For CBX treatment, E1-GFP cells and MLO-Y4 cells were pre-cultured in growth medium in the presence or absence of CBX (100 μM) for 1 h, and then co-cultured for 12 h.

2.7. Cell counting assay

After co-culture, E1-GFP cells were isolated with a flow cytometer, seeded onto 100 mm culture dishes (1×10^4 cells/dish) and cultured for the indicated periods in the complete medium. The cells were then trypsinized and resuspended in the media, and the number of cells was determined using a microscope counting chamber (hemocytometer).

2.8. Cell cycle analysis

Cell cycle analysis was performed using Click-iT™ EdU Flow Cytometry Assay Kits (Invitrogen/Molecular Probes, Eugene, OR) according to the manufacturer's instructions. In brief, co-cultured cells were treated with 10 μM EdU (5-ethynyl-2′-deoxyuridine) for 1 h. EdU-incorporated cells were fixed with paraformaldehyde for 15 min and permeabilized for 30 min with a saponin-based buffer. The cells were treated for 30 min with the click-reaction mixture containing pacific blue azide, and resuspended in PBS buffer. GFP-positive cells were analyzed on a FACS Aria.

2.9. Alizarin Red S staining

After co-culture, E1-GFP cells were isolated with a FACS Aria and then cultured in 12-well plates with osteogenic medium for the indicated days. The osteogenic medium was composed of α-MEM supplemented with 10% FBS, 50 μg/ml L-ascorbate phosphate (Sigma), and 10 mM β-glycerophosphate (Sigma). Fresh osteogenic medium was added every 2 days. Thereafter, cultures were fixed in 4% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.3) for 30 min, washed with 0.1 M cacodylate buffer (pH 7.3), and stained for 5 min with a saturated solution of Alizarin Red S (pH 4.0). The wells were then washed with sterilized distilled water, dried, and examined under an EPSON GT-X800 scanner.

2.10. Statistical analysis

Results are presented as the means ± SD of triplicate cultures, and statistical differences were assessed using Student's *t*-test. Significant differences ($P < 0.05$) are indicated.

3. Results

3.1. The effect of cell-to-cell interaction on ALP and BSP mRNA expression in E1-GFP cells co-cultured with MLO-Y4 cells

To examine the direct cell-to-cell interaction between MC3T3-E1 and MLO-Y4 cells, GFP-expressing MC3T3-E1 (E1-GFP) cells were established. Equal numbers of E1-GFP and MLO-Y4 cells were then mixed and co-cultured for 24 h, and E1-GFP cells were sorted by a FACS Aria (Fig. 1A–C). First, the effect of co-culture with MLO-Y4 cells on the transcription of osteoblast differentiation markers in E1-GFP cells was investigated. Real-time RT-PCR showed drastic time-dependent increases in the mRNA expression levels of ALP and BSP in E1-GFP cells co-cultured with MLO-Y4 cells compared to mono-cultured E1-GFP cells, but no significant difference in the mRNA expression levels of the osteoblastic transcription factors Runx2, Osterix, Dlx5, or Msx2 (Fig. 1D). We then determined the ratio of MLO-Y4 cells to E1-GFP cells that was most effective for inducing ALP and BSP expression in co-cultured E1-GFP cells. E1-GFP cells were co-cultured with MLO-Y4 cells at various ratios, as shown in Supplemental Table 1. Significant increases in the mRNA expression levels of ALP and BSP were observed in E1-GFP cells in co-culture containing 1% MLO-Y4 cells compared to mono-cultured E1-GFP cells (approximately 2 or 3-fold, respectively) (Fig. 1E). Increased levels of ALP and BSP mRNA expression in co-cultured E1-GFP cells reached a peak in co-culture containing 30% or more MLO-Y4 cells (Fig. 1E). Osteogenic terminal differentiation is characterized by the formation of mineralized nodules. Thus, we next investigated the effect of co-culture on mineralization. After co-culture with MLO-Y4 cells or in mono-culture, E1-GFP cells were isolated and then cultured in osteogenic medium for the indicated times. The numbers of Alizarin Red S-positive mineralized nodules were higher in E1-GFP co-culture than in E1-GFP mono-culture (Fig. 2A). Similarly, Ca^{2+} levels were also increased by co-culture with MLO-Y4 cells at day 7 and day 14 (Fig. 2B).

