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# Reciprocal Osteoblastic and Osteoclastic Modulation in Co-Cultured MG63 Osteosarcoma Cells and Human Osteoclast Precursors

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## ABSTRACT

Osteosarcoma is usually associated with a disturbed bone metabolism. The aim of this work was to characterize the reciprocal interactions between MG63 osteosarcoma cells and osteoclasts, in a co-culture system. Co-cultures were characterized throughout 21 days for the osteoclastogenic response and the expression of osteoblastic markers. Monocultures of MG63 cells and peripheral blood mononuclear cell (PBMC) and co-cultures of PBMC + human bone marrow cells (hBMC) were also performed. Compared to PBMC cultures, co-cultures yielded significantly increased gene expression of osteoclast-related markers, tartarate-acid resistant phosphatase (TRAP) activity, TRAP-positive multinucleated cells, cells with actin rings and vitronectin receptors (VNR) and calcitonin receptors (CTR) and calcium phosphate resorbing ability. Results showed that the development of functional osteoclasts required a very low number of MG63 cells, suggesting a high osteoclastogenic-triggering capacity of this cell line. Subjacent mechanisms involved the pathways MEK and NF-kB, although with a lower relevance than that observed on PBMC monocultures or co-cultures of hBMC + PBMC; PGE2 production also had a contribution. Compared to MG63 cell monocultures, the co-culture expressed lower levels of COL1 and ALP, and higher levels of BMP-2, suggesting that PBMC also modulated the osteoblastic behavior. While M-CSF appeared to be involved in the osteoclastogenic response on the MG63 + PBMC co-cultures, RANKL does not seem to be a key player in the process. On the other hand, sphingosine-1-phosphate production might contribute to the modulation of the osteoblastic behavior. Results suggest that the reciprocal modulation between osteosarcoma and osteoclastic cells might contribute to the disturbed bone metabolism associated with bone tumors. J. Cell. Biochem. 112: 3704–3713, 2011.



**KEY WORDS:** OSTEOCLAST; OSTEOSARCOMA; CELL-TO-CELL COMMUNICATION; CO-CULTURES

In the bone microenvironment, the reciprocal osteoblast–osteoclast communication and the corresponding coordinated bone formation and resorption events are highly complex and regulated processes responsible for the life-long bone remodeling in order to ensure a healthy tissue [Matsuo and Irie, 2008]. Bone tumors are usually associated with disturbed bone metabolic activities. Among the different types of bone tumors, osteosarcoma appears as the most frequent one [San-Julian et al., 2003]. Osteosarcoma cells are osteoblast-like cells that proliferate in the bone tissue, leading to the formation of woven bone [San-Julian et al., 2003], and also to an increase in bone destruction rates [Avnet et al., 2008]. Regarding the underlying mechanisms, the osteoblast–osteoclast communication is thought to play a relevant role. Osteoblasts synthesize a variety of molecules important in the recruitment and survival of osteoclast precursors [Yoshida et al., 1990; Lagasse and Weissman, 1997; Costa-Rodrigues et al., 2010] and also engaged with the later steps of osteoclastogenesis [Legendre et al., 2003; Motokawa et al., 2005; Hashizume et al., 2008; Costa-Rodrigues et al., 2010]. Osteosarcoma cells display many osteoblastic features, namely the expression of several pro-osteoclastogenic molecules, including M-CSF and RANKL [Saunders et al., 2006], and several studies suggest that these paracrine mechanisms might mediate bone destruction by stimulating osteoclast development [Miyamoto et al., 2002; Michael et al., 2007; Mori et al., 2007; Knowles and Athanasou, 2008]. While recognizing the relevance of osteosarcoma cells in the osteoclast activity, the reverse interaction, that is, the influence of osteoclastic

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cells in the behavior of osteosarcoma cells has received little attention. However, in the normal bone microenvironment, osteoclasts–osteoblasts crosstalks appear to have a direct influence in the bone formation events [Gray et al., 1996; Datta et al., 2008]. Subjacent mechanisms might involve tartarate-acid resistant phosphatase (TRAP), an enzyme required for bone matrix resorption [Hayman et al., 1996; Roberts et al., 2007], but that also appears to be associated with high rates of bone formation [Angel et al., 2000; Matsuo and Irie, 2008]. In addition, some factors produced by osteoclasts seem to stimulate osteogenesis, including sphingosine 1-phosphate, myb-induced myeloid protein-1, hepatocyte growth factor, and a B polypeptide chain platelet-derived growth factor homodimer [Matsuo and Irie, 2008]. Regarding the eventual relevance of osteoclasts on the behavior of osteosarcoma cells, information is still scarce. The only related results are from a study showing that conditioned medium from chicken osteoclastic cell cultures affects the osteoblastic behavior of the rat osteosarcoma cell line UMR-106-01 [Galvin et al., 1994].

In this context, the aim of the present work is to characterize the reciprocal interactions involving the osteosarcoma MG63 cell line and human osteoclast precursors, in a co-culture system. The MG63 cell line, widely used as an osteoblast-like cell model, was co-cultured (at two-cell densities) with human peripheral blood mononuclear cells (PBMC), and co-cultures were assessed for osteoblastic and osteoclastic parameters. For comparison, monocultures of MG63 cells and PBMC, as well as co-cultures of PBMC and human bone marrow cells (hBMC) were also performed.

## MATERIALS AND METHODS

### CO-CULTURE OF hBMC OR MG63 CELLS WITH HUMAN PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMC)

**hBMC and MG63 cells.** hBMC (obtained from patients of 25- to 35-year old undergoing orthopedic surgery procedures, after informed consent) and MG63 cell line were maintained in α-minimal essential medium (α-MEM) containing 10% fetal bovine serum, 100 IU/ml penicillin, 2.5 μg/ml streptomycin, 2.5 μg/ml amphotericin B, and 50 μg/ml ascorbic acid. At about 70–80% confluence, cells were enzymatically detached with 0.05% trypsin and 0.5 mM EDTA and seeded at the densities described below. Cultures were maintained in a 5% $CO_2$ humidified atmosphere at 37°C.

**PBMC.** Cells were isolated from blood of healthy male donors with 25- to 35-year old, as described previously [Costa-Rodrigues et al., 2011]. Briefly, after dilution with PBS (1:1), blood was applied on top of Ficoll-Paque™ PREMIUM (GE Healthcare Bio-Sciences) and centrifuged at 400g for 30 min. PBMC were collected and washed twice with PBS. On average, for each 100 ml of processed blood about $70 \times 10^6$ PBMC were obtained.

