Int J Clin Exp Med 2016;9(2):1062-1072
www.ijcem.com /ISSN:1940-5901/IJCEM0015051

Original Article

Effect of osteoclasts on murine osteoblastic differentiation in early stage of co-culture

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Received August 25, 2015; Accepted December 22, 2015; Epub February 15, 2016; Published February 29, 2016

Abstract: Interaction between osteoclasts and osteoblasts is known to play critical roles in bone formation. However, the biological significance of this interaction remains incompletely understood. This study aimed to investigate the early-stage effects of osteoclasts on the differentiation of murine osteoblasts. Osteoclasts were isolated from mouse bone marrow, and osteoblasts were harvested from neonatal mouse calvaria. Both cell types were co-cultured using a Millipore cell culture system. Expression of osteoblast marker genes was assessed using real-time polymerase chain reaction. Then changes in the protein expression profile of osteoblasts co-cultured with osteoclasts were examined using antibody array analysis and western blotting. The osteoblastogenic activities of cells under these conditions were also investigated using an *in vitro* assay. Osteoblasts and osteoclasts were successfully isolated, as confirmed by alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP) staining, respectively. The expression of osteoblast specific marker genes, such as RUNX-2, ALP, and collagen-1a, was significantly increased. In addition, six proteins were found to be up-regulated and another six were down-regulated in osteoblasts co-cultured with osteoclasts compared to expression levels in osteoblasts cultured alone. In particular, the expression levels of basic fibroblast growth factor and insulin-like growth factor-1 were remarkably altered. Indeed, osteoclasts grown in co-culture with osteoblasts significantly promoted osteoblastogenesis *in vitro* ($P<0.005$). In conclusion, osteoclasts may up-regulate osteoblastogenesis-related gene expression in osteoblasts and enhance osteoblastogenesis. Together, the results of this study indicate that osteoclasts play a critical role in the differentiation of osteoblasts during the early stage of co-culture.

Keywords: Osteoblasts, osteoclasts, co-culture, osteoblastogenesis, antibody array analysis

Introduction

Bone tissue maintains its normal structure and metabolic balance through bone repair and regeneration [1, 2]. Osteoblasts and osteoclasts are the two main cell types in bone tissue, and interactions between these two cell types determine the processes of bone regeneration [3]. Therefore, modulation of the interaction between osteoblasts and osteoclasts may represent a potential strategy for treating various bone diseases. Many *in vitro* co-culture models have been used to study the interactions between these two types of cells; however, previous research has focused primarily on the influence of osteoblasts on the behavior of osteoclasts, and specifically, the role of the osteoprotegerin (OPG)-receptor activator of nuclear factor κB ligand (RANKL)-RANK axis in the differential development of osteoclasts [4-6]. In contrast, few studies have investigated how osteoclasts influence the biological functions of osteoblasts.

Osteoclasts, as the only cells of the body that resorb bone matrix, are derived from bone marrow monocyte-macrophage cell lineages, also known as hematopoietic stem cells [7, 8]. Multiple mononuclear macrophages are fused to form multinucleated macrophages, i.e., osteoclasts. Various cytokines and hormones such as tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6, and parathyroid hormone, participate in the formation of osteoclasts [9-11].

Two specific activators of osteoclastogenesis include macrophage colony stimulating factor (M-CSF) and RANKL [12]. M-CSF is important for osteoclast precursor migration, survival, and cytoskeletal reorganization, whereas RANKL binds to its receptor (RANK) on the surface of osteoclast precursor cells, leading to the activation of several signaling pathways, including the nuclear factor (NF)-κB and mitogen-activated protein kinase (MAPK) pathways [13, 14], which are crucial for the development of mature osteoclasts. OPG is secreted by osteoblasts and acts as a decoy receptor to inhibit the effect of RANKL on osteoclasts [15, 16]. In order to induce the expression of genes characteristic of the osteoclast lineage, such as tartrate-resistant acid phosphatase (TRAP), osteoclast-associated receptor (OSCAR), calcitonin receptor (CALCR), and cathepsin K (CTSK), both M-CSF and RANKL must be present [17]. *In vivo* and *in vitro* cells of the osteoblastic lineage as well as osteoblasts express and secrete M-CSF and RANKL and induce osteoclastogenesis primarily via interaction between the two types of cells [18, 19]. Although the effects of osteoclasts on osteoblasts have not been studied extensively and remain relatively unknown, some researchers have proposed that osteoclasts promote the proliferation and differentiation of osteoblasts [20]. However, others have reported an opposing view, proposing that osteoclasts may inhibit osteoblast functions [21].

Compared to cultures of individual cell types, co-culture systems are one step closer to natural conditions and are thus attractive for testing the properties and effects of biomaterials *in vitro*. Co-culture systems allow for the elucidation of various aspects of the complex interactions between osteoblasts and osteoclasts through direct observation of cell-cell interactions. One previous study in which osteoblasts and osteoclasts were cultured together reported that the two cells types mutually influence the generation and activity of the other cell type through direct physical contact and the secretion of cytokines [22].