3.2. The effect of cell-to-cell interaction on cell proliferation and cell cycle progression in E1-GFP cells co-cultured with MLO-Y4 cells

We next examined the effect of co-culture with MLO-Y4 cells on cell proliferation. After 24 h of mono-culture or co-culture with MLO-Y4 cells, isolated E1-GFP cells were cultured for the indicated time in growth medium and cell numbers were then measured. Both co-cultured and mono-cultured E1-GFP cells rapidly proliferated from day 3 to day 5, and the proliferation of co-cultured

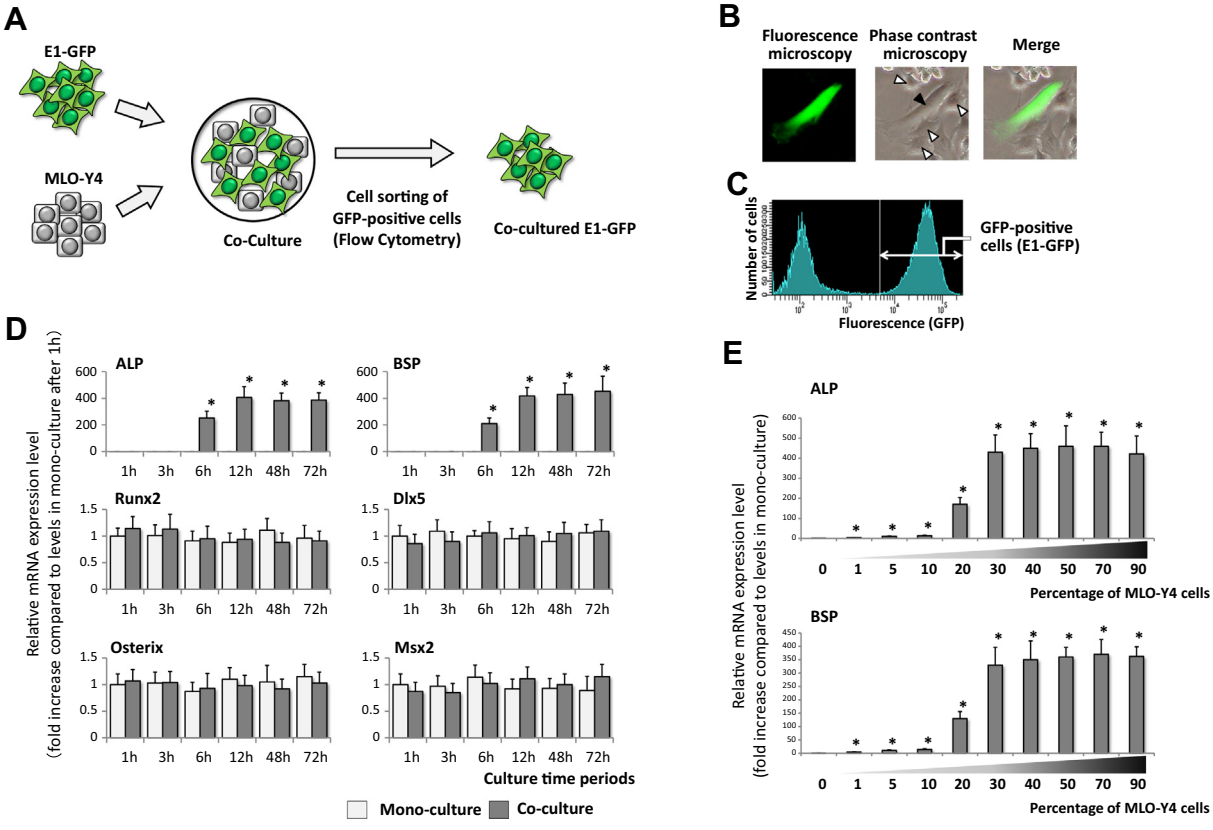

Fig. 1. Effect of co-culture on mRNA expression of osteogenic markers and transcription factors. (A) Schematic representation of the co-culture system used in this study. Single cell suspension consisted of E1-GFP and MLO-Y4 cells seeded onto a dish and co-cultured for 1–72 h. Subsequently, E1-GFP cells were separated by flow cytometry for analysis. Single culture (mono-culture) of E1-GFP cells was also used as a control. (B) White and black arrowheads indicate GFP-negative cells (MLO-Y4) and GFP-positive cells (E1-GFP), respectively, in co-culture. (C) Flow cytometry profile of E1-GFP and MLO-Y4 cells. (D) The mRNA expression levels of osteogenic markers (ALP and BSP) and osteogenic transcription factors (Runx2, Osterix, Dlx5, and Msx2). E1-GFP cells were cultured alone or co-cultured with MLO-Y4 cells for the indicated times, and mRNA expression levels were determined by real-time RT-PCR. * significantly different from mono-cultured cells, mean ± S. D. ($n = 3$, $P < 0.05$). (E) Ratio-dependent effect of co-culture with MLO-Y4 cells on ALP and BSP expression in E1-GFP cells. E1-GFP and MLO-Y4 cells were co-cultured at various ratios for 24 h as detailed in Supplemental Table 1. After co-culture, E1-GFP cells were isolated by flow cytometry and subjected to real-time RT-PCR. Results are given as the mean ± S. D. ($n = 3$, $P < 0.05$). *, significantly different from E1-GFP cells cultured alone (mono-culture).

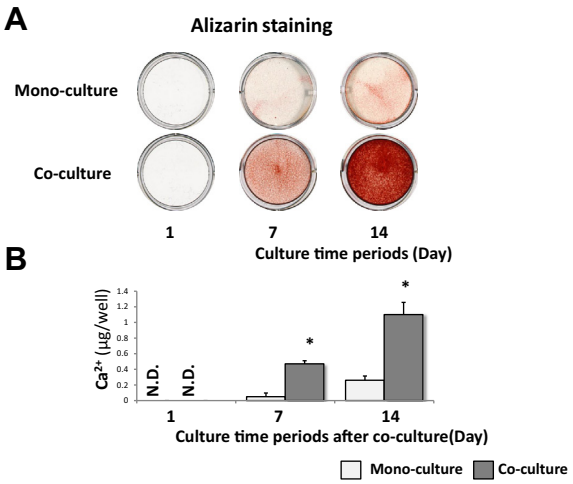