**Co-cultures of MG63 cells with PBMC and hBMC and PBMC.** MG63 cells were seeded at $1 \times 10^2$ and $1 \times 10^3$ cells/cm$^2$ (MG63 I and MG63 II, respectively) and cultured in α-MEM containing 10% fetal bovine serum, 100 IU/ml penicillin, 2.5 μg/ml streptomycin, 2.5 μg/ml amphotericin B, and 50 μg/ml ascorbic acid for 24 h. Following, PBMC were added at $1.5 \times 10^6$ cells/cm$^2$ and cultures were maintained in α-MEM supplemented with 20% human serum (from the same donor from which PBMC were collected), 100 IU/ml penicillin, 2.5 μg/ml streptomycin, 2.5 μg/ml amphotericin B, and 2 mM L-glutamine.

In parallel, monocultures of MG63 cells and PBMC, as well as co-cultures of PBMC and hBMC ($1.5 \times 10^6$ cells/cm$^2$: $10^3$ cells/cm$^2$), were used as controls, and were performed at the experimental conditions used in the co-cultures. Lower seeding densities of hBMC elicited a significantly lower osteoclastogenic response (data not shown) and, thus, were not used as controls.

Monocultures and co-cultures were incubated for 21 days at 37°C in a 5% $CO_2$ humidified atmosphere. Culture medium was replaced once a week. Cultures were characterized throughout the culture time, as described below.

### CELL CULTURES CHARACTERIZATION

**Protein quantification.** Total protein content of cell cultures was quantified at days 4, 7, 14, and 21 by Bradford's method [1976], using bovine serum albumin as a standard. After being washed twice with PBS, cell cultures were solubilized with 0.1 M NaOH, and were treated with Coomassie® Protein Assay Reagent (Fluka) for 2 min at room temperature. The 600 nm absorbance was determined in an ELISA plate reader (Synergy HT, Biotek).

**Cell viability/proliferation.** Cellular viability/proliferation was assessed by the MTT assay at days 4, 7, 14, and 21, as described before [Gomes et al., 2008]. Cells were incubated for 3 h at 37°C in the presence of 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. Culture medium was removed and formazan crystals were solubilized with DMSO. The absorbance of the samples was evaluated at 600 nm in an ELISA plate reader (Synergy HT, Biotek).

**RT-PCR analysis.** Cultures with 21 days were analyzed for RT-PCR, except MG63 I cell monocultures that did not yield sufficient amounts of mRNA. RNA was extracted with Rneasy® Mini Kit (Qiagen) according to manufacturer's instructions. RNA was quantified by UV spectrophotometry at 260 nm and analyzed by RT-PCR for the expression of the housekeeping gene GAPDH, the osteoblast-related genes collagen type 1 (COL1), alkaline phoshpatase (ALP), bone morphogenetic protein 2 (BMP-2) [Datta et al., 2008], the osteoclast-associated differentiation and activation factors, c-myc and c-src, respectively [Zhao et al., 2007], and the osteoclast functional genes TRAP, cathepsin K (CATK), and carbonic anhydrase 2 (CA2) [Zhao et al., 2007]. For that, 0.5 μg of RNA was reverse transcribed and amplified (25 cycles) with the Titan One Tube RT-PCR System (Roche), with an annealing temperature of 55°C. The primers used are listed in Table I. RT-PCR products were electophoretically separated on a 1% (w/v) agarose gel and subjected to densitometric analysis with ImageJ 1.41 software. Values were normalized to the corresponding GAPDH value of each experimental condition.

**TRAP activity.** TRAP activity was determined by the *p*-nitrophenilphosphate (pNPP) hydrolysis assay, at days 7, 14, and 21, as described before [Costa-Rodrigues and Fernandes, 2011]. Shortly, cell layers were washed twice with PBS and solubilized with 0.1% (v/v) Triton X-100. After that, cellular extracts were incubated with 12.5 mM pNPP in 0.04 M tartaric acid and 0.09 M citrate (pH 4.8) for 1 h at 37°C. The reaction was stopped with 5 M NaOH, and the absorbance of the samples at 405 nm was measured in an ELISA

TABLE I. Primers Used on RT-PCR Analysis of PBMC Cultures

| Gene | 5′ Primer | 3′ Primer |
|---|---|---|
| GADPH | 5′-CAGGACCAGGTTCACCAACAAGT-3′ | 5′-GTGGCAGTGATGGCATGGACTGT-3′ |
| COL1 | 5′-TCCGGCTCCTGCTCCTCTTA-3′ | 5′-ACCAGCAGGACCAGCATCTC-3′ |
| ALP | 5′-ACGTGGCTAAGAATGTCATC-3′ | 5′-CTGGTAGGCGATGTCCTTA-3′ |
| BMP-2 | 5′-GACGAGGTCCTGAGCGAGTT-3′ | 5′-GCAATGGCCTTATCTGTGAC-3′ |
| c-myc | 5′-TACCCTCTCAACGACAGCAG-3′ | 5′-TCTTGACATTCTCCTCGGTG-3′ |
| c-src | 5′-AAGCTGTTCGGAGGCTTCAA-3′ | 5′-TTGGAGTAGTAGGCCACCAG-3′ |
| TRAP | 5′-ACCATGACCACCTTGGCAATGTCTC-3′ | 5′-ATAGTGGAAGCGCAGATAGCCGTT-3′ |
| CATK | 5′-AGGTTCTGCTGCTACCTGTGGTGAG-3′ | 5′-CTTGCATCAATGGCCACAGAGACAG-3′ |
| CA2 | 5′-GGACCTGAGCACTGGCATAAGGACT-3′ | 5′-AAGGAGGCCACGAGGATCGAAGTT-3′ |

plate reader (Synergy HT, Biotek). Results were normalized to total protein content of cultures and expressed as nmol/min/$mg_{protein}$.