To investigate the biological effects of osteoclasts on osteoblast differentiation at early stage, we first established an *in vitro* co-culture system for osteoclasts and osteoblasts using a Millipore cell co-culture system. We then observed the proliferation and differentiation of osteoblasts as well as used RayBiotech antibody arrays to identify changes in the complete protease and inhibitor expression profiles of osteoblasts that occur in the co-culture conditions. Our results reveal a previously unknown function of osteoclasts in osteoblastogenesis.

Materials and methods

Reagents

Recombinant murine M-CSF and recombinant murine sRANK-L were purchased from Peprotech Systems, Inc. (USA). Fetal bovine serum (FBS), α-minimal essential medium (MEM), and antibiotics (100 U/ml penicillin and 100 g/ml streptomycin) were purchased from Gibco (USA). The TRAP staining solution 387-A was purchased from Sigma-Aldrich (USA), and the Cell Counting Kit-8 and Alkaline Phosphatase Colour Development kit were both purchased from Beyotime Institute of Biotechology (China). For real-time reverse transcription (RT) polymerase chain reaction (PCR), Inventoried TaqMan® Gene expression Assays were used with Reverse Transcriptase qPCR™ Mastermix No ROX from Promega (USA).

Preosteoclast isolation and in vitro differentiation

C57BL/6 mice were obtained from the Animal Experiment Lab of Daping Hospital. All experiments involving mice were approved by the Institutional Animal Care and Use Committee at the Third Military Medical University. Briefly, bone marrow cells (BMCs) were flushed from mouse tibiae and femurs of young (4-6-week-old) mice, treated with red blood cell lysis buffer (150 mm ammonium chloride, 10 mm potassium bicarbonate, and 0.1 mm EDTA, pH 7.4), and cultured in α-MEM supplemented with 10% FBS and 10 ng/ml M-CSF on tissue culture-treated plastic. After overnight incubation, non-adherent bone marrow monocytes were collected and replated in 24-well plates in α-MEM with 10% FBS and 10 ng/ml M-CSF for a further 48 h to generate bone marrow macrophages (BMMs). Osteoclast differentiation was then stimulated by addition of 30 ng/ml M-CSF and 50 ng/ml RANKL for 4 days.

Osteoblast isolation and culture

Osteoblasts were enzymatically isolated from calvariae of neonatal (24 h) C57Bl/6 mice.

Table 1. Description of test groups, cell types and medium used within each test group

Test group	Culture medium
OB	α-MEM+10% FBS+100 IU/ml penicillin+100 mg/ml streptomycin
OC	OB medium supplemented with M-CSF (30 ng/ml) and RANKL (50 ng/ml)
OBOC	α-MEM+10% FBS+100 IU/ml penicillin+100 mg/ml streptomycin

Abbreviations: OB, osteoblast; OC, osteoclast; OBOC, osteoblast/osteoclast co-culture.

Table 2. Primer sequences used for real-time PCR

Gene	Primer sequence		Accession number
ALP	F	AGATTGACAGGGCAGAAACTCG	NM_016798.3
	R	CTGTGGTTCAGCTTCTAAGTTGA	
OCN	F	GCAGAACAGACAAGTCCCACA	NM_001037939.2
	R	CTGTGAGGTCAGAGAGACAGAGC	
COL-1a	F	CCCTGAAGTCAGCTGCATACA	NM_007742.3
	R	TTTGGTGATACGTATTCTTCCG	
RUNX-2	F	TTGTAGCCCTCGGAGAGGTA	NM_001145920.2
	R	AAACTCTTGCCTCGTCCGCT	
GAPDH	F	ATTTGGCCGTATTGGGCG	NM_001289726.1
	R	TGGTGGTGAAGACACCAGTAGA	

Abbreviation: OCN, osteocalcin; COL-1a, collagen 1a; RUNX-2, Runt-related transcription factor 2; GAPDH, glyceraldehyde 3-phosphate dyhydrogenase; F, forward; R, reverse.

Briefly, skin and soft connective tissue were removed. The bone was cut into 1-mm^2 square pieces and washed twice in phosphate-buffered saline (PBS; pH 7.4). The bone pieces were sequentially digested in trypsin (25 mg)/collagenase I (30 mg)/collagenase II (30 mg) in 25 mL PBS at 37°C for 20 and 70 min. Osteoblasts obtained from the final two digestions were pooled and plated together in 25-cm^2 cell culture bottles. The cells were cultured until confluence in α-MEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin.

Co-culture system

Millipore cell culture devices were used to establish the co-culture system for the two types of cells. Osteoclasts (1×10^5 cells/well) were placed in the top well, and osteoblasts (1×10^5 cells/well) were applied to the bottom well. The culture media used for all test groups are listed in **Table 1**.