Fig. 2. Effect of co-culture with MLO-Y4 cells on mineralized nodule formation. (A) E1-GFP cells were co-cultured with MLO-Y4 cells or mono-cultured for 24 h and isolated by a flow cytometry. Isolated cells were then cultured in osteogenic medium for the indicated days. After culture, Alizarin Red S staining was performed. (B) Quantitation of calcium deposition. Calcium content of the cell layers, which were cultured under the same conditions described in panel A, were measured using Calcium *E*-test. Results are presented as the mean ± S. D. ($n = 3$, $P < 0.05$). *, significantly different from mono-cultured cells.

E1-GFP cells was lower than that of mono-cultured E1-GFP cells at day 5 (Fig. 3A). We then analyzed the effect of co-culture with MLO-Y4 cells on cell cycle progression and found that co-cultured E1-GFP cells exhibited an increased number of cells in G1, G0-phase (Fig. 3B).

3.3. Increased ALP and BSP mRNA expression is independent of BMP signaling

To investigate whether a soluble factor, such as bone morphogenetic proteins (BMPs) released from MLO-Y4 cells, was responsible for the increase in ALP and BSP expression in co-cultured E1-GFP cells, separated co-culture was performed using a filter membrane to prevent direct contact between E1-GFP and MLO-Y4 cells (Fig. 4A). Although the cells were maintained in co-culture for 3 days, no increase in mRNA expression of the osteoblastic markers (Fig. 4B) or transcription factors (data not shown) was detected in E1-GFP cells.

We next investigated the transcriptional activity of Smads 1, 5, and 8, which are known as essential factors involved in intracellular signaling of BMPs in the differentiation of MC3T3-E1 cells toward mature osteoblasts. As shown in Fig. 4C, no significant changes in the mRNA expression levels of Id-1 and Smad6, which are direct targets of phosphorylated Smads, were detected in E1-GFP cells co-cultured with MLO-Y4 cells during any culture period. In addition, no inhibitory effect on ALP or BSP mRNA expression was observed in the presence of the BMP-2 antagonist, noggin, in E1-GFP cells co-cultured with MLO-Y4 cells (Fig. 4D).