**Number of TRAP-positive multinucleated cells.** At days 14 and 21, PBMC monocultures and the co-cultures were washed twice with PBS, fixed with 3.7% formaldehyde for 10 min, rinsed with distilled water, and stained for TRAP with acid phosphatase, leukocyte (TRAP) kit (Sigma), according manufacturer's instructions. Briefly, cells were incubated with naphtol AS-BI 0.12 mg/ml in the presence of 6.76 mM tartarate and 0.14 mg/ml Fast Garnet GBC at 37°C for 1 h in the dark. After incubation, cell layers were washed and stained with hematoxylin. Multinucleated (>2 nuclei) and TRAP-positive (purple/dark red) cells were counted.

**Confocal laser scanning microscopy (CLSM).** After being washed twice with PBS, 21 days cultures were fixed with 3.7% (v/v) *p*-formaldehyde for 15 min. After that, they were permeabilized with 0.1% (v/v) Triton X-100 for 5 min and stained for actin with 5 U/ml Alexa Fluor® 647-Phalloidin (Invitrogen), for nucleus with 500 nM propidium iodide, and for VNR and CTR with 50 μg/ml mouse IgGs anti-VNR and IgGs anti-CTR (R&D Systems), respectively. Anti-VNR and anti-CTR detection was performed with 2 μg/ml Alexa Fluor® 488-Goat anti-mouse IgGs.

**Osteoclastic resorption activity.** After 21 days of culture on BD BioCoat™ Osteologic™ Bone Cell Culture Plates (BD Biosciences), PBMC monocultures and the co-cultures were bleached with 6% NaOCl and 5.2% NaCl, according to manufacturer's instructions. Calcium phosphate layers were visualized by phase contrast light microscopy. Image analysis of the resorpted areas was performed with ImageJ 1.41 software.

**Osteoclastogenic signaling pathways.** PBMC monocultures and the co-cultures were treated with the MEK signaling pathway inhibitor, U0126, the NFkB signaling pathway inhibitor, PDTC and the PGE2 production inhibitor, indomethacin. U0126 was tested at 1 and 10 μM, because some authors have proposed a stimulatory effect of this molecule on osteoclastogenesis, at low concentrations [Hotokezaka et al., 2002; Yamashita et al., 2010], while other reports have revealed an inhibitory effect [Kim et al., 2007; Yang et al., 2008]. PDTC was used at 10 and 100 μM, because the lower concentration has been previously described as the $IC_{50}$ on rat osteoclastic differentiation [Hall et al., 1995]. Indomethacin (1 μM) inhibits the synthesis of PGE2, a molecule known to promote osteoclastogenesis [Kawashima et al., 2009]. Cultures were characterized for TRAP activity (days 7, 14, and 21) and number of TRAP-positive multinucleated cells (days 14 and 21).

**M-CSF, RANKL, and sphingosine-1-phosphate quantification.** M-CSF, RANKL, and sphingosine-1-phosphate quantification in the culture medium from the tested cultures was performed with the Human M-CSF Quantikine ELISA Kit (R&D Systems), the sRANKL (total) Human ELISA (Osteoprotegerin Ligand; BioVendor), and the Sphingosine 1 Phosphate Assay Kit (Echelon Biosciences Inc.), respectively, following manufacturer's instructions. After detection, the absorbance of the samples was measured at 450 nm in an ELISA plate reader (Synergy HT, Biotek). Results were expressed as ng/ml.

### STATISTICAL ANALYSIS

All the data presented in this work was obtained from three replicas from three separate experiments using cell cultures from three different donors. Groups of data were evaluated using a two-way analysis of variance (ANOVA) and no significant differences in the pattern of the cell behavior were found. Statistical differences found between controls and experimental conditions were assessed by Bonferroni's method. Values of $P \leq 0.05$ were considered significant. Error bars in figures represent standard deviations.

## RESULTS

### TOTAL PROTEIN CONTENT. CELLULAR VIABILITY/PROLIFERATION

MG63 I cell cultures displayed low and approximately constant levels of protein content, as a function of culture period. MG63 II cell cultures revealed an increase of about 80% on cellular protein content during the culture period, being that increase mainly obtained between days 7 and 14. By contrast, protein content in PBMC cultures decreased from the first week onwards (at day 21, levels were approximately half of those found at days 4–7). Co-cultures of MG63 I + PBMC presented high levels of protein in the first week and lower (~20–25%) and stationary values on the second and third weeks, and values were higher than those found in the isolated cultures. Co-cultures of MG63 II + PBMC presented a similar response. Co-cultures of MG63 cells + PBMC presented a similar behavior to that of co-cultures of hBMC + PBMC. Results are shown in Figure 1A.

Regarding cell viability/proliferation (MTT assay; Fig. 1B), MG63 I cultures presented low values and a tendency for a slow increase with culture time. MG63 II showed higher values, with a lag phase during the first week and a significant increase during the second week. PBMC cultures showed relatively low and similar values during the 3-week incubation time. Co-cultures of MG63 I + PBMC