Measurement of TRAP activity and TRAP staining

TRAP staining was conducted on samples at the conclusion of the study. Preosteoclast cells (1×10^5 cells/well) were seeded in a 24-well plate and incubated for 24 h. Cells were treated with M-CSF (10 ng/ml) for 2 days and then M-CSF (30 ng/ml) plus RANKL (50 ng/ml) in media containing 10% FBS for 4 days. After incubation, the cells were fixed with 3.7% formaldehyde, permeabilized with 0.1% Triton X-100 and incubated with reagents from the TRAP staining solution 387-A in the dark at 37°C. After rinsing the cells, TRAP-positive multinucleated cells were visualized by phase-contrast light microscopy (Olympus Optical, Japan). TRAP-positive multinucleated cells with more than five nuclei were considered to be osteoclasts.

Osteoblast proliferation and viability

Osteoblast proliferation upon exposure to osteoclasts was measured using the xCELLigence RTCA System (ACEA Biosciences Inc., USA). Briefly, osteoblasts (1×10^4 cells/well) were seeded in a 96-well plate and incubated for 24 h. Then the cells were co-cultured with osteoclasts (1×10^4 cells/well) using Millipore cell culture inserts for 7 days.

In addition, cell viability was measured using the Cell Counting Kit-8 (Beyotime Institute of Biotechology), according to the manufacturer's instructions. The method of co-culture was the same as described above, and the absorbance at 450 nm was measured using the Biotek ELX-800 plate reader (Biotek, USA). All experiments were performed in triplicate.

Alkaline phosphatase (ALP) staining and activity assay

Osteoblasts (1×10^5 cells/well) were seeded in a 24-well plate and incubated for 24 h. Then they were cultured with osteoclasts (1×10^5 cells/

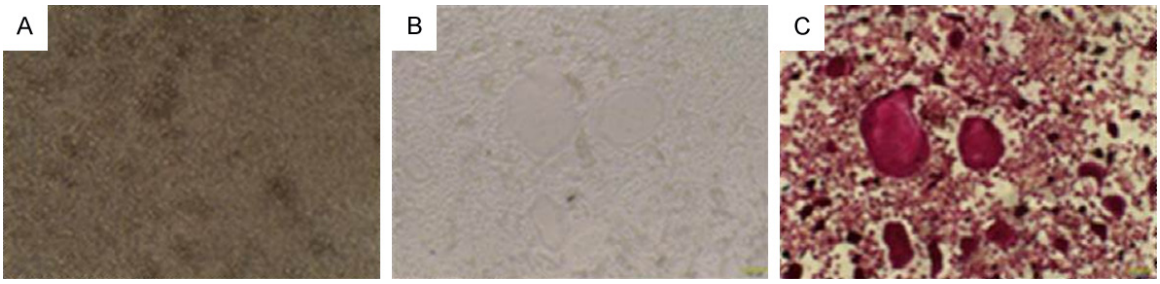

Figure 1. Cultured osteoclasts and TRAP staining. A. After BMCs were cultured for 1 day in tissue culture dishes, the non-adherent cells were transferred to new 6-well plates and cultured in media containing 10 ng/ml M-CSF for 2 day (magnification, 100×). B. Large multinucleated cells formed after culture with 30 ng/ml M-CSF and 50 ng/ml RANKL for 6 days (magnification, 100×). C. TRAP staining of generated osteoclasts (magnification, 100×).

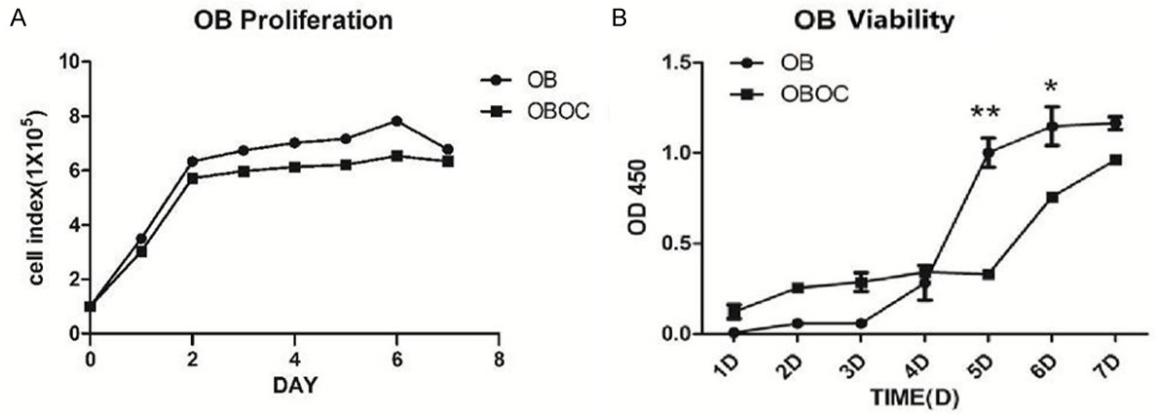

Figure 2. Effect of osteoclasts on the proliferation and viability of osteoblasts. A. Numbers of osteoblasts in the control and co-culture groups over 7 days in culture. B. Viability of osteoblasts in the control and co-culture groups over 7 days in culture. *P<0.05.