3.4. Gap junctions with MLO-Y4 are required for terminal differentiation of E1-GFP cells

Our results suggest that direct cell-to-cell contact between both cells is required for the terminal differentiation of E1-GFP. Thus, we studied the relationship between E1-GFP cells and MLO-Y4 cells using electrophysiological techniques. Patch clamp analyses were performed in combined cultures of E1-GFP and MLO-Y4 cells, as shown in Fig. 4E. A pair of neighboring cells was selected using a fluorescence microscope, and current was recorded from one side cell following voltage-step pulse injection in the other side cell under constant voltage-clamp conditions. As shown in the bottom of Fig. 4E, passage of electric currents was observed in pairs of E1-GFP and MLO-Y4 cells (right) but not in pairs of two E1-GFP cells (left). To further understand the role of gap junctions between E1-GFP and MLO-Y4 cells, an inhibition assay was performed in the presence of CBX, which inhibits the formation of intercellular gap junctions. Expectedly, increased expression of ALP and BSP mRNA was strongly suppressed in CBX-treated E1-GFP cells co-cultured with MLO-Y4 cells (Fig. 4F).

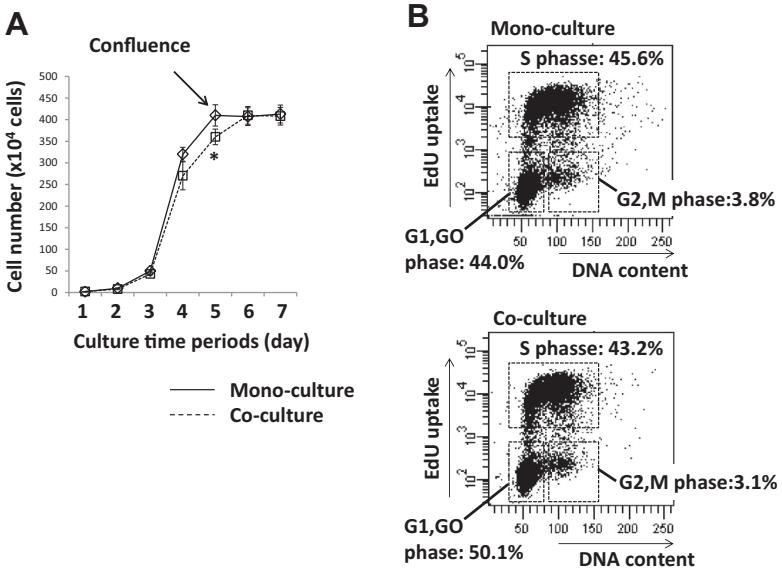

Fig. 3. Effect of co-culture with MLO-Y4 cells on proliferation and cell cycle progression in E1-GFP cells. (A) Cell proliferation. E1-GFP cells were co-cultured with MLO-Y4 cells for 24 h and isolated by flow cytometry. The isolated cells were then cultured in growth media for the indicated days, and cell numbers were counted. Data are presented as the mean ± S. D. ($n = 3$, $P < 0.05$). *, significantly different from mono-cultured cells. (B) Cell cycle progression. E1-GFP cells were co-cultured with MLO-Y4 cells for 24 h and then treated with EdU for 1 h. EdU-incorporated cells were labeled by pacific blue azide and GFP-positive cells (E1-GFP) were detected by FACS analysis using 407 nm excitation with a 450/50 nm bandpass.