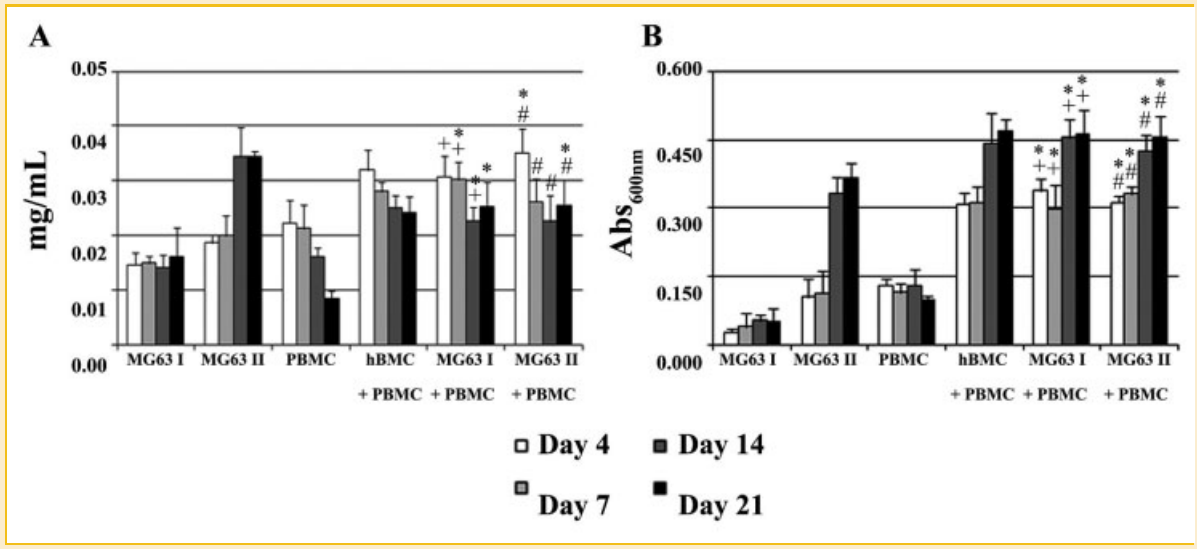


Fig. 1. Total protein content (A) and cellular viability/proliferation (B) of monocultures of MG63 I, MG63 II, and PBMC, and co-cultures of hBMC + PBMC and MG63 + PBMC. MG63 I: $10^2$ cells/cm$^2$; MG63 II: $10^3$ cells/cm$^2$. The +, #, and * indicate significantly different from MG63 I, MG63 II, or PBMC monocultures, respectively.

presented stationary values during the first week and a significant increase during the second week of culture; in addition, values were higher than those found in the isolated cultures. Comparatively, co-cultures of MG63 II + PBMC displayed a similar behavior. Once again, cellular response on MG63 + PBMC co-cultures followed the same pattern as hBMC + PBMC co-cultures.

## GENE EXPRESSION BY RT-PCR

Gene expression regarding MG63 cells and PBMC monocultures and co-cultures is presented in Figure 2. GAPDH was highly expressed by every cell culture. MG63 II cell cultures expressed the osteoblastic-related genes COL1, ALP, and BMP-2, and residual amounts of c-myc and c-src. COL1 and ALP expression was slightly higher on day 21. PBMC cultures expressed the osteoclastic-related genes c-myc, c-src, TRAP, CATK, and CA2 at day 14, and that expression was slightly higher at the end of culture period (day 21). Comparatively, co-cultures of MG63 I + PBMC and MG63 II + PBMC showed a similar increase in the expression of all osteoclastic genes, at the two analyzed time points. The co-culture MG63 I + PBMC did not express osteoblastic genes. Compared to MG63 II cultures, co-cultures of MG63 II + PBMC showed significantly lower expression levels of COL1 and ALP and higher levels of BMP-2. These differences were more pronounced at day 21 than at day 14 of culture. The behavior of MG63 II + PBMC was identical to that of hBMC + PBMC co-cultures, despite the fact that ALP and BMP-2 expression levels were higher in the latter condition.

## TRAP ACTIVITY AND NUMBER OF TRAP-POSITIVE MULTINUCLEATED CELLS

TRAP activity was not detected in MG63 cell cultures. In PBMC cultures, TRAP activity increased progressively during the 21-day

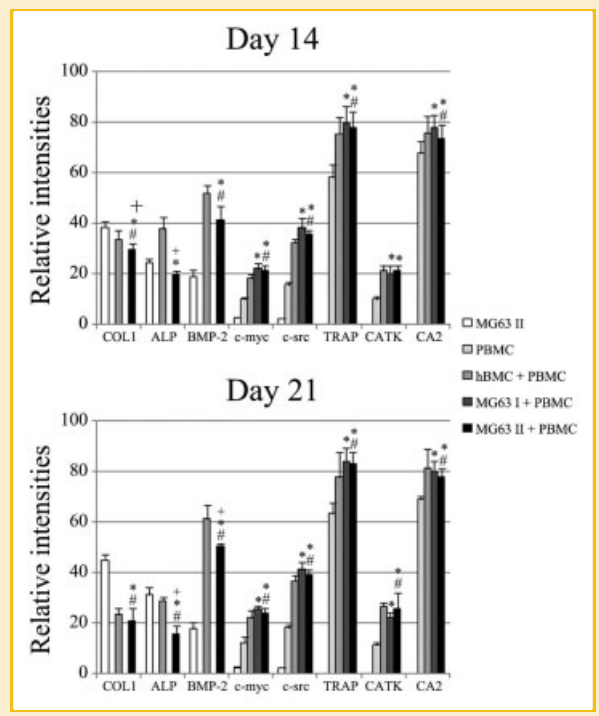


Fig. 2. RT-PCR analysis of monocultures of MG63 cells and PBMC and co-cultures of hBMC + PBMC and MG63 + PBMC. Cell layers were assessed for the expression of GAPDH, COL1, BMP-2, ALP, TRAP, CATK, CA2, c-myc, and c-src, and the PCR products were subjected to a densitometric analysis and normalization to the value obtained for GAPDH. MG63 I: $10^2$ cells/cm$^2$; MG63 II: $10^3$ cells/cm$^2$. The #, *, and + indicate significantly different from MG63 II monocultures, PBMC monocultures, and co-cultures of hBMC + PBMC, respectively.

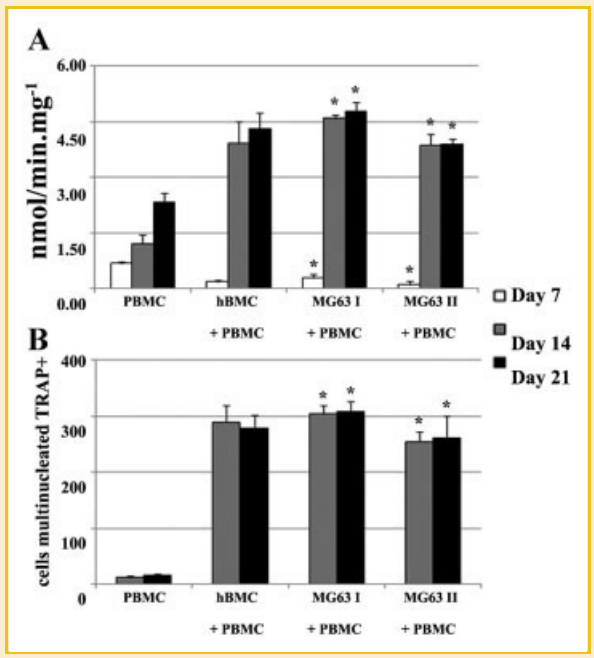


Fig. 3. TRAP activity (A) and number of TRAP-positive multinucleated cells (B) of PBMC monocultures and co-cultures of hBMC + PBMC and MG63 cells + PBMC. MG63 I: $10^2$ cells/cm$^2$; MG63 II: $10^3$ cells/cm$^2$. *Significantly different from PBMC monocultures.

culture period (Fig. 3A). Comparatively, co-cultures displayed significantly higher TRAP activity, that displayed a sharp increase between days 7 and 14. The two co-culture systems displayed a similar behavior, but MG63 I + PBMC showed slightly higher values (~20%; Fig. 3A). Compared to hBMC + PBMC co-cultures, MG63 cells + PBMC displayed a similar behavior.