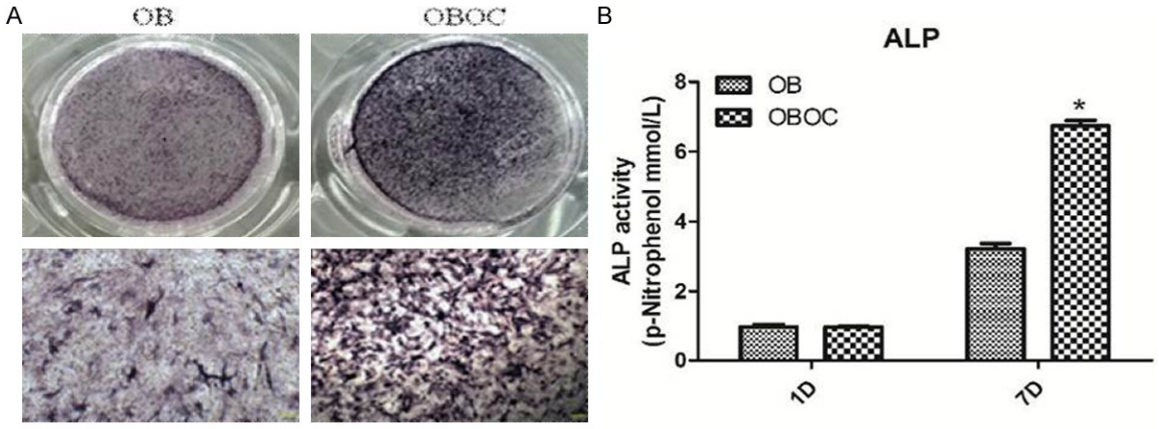

Figure 3. Potential of osteoclasts to induce osteogenic differentiation of osteoblasts at the initial co-culture period. A. Photographs of ALP staining in osteoblasts cultured (OB) alone or with osteoclasts (OBOC) for 7 days. B. ALP activity assay results for osteoblasts cultured (OB) alone or with osteoclasts (OBOC) after 1 and 7 days in culture. *P<0.05.

well) in media containing 10% FBS, with media exchanged every 3 days. After 7 days, cells were fixed with 3.7% formaldehyde, rinsed with PBS, and stained with the Alkaline Phosphatase

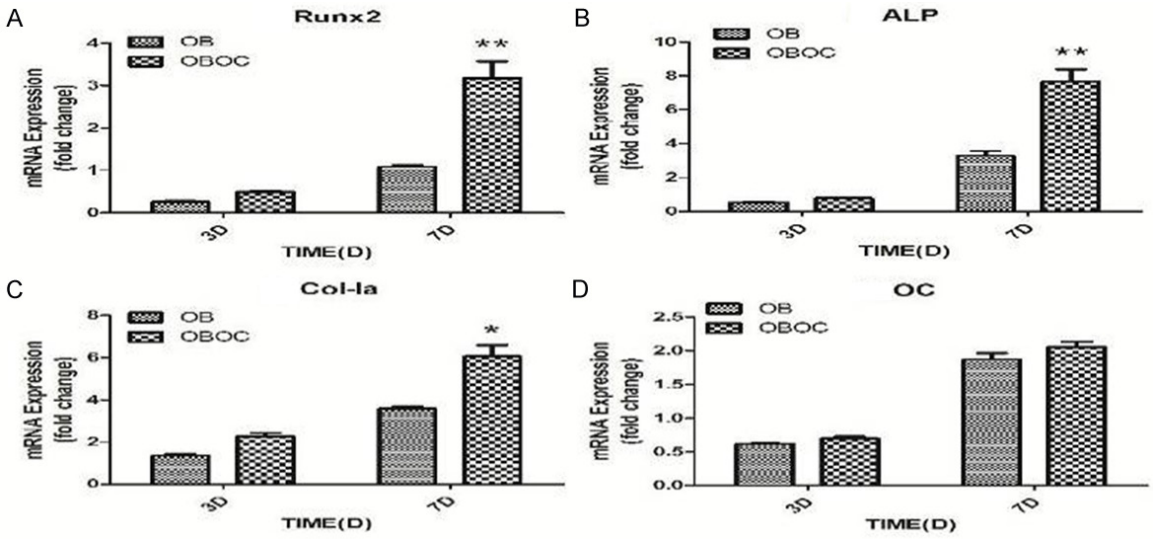

Figure 4. Real-time PCR analysis of the expression of different osteogenic markers in osteoblasts co-cultured with osteoclasts at days 3 and 7 in culture: A. RUNX-2; B. ALP; C. COL-1a; D. OC. *P<0.05; **P<0.01.