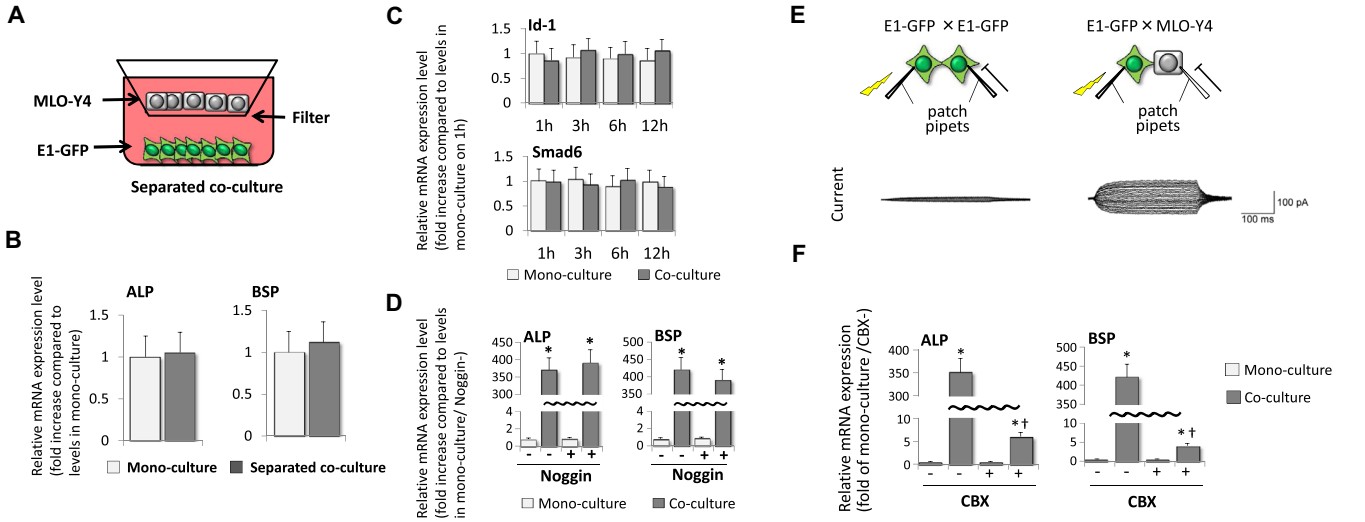

Fig. 4. Gap junction-mediated ALP and BSP mRNA expression in E1-GFP cells. (A) Experimental model of separate culture of E1-GFP and MLO-Y4 cells. E1-GFP cells were placed in a lower well covered by a culture chamber with a filter membrane (1 μm pore size), in which MLO-Y4 cells were seeded. (B) mRNA expression of ALP and BSP. E1-GFP cells were co-cultured with MLO-Y4 cells or mono-cultured for 24 h using the separated culture system and real-time RT-PCR was performed to measure mRNA expression levels of ALP and BSP. No significant differences were observed between co-cultured and mono-cultured cells. Data are presented as the mean ± S. D. ($n = 3$, $P < 0.05$). (C) The mRNA expression levels of Id-1 and Smad6. E1-GFP cells were co-cultured with MLO-Y4 cells or mono-cultured for 1, 3, 6, or 12 h, and real-time RT-PCR was performed. No significant differences were observed between co-cultured and mono-cultured cells. Data are presented as the mean ± S. D. ($n = 3$, $P < 0.05$). (D) Effect of noggin on the increased mRNA expression levels of ALP and BSP. E1-GFP cells were co-cultured with MLO-Y4 cells in the presence (+) or absence (−) of noggin (1 mg/ml) for 12 h, and real-time RT-PCR was performed. No significant differences were observed between cells cultured in the presence (+) or absence (−) of noggin. Data are presented as the mean ± S. D. ($n = 3$, $P < 0.05$). *, significantly different from mono-cultured cells. (E) Patch clamp assay. E1-GFP cells were co-cultured with MLO-Y4 cells or mono-cultured for 24 h and two neighboring cells were attached by patch pipette. Current was recorded between the cells as described in the Materials and Methods. (F) Effect of carbenoxolone (CBX) on mRNA expression levels of ALP and BSP. E1-GFP cells were pre-treated with (+) or without (−) 100 μM CBX for 1 h and co-cultured with MLO-Y4 cells or mono-cultured for 12 h, followed by real-time RT-PCR to measure the mRNA expression levels of ALP and BSP. Data are presented as the mean ± S. D. ($n = 3$, $P < 0.05$). *, significantly different from the cells co-cultured with MLO-Y4 cells in the absence (−) of CBX; †, significantly different from mono-cultured cells in the presence (+) or absence (−) of CBX.

4. Discussion

The data presented in this study showing that GFP-expressing MC3T3-E1 cells exhibited drastically increased mRNA levels of ALP and BSP when co-cultured with MLO-Y4 cells suggests that several signaling molecules are activated intracellularly through gap junctional interaction between the two cell types. BMP-2-associated signaling molecules, including Smads, are thought to regulate ALP and BSP transcription together with osteogenic transcription factors, such as Runx2 and Osterix [8,9]. However, no increases in the mRNA expression levels of Smad6 and Id-1 [10] were observed in E1-GFP cells co-cultured with MLO-Y4 cells. In addition, the BMP-antagonist, noggin [11], failed to suppress the increase in ALP and BSP expression levels in E1-GFP cells co-cultured with MLO-Y4 cells. These results suggest that the increase in ALP and BSP mRNA expression levels in co-cultured E1-GFP cells is not mediated via BMP-2 signaling.