MG63 cell cultures did not show any TRAP-positive multinucleated cells, while PBMC revealed a low number (Fig. 3B). The co-cultures of MG63 cells and PBMC showed a sharply increase in the number of these cells (~17–23 times), similar to that observed in hBMC + PBMC co-cultures. Co-cultured MG63 I + PBMC revealed a slightly higher response (~16%).

## CONFOCAL LASER SCANNING MICROSCOPY

After 21 days of culture, cell layers were stained for nuclei, actin, and VNR and CTRs (Fig. 4A,B), and were observed by CLSM. PBMC monocultures showed few, isolated, cells displaying osteoclastic features, that is, multinucleated cells with actin rings and expressing VNR and CTRs. PBMC co-cultured with MG63 I cells revealed high numbers of cells with these features, and MG63 cells were hardly seen. Co-cultures of MG63 II + PBMC also presented high number of osteoclastic cells, although in this case they also displayed well-organized MG63 cell layers. hBMC + PBMC revealed an identical behavior (data not shown).

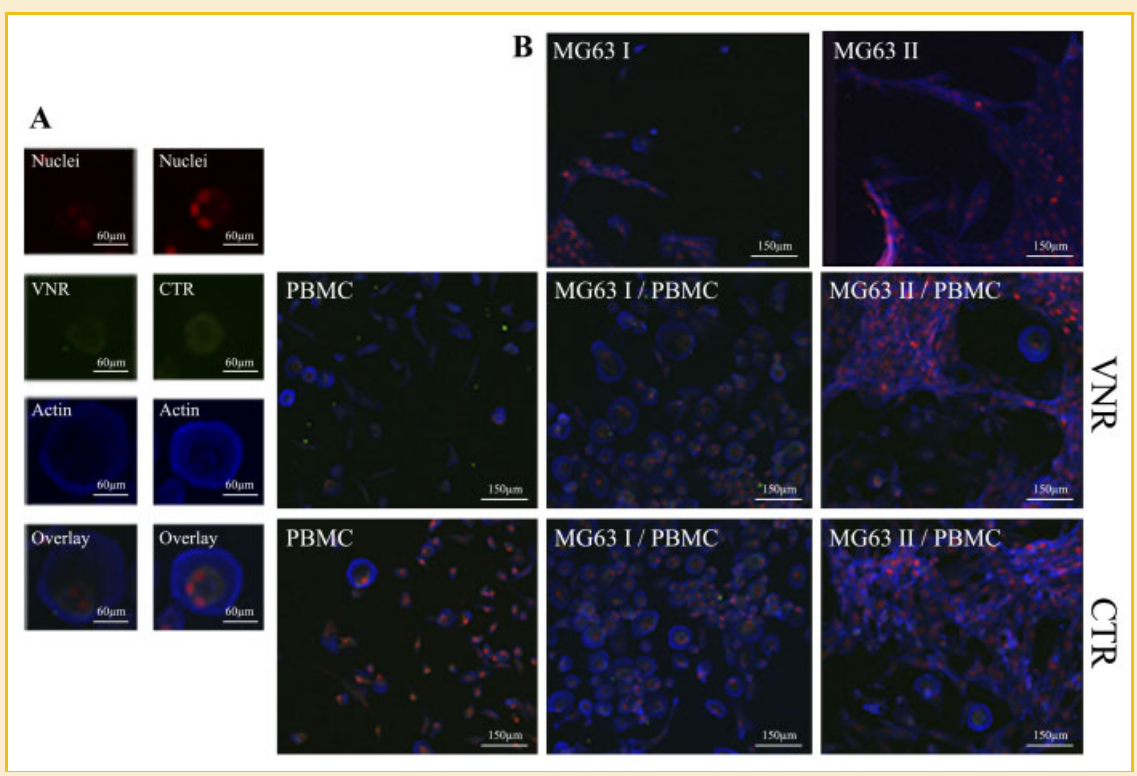


Fig. 4. CLSM visualization of monocultures or co-cultures of MG63 cells and PBMC, stained for nuclei, actin cytoskeleton, and vitronectin and calcitonin receptors (VNR and CTR). A: Representative images. B: Monocultures or co-cultures. MG63 I: $10^2$ cells/cm$^2$; MG63 II: $10^3$ cells/cm$^2$. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

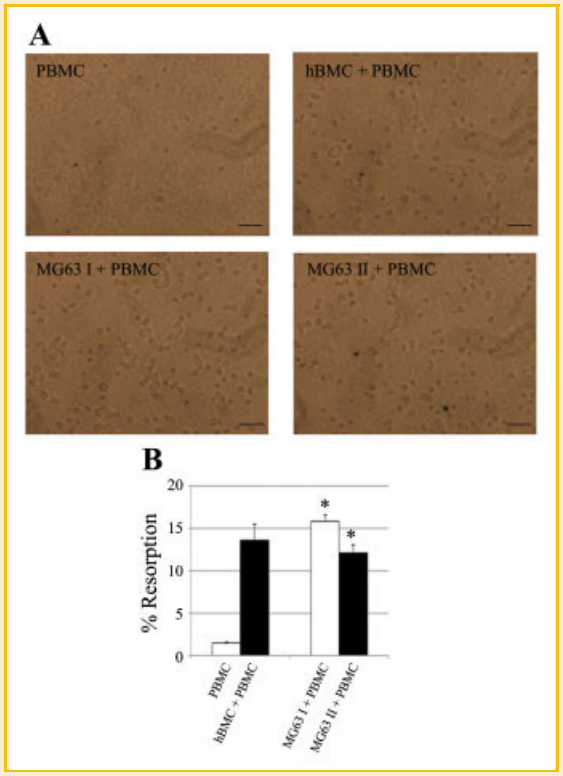


Fig. 5. Calcium phosphate resorbing ability of PBMC cultures or co-cultures of hBMC + PBMC and MG63 cells + PBMC. Calcium phosphate layers were analyzed after cell removal (A), and resorpted areas were quantified as a function of total area (B). MG63 I: $10^2$ cells/cm$^2$; MG63 II: $10^3$ cells/cm$^2$. Black bars = 600 μm. *Significantly different from PBMC monocultures. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