Table 3. Twelve proteins most significantly up- and down-regulated in osteoblasts co-cultured with osteoclasts

Protein	Description	Fold change
Up-regulated		
IGFBP5	Insulin-like growth factor binding protein 5	4.788
CDH1	Cadherin 1	4.729
TROY	Tumor necrosis factor receptor superfamily, member 19	4.481
IGFBP3	Insulin-like growth factor binding protein 3	3.709
GAS1	Gas1 protein	3.26
HGF	Hepatocyte growth factor	3.023
Down-regulated		
β-FGF	Basic fibroblast growth factor	0.197
CD40	CD40 antigen	0.174
NPTX2	Neuronal pentraxin-2	0.146
IGF-I	Insulin-like growth factor 1	0.139
MCP1	Chemokine (C-C motif) ligand 2	0.092
GITR Ligand	Tumor necrosis factor receptor superfamily, member 18	0.001

Color Development kit, and ALP-positive cells were visualized by phase-contrast light microscopy (Olympus Optical, Japan).

After 7 days of co-culture, osteoblasts were washed with PBS and lysed with 0.1% Triton X-100 using three cycles of freezing and thawing to verify that the cells were completely lysed. The cell lysates were centrifuged at 15,000× g for 5 minutes at 4°C, and the supernatants were collected. Next, 5-mL aliquots of the cell lysates were transferred to 96-well plates, and 150 mL of p-nitrophenyl phosphate (Jiancheng Biotechnology, Nanjing, China) was added to each well as a substrate for 20 minutes at 37°C. p-Nitrophenyl phosphate was quantified based on the spectrophotometric absorbance at 450 nm.

Expression of common bone-related genes by real-time PCR

Osteoblasts were seeded in 6-well plates at a density of 1×10^5 cells/well and cultured with osteoclasts for 7 days. Briefly, total RNA was extracted using TRIzol reagent (Invitrogen), and the purified total RNA was used for cDNA synthesis with M-MLV reverse transcriptase and oligo (dT) primers. The specific primers used to detect the mRNA transcripts of the Runx^2, Col1a1, ALP, and OC genes are shown in **Table 2**. The transcript levels were normalized by the GAPDH transcript levels. The PCR conditions were: 95°C for 3 minutes followed by 40 cycles of 94°C for 10 seconds and 60°C for 30 seconds.

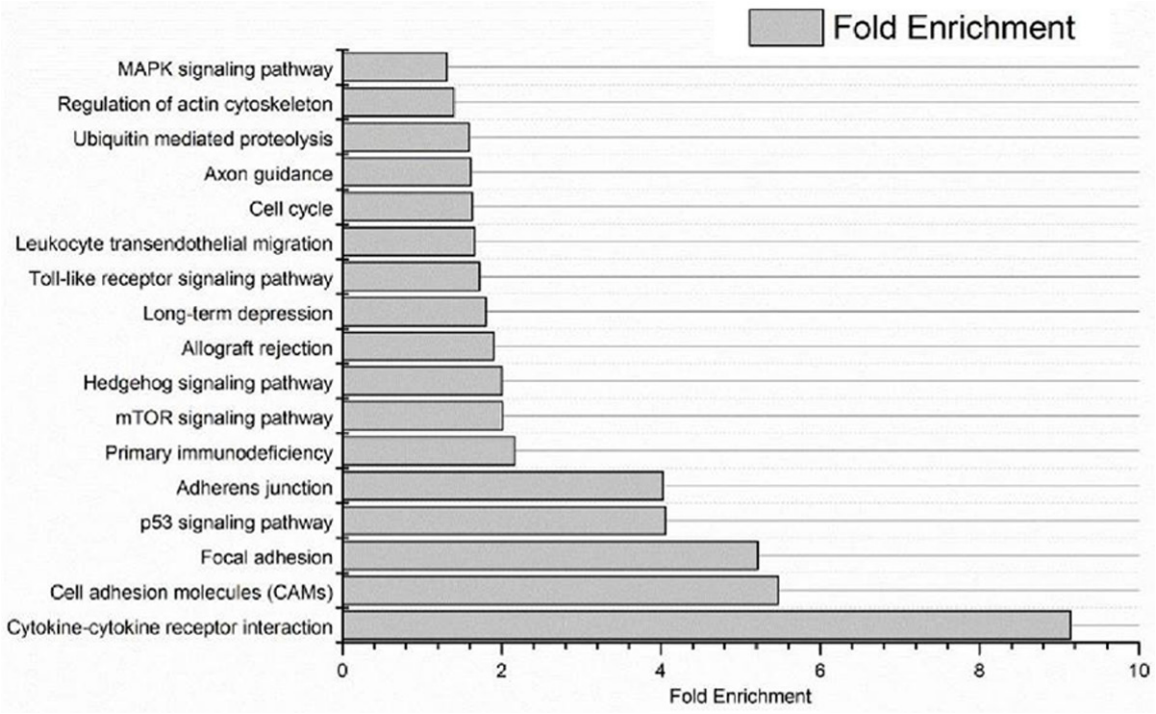

Figure 5. Pathways identified by GO analysis as significantly represented by the differentially expressed proteins in osteoblasts co-cultured with osteoclasts. *Pathways identified by GO ontological analysis.