The present study clearly demonstrated the existence of gap junctions between MLO-Y4 and E1-GFP cells, and inhibition with the gap junction inhibitor CBX suppressed increased mRNA expression of ALP and BSP in E1-GFP cells co-cultured with MLO-Y4 cells. Gap junctions regulate various cellular activities through the transport of small molecules, such as calcium ions [12,13]. Calcium ions (Ca^{2+}) affect the expression of osteoblastic markers, including ALP and BSP, as well as the formation of mineralized matrix of osteoblasts [14]. Thus, calcium ions may play an important role in the observed increase in ALP and BSP expression levels in E1-GFP cells co-cultured with MLO-Y4 cells. In addition, since transcription factors that have a zinc finger domain regulate the transcription of a variety of genes in a zinc-dependent manner [15], it is possible that small changes in the concentration of zinc ions is critical for the increase in ALP and BSP mRNA expression levels in E1-GFP cells co-cultured with MLO-Y4 cells.

It has been reported that BMP-2 increases the expression of ALP and BSP by inducing the expression of the osteoblastic transcription factors Runx2, Osterix, Dlx5, and Msx2 during osteoblast differentiation [8,9]. However, no significant differences in the mRNA expression levels of Runx2, Osterix, Dlx5, and Msx2 was shown. This may suggest the existence of a novel signaling pathway that plays a role in the mechanism by which co-culture with MLO-Y4 cells induces terminal differentiation of MC3T3-E1 cells. On the other hand, the effect of co-culture with MLO-Y4 cells on cell proliferation, which is closely related to cell differentiation [16], was also investigated. Proliferation assays revealed that cell numbers in co-cultured cells were significantly decreased at day 5 compared to mono-cultured cells although, the difference between them was slightly. In contrast, mineralized nodule formation and osteogenic gene transcription were drastically increased in co-cultured E1-GFP cells compared to mono-cultured cells, and analysis of cell cycle progression revealed in increased proportion of co-cultured cells in G1, G0 phase. These results suggest that MLO-Y4 cells do not induce terminal differentiation of E1-GFP cells by regulating cell proliferation. It appears that different signaling pathways regulate cell proliferation and terminal differentiation of co-cultured E1-GFP cells. To our knowledge, this is the first report to demonstrate the molecular mechanism of cell-to-cell interaction between pre-osteoblasts and osteocytes via gap junctions in the terminal differentiation of pre-osteoblasts. However, some issues remain to be clarified. The first of these is the identity of the molecules that move across the gap junction from MLO-Y4 to E1-GFP cells. It is thought that the majority of proteins are unable to pass through gap junctions, since the diameter of the gap junction opening is only approximately 1.5 nm [17]. Thus, as described above, the intercellular transport of ions, including calcium and/or magnesium, is likely to play an important role. On the other hand, it has been reported that micro (mi) RNA, a signal-strand RNA approximately 20–25 bases long that has been

shown to regulate gene expression, is also transported through gap junctions [18], making it a candidate mediator of intercellular communication between pre-osteoblasts and osteocytes. The second question that must be addressed is what directly induces the transcription of ALP and BSP. In this study, increased mRNA expression of ALP and BSP was observed in E1-GFP cells co-cultured with MLO-Y4 cells. However, no change was observed in the mRNA expression of the transcription factors Dlx5, Runx2, Osterix, and Msx2. A number of transcription factors, such as STAT3 and NF-κB, are well known to interact with other intracellular signaling molecules and regulate ALP and BSP expression [7], suggesting that other factors may contribute to the observed increase in mRNA expression.

In the present study, evidence of cell-to-cell interaction between pre-osteoblasts and osteocytes through gap junctions provides important insight into the complex mechanism of terminal differentiation of pre-osteoblasts *in vitro*. However, further analysis will be needed to elucidate the role of gap junctions in the differentiation of osteoblasts.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.10.128.

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