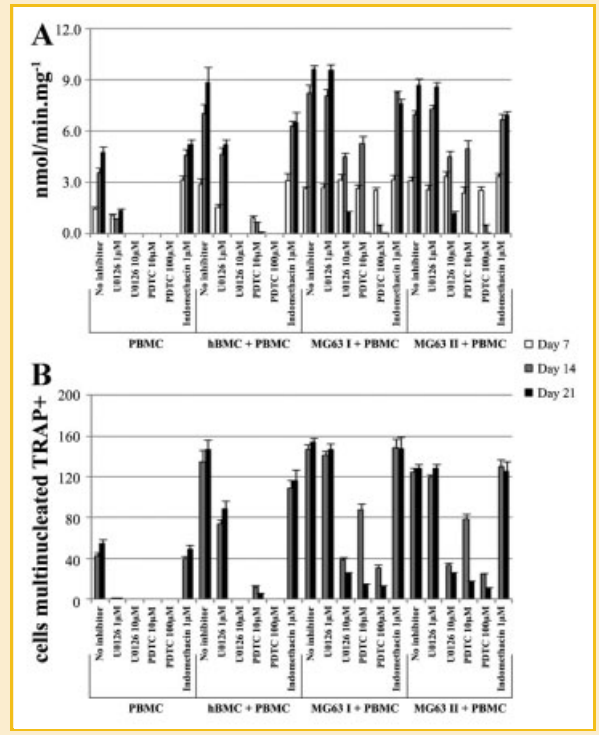


Fig. 6. Characterization of the intracellular mechanisms involved in the osteoclastogenic response observed in PBMC cultures and co-cultures of hBMC + PBMC and MG63 cells + PBMC. Cell cultures were performed in the presence of U0126, PDTC, and indomethacin, and were assessed for TRAP activity (A) and number of TRAP-positive multinucleated cells (B). MG63 I: $10^2$ cells/cm$^2$; MG63 II: $10^3$ cells/cm$^2$.

## CALCIUM PHOSPHATE RESORBING ACTIVITY

Culture plates maintained for 21 days in the absence of any cell type or cultured with only MG63 cells did not reveal the presence of resorption lacunae (data not shown). PBMC cultures displayed the presence of few, isolated lacunae (Fig. 5A,B), while the co-cultures (both with hBMC or MG63 cells) showed a significant increase (5–6 times) in the total resorbed area. Co-cultures of MG63 I + PBMC revealed a higher (~20%) resorbing activity compared to co-cultures performed with MG63 II cells.

## OSTEOCLASTOGENIC INTRACELLULAR MECHANISMS

In order to evaluate the possible involvement of MEK and NF-kB signaling pathways, as well as PGE2 production, on the observed osteoclastogenic behavior, cell cultures were treated with the signaling pathway inhibitors U0126, PDTC, and indomethacin, and the osteoclastogenic response was accessed for TRAP activity and number of TRAP-positive multinucleated cells (Fig. 6).

In PBMC monocultures and co-cultures with hBMC, the presence of 1 μM U0126 significantly decreased TRAP activity (the decrease was more pronounced in PBMC cultures), and, at 10 μM, the enzyme was not detected. In addition, PDTC completely abolished TRAP activity in PBMC cultures, even at a low concentration, while in the co-cultures of hBMC + PBMC, the lower concentration of PDTC caused a decrease to barely undetectable levels (but not null) of TRAP activity. Indomethacin did not affect the response of PBMC cultures, but significantly inhibited the response of hBMC + PBMC cultures. In the co-cultures of MG63 cells + PBMC, 1 μM U0126 did not affect significantly TRAP activity, while 10 μM caused a time-dependent decrease (~85%, at day 21). The presence of PDTC caused a concentration and time-dependent decrease on TRAP activity. Indomethacin decreased TRAP activity by about 20% on the end of the culture period. Quantification of TRAP-positive multinucleated cells provided similar information (Fig. 6B).

## CYTOKINE QUANTIFICATION ON CULTURE MEDIA

The concentrations of M-CSF, RANKL, and sphingosine-1-phosphate on culture media were quantified on all the tested cell cultures (Fig. 7). M-CSF expression was higher on MG63 than on PBMC monocultures, particularly at later culture periods. However, hBMC or MG63 + PBMC co-cultures exhibited higher and similar levels, which increased over the culture period. Regarding RANKL expression, monocultures displayed low values, and those observed for MG63 + PBMC co-cultures were only slightly higher. On the other hand, hBMC + PBMC co-cultures displayed a significant different behavior, with a RANKL concentration about three times

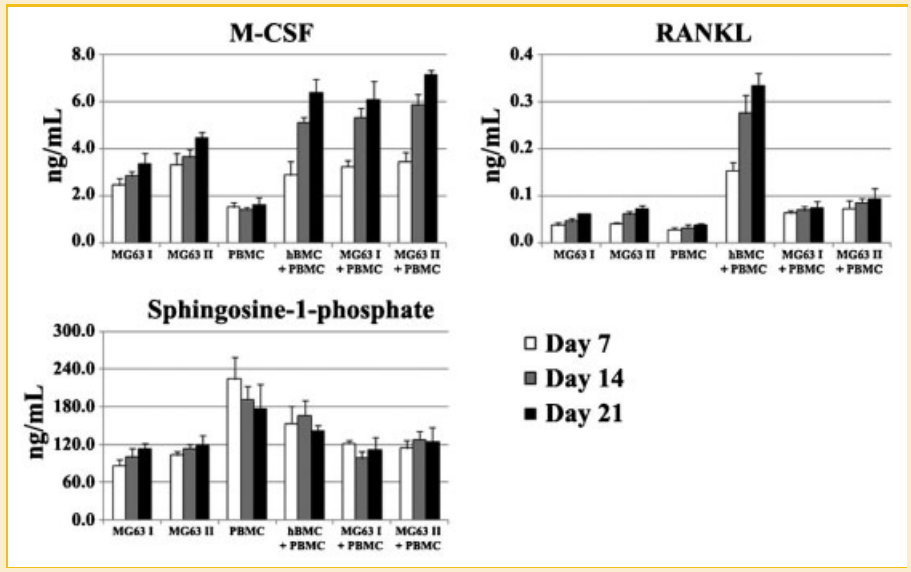


Fig. 7. Quantification of the levels of M-CSF, RANKL, and sphingosine-1-phosphate present in the culture media of PBMC cultures and co-cultures of PBMC and hBMC or MG63 cells.

higher than on the other cell cultures. Sphingosine-1-phoshphate concentration was identical in MG63 mono- and co-cultures, and only slightly higher on hBMC + PBMC co-cultures, while PBMC cultures displayed the highest values.