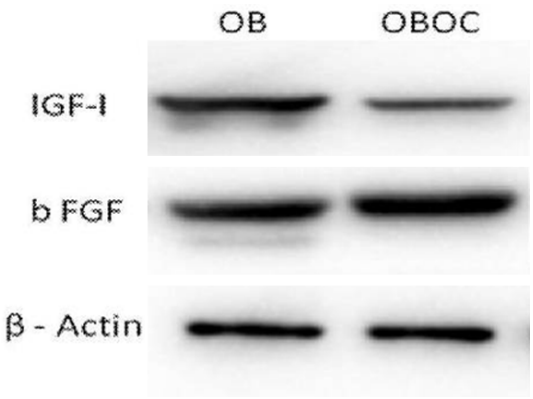

Figure 6. Western blot analysis of IGF-I and β-FGF expression in osteoblasts co-cultured with osteoclasts.

Antibody arrays

The presence of soluble proteins in the medium of osteoblasts was measured using the Mouse Cytokine Antibody Array (RayBio, AAM-CYT-G2000), according to the recommended protocols. These arrays can detect 144 proteins, respectively. Osteoblasts were plated for 3 days before the experiment in Dulbecco's Modified Eagle Medium containing 10% FBS and were 75-90% confluent when the media were collected and filtered. Medium containing 10% FBS was also hybridized to the arrays and used later for normalization. Ten technical and biological replicates were carried out, and both showed a very high correlation. Hybridization was carried out overnight at 4°C. All slides were scanned using a GenePix 2000B Microarray Scanner (Axon) and analyzed using the software GenePix Pro 6.0. The F532 median 2 B532 score was used and averaged across triplicates on each array. The results were then normalized using internal controls, and the values for cytokines in clear medium containing 10% FBS were subtracted.

Western blot analysis

According to the results of antibody arrays, two main factors changed significantly, insulin-like growth factor (IGF)-1 and basic-fibroblast growth factor (β-FGF). Then western blot analysis was conducted to confirm the changes in the levels of these two factors. Osteoblasts were collected after 7 days in co-culture or culture alone. The cells were lysed in lysis buffer containing protease inhibitors, and 15-mg aliquots were subjected to western blot analysis using the specified primary antibodies against IGF-I

and β-FGF. Then the secondary antibody labeled with horseradish peroxidase (Boston Biotechnology, Wuhan, China) was used. An anti-β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used for β-actin evaluation as an internal control. The bound antibodies were detected with an enhanced chemiluminescence detection reagent (Pierce Biotechnology Inc., Rockford, IL, USA), and the intensities of the bands were measured using Image Software Pro Plus 6.0 software.

Statistical data analysis

Statistical analyses were performed using SPSS 17.0 software (Chicago, IL, USA). All data were analyzed by analysis of Student's t-test and analysis of variance (ANOVA), and differences were considered statistically significant with values of $P<0.05$. All experiments were carried out at least three times independently.

Results

Differentiation and identification of osteoclasts

To obtain and identify mature osteoclasts, primary mouse BMCs were generated *in vitro* by growing whole bone marrow in the presence of M-CSF for 1 day. Then non adherent cells were moved to new 6-well plates and cultured in media containing 10 ng/ml M-CSF for another 2 days (**Figure 1A**). Finally, the cells were cultured with 30 ng/ml M-CSF and 50 ng/ml RANKL for 6 days and formed multinucleated huge cells. The addition of RANKL to these cultures initiated a differentiation program that evoked the terminal differentiation of monocytes into mature, multinucleated huge osteoclasts. As shown in **Figure 1B** and **1C**, RANKL strongly stimulated the differentiation of BCMs into mature osteoclasts at 6 days.

Effects of osteoclasts on the proliferation and viability of osteoblasts

To assess the effects of osteoclasts on the proliferation of osteoblasts, the xCELLigence RTCA system was used to quantitate the numbers of cells in co-culture over 7 days. The numbers of osteoblasts cultured with or without osteoclasts increased greatly during the first 2 days. On days 5 and 6, the number of cells in the control group appeared to be greater than that in the co-culture group, but the differences were not statistically significant (**Figure 2A**). However, using the Cell Counting Kit-8, we observed significantly greater viability of osteoblasts in control cultures compared to that of osteoblasts in co-cultures on days 5 and 6 ($P<0.05$; **Figure 2B**).