## DISCUSSION

Osteoblast–osteoclast communications, involving cell–cell contacts and paracrine mechanisms, have a central role in the maintenance of a proper bone structure [Matsuo and Irie, 2008; Costa-Rodrigues et al., 2010], and the presence of osteosarcoma cells usually led to disturbed bone metabolism [San-Julian et al., 2003; Avnet et al., 2008]. In the present work, osteosarcoma MG63 cells were co-cultured with osteoclast precursor cells aiming to assess the reciprocal cellular interactions. For comparison, monocultures of MG63 cells and PBMC and co-cultures of hBMC + PBMC were also performed.

MG63 and PBMC monocultures presented characteristic phenotype features. MG63 cells plated at the lower density ($10^2$ cell/cm$^2$) showed approximately constant values of protein content and cell viability/proliferation during the culture period, suggesting that the very low seeding density difficult the cell–cell communications important for their proliferation. Accordingly, MG63 cells seeded at a higher cell density ($10^3$ cell/cm$^2$), after a lag phase of few days, exhibited a significant increase in cell proliferation followed by a stationary phase, as shown by the protein content and MTT assay. MG63 cells expressed the osteoblast-related genes COL1, ALP, and BMP2 [Datta et al., 2008]. They also expressed vestigial amounts of c-myc and c-src genes, which is in agreement with previous findings [Womer et al., 1987; Izbicka et al., 1994], as these genes are not exclusively involved in the osteoclastogenic process. On the other hand, PBMC cultures presented a time-dependent decrease in protein content after the first week of culture. As PBMC do not proliferate, this decrease might be associated with the progressive removal of non-adherent cells, and also with the fusion of mononuclear cells. However, cell viability/proliferation measured by the MTT assay, which is based on a mitochondrial activity, remained constant. As osteoclasts are very rich in mitochondria [Oursler et al., 2005], together, protein content and MTT assay results, might suggest an increased metabolic activity due to the formation of osteoclastic cells. PBMC expressed the osteoclast differentiation and activation genes c-myc and c-src [Zhao et al., 2007], and, also, the functional related genes TRAP, CATK, and CA2 [Zhao et al., 2007; Datta et al., 2008]. However, in spite of the increasing values of TRAP activity throughout the culture time, this cell population displayed low number of TRAP-positive multinucleated cells and limited calcium phosphate resorbing activity, which is in line with previous studies showing that development of osteoclast cells in vitro is low in the absence of osteoclastogenic inducing factors [Costa-Rodrigues et al., 2010].

Co-cultured MG63 I + PBMC revealed total protein content higher than that found in the corresponding monocultures, as expected due to the presence of the two-cell populations. The decrease in the protein from the first to the second week might be related to the removal of non-adherent PBMC and/or the fusion of mononuclear cells, as referred above. Considering the protein content and MTT assay of MG63 and PBMC monocultures, the behavior of the co-culture during the second and third weeks (with stationary protein levels and increased MTT values) suggests an increase in the proliferation of MG63 cells and/or in the number of osteoclastic cells. CLSM observation favors the later hypothesis, as co-cultures showed essentially an increased number of cells with osteoclastic features. In line with this, expression of the osteoclast-related genes in the co-culture was greatly induced compared to that found in PBMC monocultures, particularly for the

differentiation and activation genes c-myc and c-src. Also, functional features as TRAP activity, number of multinucleated cells and resorbing activity were significantly increased. However, in spite of the two populations in culture, expression of osteoblast-related genes was not detected in this co-culture, most probably due to the very low number of seeded MG63 cells, followed by a low proliferation rate, yielding an insufficient amount of MG63 cellular mRNA.

Regarding the co-culture MG63 II + PBMC, total protein content was lower than the sum of the values found in the MG63 and PBMC monocultures and decreased during the first days, being approximately constant afterwards. However, cell viability/proliferation measured by the MTT assay revealed a significant increase from days 7 to 14. Thus, the behavior of the co-culture following the first days might result from increased osteoclast formation and/or MG63 cell proliferation, as suggested by CLSM observation at day 21, showing the presence of the two-cell populations. Compared to PBMC monocultures, this co-culture showed a significant induction of the osteoclastogenic response regarding all the parameters analyzed. This response was slightly lower than that of the co-culture MG63 I + PBMC, which might be explained by the higher number of MG63 cells in culture, lowering the final osteoclast-related parameters after normalization. In both systems, maximal osteoclastogenic response was achieved by day 14, earlier than that found in the PBMC monocultures, suggesting an earlier differentiation process. Furthermore, the osteoclastogenic behavior of both co-cultures was identical to that observed for hBMC and PBMC co-culture.

In addition, co-cultured MG63 II + PBMC also expressed osteoblastic-related markers. Compared to MG63 II monocultures, the co-culture expressed lower levels of COL1 and ALP and higher levels of BMP-2, showing that the presence of PBMC greatly affected the expression of osteoblastic genes. A similar behavior was observed on hBMC + PBMC co-cultures. Results suggested a decreased ability to produce an extracellular collagenous matrix in the presence of PBMC that, together with a lower expression of ALP, might anticipate impairment in the matrix mineralization events [Torii et al., 1996; Hessle et al., 2002]. On the other hand, BMP-2 induces osteoblastic differentiation but also appears to be important for osteoclastic activity [Chen et al., 2004]. For instance, it has been suggested that osteoclasts play a role in the regulation of osteoblast differentiation through increased activation of Wnt/BMP pathway [Pederson et al., 2008]. In addition, osteoclasts express receptors for BMP-2, and BMP-2 enhances the resorptive activity of osteoclastic cells [Kaneko et al., 2000]. Finally, although BMP-2 expression on PBMC monocultures was not detected, BMP-2 is also known to be expressed by osteoclastic cells [Garimella et al., 2008]. Taken together, the increased expression of BMP-2 in the co-cultured osteoblastic and PBMC system observed in the present work might suggest the possibility of an alteration of both osteoblastic and osteoclastic activities leading to a modulation of bone remodeling.

Results showed that the presence of MG63 cells greatly induced the osteoclastogenic response of PBMC. The osteoclastogenic pathways MEK, NF-kB, and PGE2 were assessed, with the inhibitors U0126, PDTC, and indomethacin, respectively. U0126 and PDTC completely blocked osteoclastogenesis in PBMC cultures or in hBMC + PBMC co-cultures, in line with other reports [Ozaki et al., 1997; Kim et al., 2007; Yang et al., 2008]. The MG63 + PBMC co-cultures were only affected by the highest concentration of U0126 and were also less sensitive to PDTC. In addition, for both inhibitors, a long exposure time was needed to observe the inhibitory effects. This suggests some contribution of these pathways on the osteoclastic response induced by MG63 cells, nevertheless with a lower relevance than that on the co-cultures of hBMC + PBMC. Both MEK and NF-kB are important osteoclastogenic signaling pathways that are activated by several factors, including RANKL [Zhao et al., 2007]. Indomethacin, that blocks PGE2 production, and, thus, has an inhibitory effect on osteoclast differentiation and activation [Kawashima et al., 2009] did not affect PBMC cultures but had an inhibitory effect on the co-cultures, probably related to the ability of osteoblastic cells to produce PGE2 [Lauledeerkind et al., 2000; Le Heron et al., 2010].

In order to evaluate the role of different cytokines in the observed cell behavior, the culture medium levels of M-CSF and RANKL, the two classic osteoclastogenic inducers [Zhao et al., 2007], as well as those of sphingosine-1-phosphate, a modulator of osteoblastic activity [Matsuo and Irie, 2008], were determined. Regarding the osteoclastogenic modulators, high levels of M-CSF were detected on MG63 cell cultures and especially on MG63 + PBMC co-cultures. This observation is in agreement with the knowledge that MG63 cells are able to express high levels of M-CSF [Taichman and Emerson, 1996; Blair et al., 2000]. On the other hand, culture media from MG63 mono- and co-cultures displayed a low RANKL concentration, which is in line with previous findings [Taichman and Emerson, 1996; Blair et al., 2000]. This may explain the apparent lower relevance of MEK and NF-kB pathways on the osteoclastogenic response induced by this cell line, when compared with the co-cultures of hBMC and osteoclast precursors (which displayed a higher RANKL expression). Thus, although M-CSF appears as a potentially important player in the osteoclast development on MG63 + PBMC co-cultures, RANKL seems not to be a key player in that process. Finally, it was observed that PBMC monocultures displayed the highest levels of sphingosine-1-phosphate, which suggests that this molecule might be involved in the observed modulation of the osteoblastic response of co-cultures.

Taken together, the present results suggest that other intracellular mechanisms may be relevant in the osteoclastogenesis induced by MG63 cells. Regarding this, MG63 are known to express different soluble molecules that can modulate osteoclast development, such as, for example, IL-3, IL-4, IL-6, and VEGF [Miyamoto et al., 2002; Takenaka et al., 2005; Benedikt et al., 2010]. A more detailed characterization of the processes involved in the complex network of cellular crosstalks involving MG63 and osteoclast precursor cells is now underway.

The present results regarding the osteoclastogenic potential of MG63 cells are in line with the previous studies performed in co-culture systems, although at different experimental conditions. Thus, co-cultured MG63 and CD14+ cells, maintained for 7 days in α-MEM, 9% FCS, 20 mM HEPES, and 0.01 μM PTH [Michael et al., 2007] and co-cultured MG63 cells and macrophages (as osteoclast

precursors) maintained for 14 days in DMEM with 10% FCS [Blair et al., 2000] also reported osteoclast formation based in TRAP activity and resorption ability [Blair et al., 2000] and expression of osteoclastic proteases [Blair et al., 2000]. In other studies, the presence of UMR-106 rat osteosarcoma cell line was able to induce osteoclast differentiation in human PBMC [Quinn et al., 1997; Buckley et al., 2002]. Interestingly, in a co-culture system composed by human PBMC and SaOS-2 cell line, the authors only observed a significant osteoclastogenesis in the presence of PTH and, particularly, with a combination of PTH and dexamethasone [Matsusaki et al., 1999]. The heterogeneity of results found among the different cell lines and culture conditions impairs the establishment of detailed comparisons among those and the present work. To the best of our knowledge, the reverse situation, that is, the assessment of the osteoblastic behavior in co-cultured MG63 and osteoclastic cells was not previously reported. However, regarding the effect of osteoclastic cells on osteosarcoma cells, a previous work showed that conditioned medium collected from chicken osteoclasts, human osteoclastoma cells, and chicken and human multinucleated giant cells inhibited collagen synthesis, and ALP activity in the rat osteosarcoma cell line UMR-106-01 cells [Blair et al., 2000].

## CONCLUSION

Co-cultured MG63 osteosarcoma cells and PBMC showed a reciprocal modulation of the osteoblastic and osteoclastic behavior. Although the behavior of MG63 + PBMC co-cultures was identical to the hBMC + PBMC co-cultures, it is important to highlight that the seeding density of hBMC was $10^3$ cells/cm$^2$, and it elicited a response similar to the co-culture performed with the same MG63 cellular density but also with the co-culture performed with 10 times less MG63 cells ($10^2$ cells/cm$^2$). Thus, it seems that the osteoclastogenic-triggering ability of MG63 cells is significantly higher than those of hBMC, which can help to understand the osteosarcoma-associated bone loss. Moreover, compared to PBMC monocultures, co-cultures presented significantly higher TRAP activity, number of TRAP-positive multinucleated cells, gene expression of osteoclast-related markers, and calcium phosphate resorption activity. Also, osteoblastic behavior was affected in co-cultures compared to MG63 cell cultures, evidenced by a decreased expression of COL1 and ALP and an increased expression of BMP-2, molecules having a central role in the osteoblastic differentiation and function. Results suggest that the reciprocal modulation between osteosarcoma and osteoclastic cells might contribute to the disturbed bone metabolism associated with bone tumors.

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