Effect of osteoclasts on ALP activity in osteoblasts

To examine the effect of osteoclasts on osteoblastogenic activity in co-culture, the ALP activity of osteoblasts co-cultured with osteoclasts was compared to that of osteoblasts cultured alone for 7 days. Osteoblasts in co-cultures showed much denser ALP staining than osteoblasts cultured alone after 7 days (**Figure 3A**), and the quantitative results of ALP activity assays were consistent with the staining results (**Figure 3B**), showing significantly greater ALP activity in osteoblasts co-cultured with osteoclasts after 7 days. These results indicate that osteoclasts can promote differentiation of osteoblasts during the early stage of co-culture.

Real-time PCR analysis of the expression of different osteoblast markers in osteoblasts after culture with osteoclasts

To gain further insight into the molecular mechanisms underlying the induction of osteoblast differentiation by osteoclasts, the expression of common bone-related genes were examined by real- time PCR. The expression level of RUNX2, which regulates osteoblast differentiation at early stages, was markedly upregulated in co-cultured osteoblasts at 7 days compared to the expression level in the control group ($P<0.05$, **Figure 4A**). In addition, ALP mRNA expression was significantly greater in co-cultured osteoblasts compared to osteoblasts cultured alone after 7 days ($P<0.05$, **Figure 4B**). COL-1a mRNA expression in the co-cultured osteoblasts also was significantly increased compared with that of the control osteoblasts after 7 days ($P<0.05$, **Figure 4C**). OCN mRNA expression was similar between the two groups of osteoblasts after 7 days (**Figure 4D**).

Antibody array analysis of the effect of osteoclasts on the biological development of osteoblasts in the early stage of co-culture

Next we performed an antibody array analysis to identify differentially expressed proteins in

osteoblasts co-cultured with osteoclasts. Our analysis identified 144 differentially expressed proteins in osteoblasts co-cultured with osteoclasts after 7 days. As controls, osteoblasts were cultured alone in the absence of osteoclasts. When a fold change in protein expression >2.0 or <0.20 was considered significant, six proteins were found to be significantly up-regulated (by 4.788-3.023-fold), and six proteins were found to be significantly down-regulated (by 0.197-0.001-fold) in osteoblasts co-cultured with osteoclasts (**Table 3**). Thus, the expression of these proteins appears to have been specifically regulated by co-culture between osteoblasts and osteoclasts, and these proteins may be relevant to osteoblast differentiation.

The proteins differentially regulated by osteoclasts were classified ontologically with the KEGG and GENMAPP classification system pathways, which also statistically determine over- or under-representation of proteins in the particular pathways (**Figure 5**). Pathway analysis showed that the differentially expressed genes are related to cytokine-cytokine receptor interaction signaling pathways such as TROY, HGF, CD40, and MCP-1. Other significantly represented pathways included the cell adhesion molecule and p53 signaling pathways. One differentially expressed protein, bFGF, is also involved in the regulation of the actin cytoskeleton and MAPK signaling.

Validation of results by western blotting

To confirm the antibody array results, we next examined the protein expression of IGF-I and bFGF in osteoblasts at the protein level. These proteins have been reported to be involved in the osteoblastogenic process and to play a crucial role in the proliferation and differentiation of osteoblasts. The protein levels of IGF-I and β-FGF were measured using western blot analysis at 7 days. The expression level of IGF-I was higher in osteoblasts under co-culture conditions than in osteoblasts cultured alone (**Figure 6**). However, the expression levels of β-FGF did not significantly differ between the two culture conditions. These results suggest that IGF-I expression in osteoblasts correlates with indirect contact between the osteoblasts and osteoclasts in the bone microenvironment.

Discussion

Previous studies have suggested a crucial role for activated osteoblasts in influencing the differentiation of osteoclasts through the secretion of soluble factors, such as OPG, RANKL, and M-CSF. However, there has been limited evidence regarding the direct regulation of osteoblasts differentiation at the early stage by osteoclasts. More recently, resident functional osteoclast populations in bone were found to serve as the most important regulators of bone homeostasis [23]. However, the mechanisms and mediators involved in these important interactions remain elusive. In the present study, we have shown that co-culture of osteoblasts with osteoclasts may promote the differentiation-of osteoblasts during the early stage of co-culture and upregulate the expression of osteoblastogenesis-related genes in osteoblasts. Our results indicate that osteoclasts can directly regulate osteogenic differentiation of osteoblasts during the early stage of co-culture.

Cell co-culture is the current best approach to investigate interactions between cells *in vitro* under conditions that model the cellular microenvironment *in vivo*. There are two co-culture methods: direct contact co-culture and indirect contact co-culture. In this study, we chose the indirect method to create a co-culture system using Millipore cell culture inserts. Only soluble factors secreted by the two types of cells could pass through the bidirectional membrane and act upon the other cell type during the course of co-culture. Nevertheless, although the formation of mature osteoclasts was induced by M-CSF and RANKL, the cells cannot survive for extended periods. Wu et al. [24] reported that the resorption activity of osteoclasts was gradually suppressed at the transitional stage and that the cysteine asparagine specific protease-3 axis induces cell death through negative feedback or estrogen induces Fas/FasL system to modulate the cell death program. Our previous study also showed that when cultured and fused, the huge multinucleated cells, i.e., the mature osteoclasts, only existed for 5-7 days before undergoing apoptosis.

ALP is an early marker of osteoblast differentiation that is relevant in matrix mineralization [25]. The differentiation and maturity of osteo-

blasts in co-culture with osteoclasts was confirmed by an increase in ALP activity. According to the results, osteoblast proliferation was not significantly influenced by co-culture with osteoclasts, whereas osteoblast viability was significantly improved at later times in the co-culture period. Additionally, when osteoblasts were co-cultured with osteoclasts, staining for ALP and ALP activity assays showed that osteoblastic differentiation was induced by the presence of osteoclasts.

As is well known, osteoblast differentiation involves three distinct phases: proliferation, maturation, and mineralization [26-28]. During the differentiation process, the expression of specific genetic markers is not the same as in other phases. RUNX2 is a transcription factor specifically expressed by osteoblast progenitor cells during the initial phase, and it is required to steer the cells away from an adipocytic or chondrocytic phenotype and toward osteoblastic differentiation [29]. This factor plays a major role in the regulation of other osteoblast-related genes, such as OCN, ALP, and COL-1 [30, 31]. To confirm our previous hypothesis and infer the probable molecular mechanism, we examined the mRNA expression levels of RUNX2, ALP, COL-1a, and OC in osteoblasts of the two groups at days 3 and 7 with RT-PCR. The results showed that the mRNA expression of all of the tested factors was up-regulated at day 7, even if not up-regulated by day 3. Specifically, the mRNA expression levels of RUNX2, ALP, and COL-1a in the co-cultured osteoblasts were much higher than that in osteoblasts cultured alone. These data suggest that the expression of genes known to be important transcriptional genes in osteoblasts, such as RUNX2, may be influenced by the presence of osteoclasts. Notably, RUNX2 plays an important role in the differentiation process of osteoblasts, enhancing the expression level of other osteoblast-related genes.

Antibody array analysis was used to identify all proteins that were differentially expressed in osteoblasts co-cultured with osteoclasts for 7 days. We identified six proteins that were up-regulated and also six that were down-regulated in osteoblasts only under co-culture conditions. Interestingly, both the up-regulated proteins (IGFBP5 and IGFBP3) and down-regulated proteins (bFGF and IGF-I) are all involved in the differentiation of osteoblasts. bFGF is known to be a potent mitogen and acts as an autocrine/paracrine factor for osteoblasts. Long-term administration of β-FGF in vivo increases osteoblast number and stimulates matrix formation, but induces hypophosphatemia and impairs matrix mineralization [32]. IGF-I can stimulate osteoblast proliferation and differentiation through positive regulation of the IR expression via the MAPK and PI3K pathways. Also, IGF-I promotes osteoblast differentiation by increasing ALP activity, type I collagen synthesis, OC expression, and mineralized nodule formation via the MAPK and PI3K pathways [33]. Based on the antibody array results, osteoclasts down-regulated the expression of β-FGF and IGF-I in osteoblasts when the two types of cells were cultured together. In particular, IGF-I expression was found to be significantly down-regulated using western blotting for confirmation. This results means that the effects of osteoclasts on the biological performances of osteoblasts at 7 days are primarily negative; that is, when osteoblasts are co-cultured with osteoclasts, their proliferative and differentiative capabilities should be down-regulated. However, this is opposite to our experimental results related to ALP viability and gene expression. Kreja et al. [34] and Bielby et al. [35] suggested that the differentiation of MSCs into osteoblasts can be affected by the presence of osteoclast -derived conditioned medium. However, Pederson and colleagues [36] concluded that osteoclast-derived conditioned medium had no impact on ALP production in MSC cultures, but the conditioned medium did positively influence cellular migration and mineralization. Therefore, when osteoblasts are cultured with osteoclasts, we can conclude that osteoclasts influence the synthesis and secretion of IGF-1 in osteoblasts.

In the present study, we have shown that interaction between osteoblasts and osteoclasts is likely a common event in advanced bone formation. We further showed when osteoblast culture with osteoclasts may enhance the differentiation of osteoblasts by up-regulating the gene expression of specific osteogenesis-related genes, such as RUNX-2, ALP, and COL-1. Additionally, IGF-I may play a critical role in this process. Finally, because the effects of osteoclasts on osteoblasts are extraordinarily complex, further studies are needed to identify fac-

tors and the underlying mechanisms of osteoclast-induced changes in osteoblasts.

Acknowledgements

We gratefully acknowledge all of the members of the laboratory for sharing reagents and advice.

Disclosure of conflict of interest

None.

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