Human Osteoclasts Enhance Osteogenic Differentiation of Bone Stromal Cells from Mandibular Tori

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Abstract

Objective: To determine the effect of human osteoclasts on osteogenic differentiation of bone stromal cells via the receptor activator of nuclear factor kappa B (RANK)-RANK ligand (RANKL) reverse signaling.

Materials and Methods: Human peripheral blood mononuclear cells were cultured with stimulating factors until they became multinucleated mature osteoclasts. After being identified for the characteristics of mature osteoclasts, their conditioned medium (OC-CM) was collected. Bone stromal cells harvested from mandibular tori of four patients were treated with OC-CM prior to assessments of osteogenic gene expressions, differentiation, and biomineralization. Both the osteoprotegerin (OPG)-pretreated bone stromal cells and the conditioned medium from GW4869-treated mature osteoclasts (GW-OC-CM) were analyzed for suppression of osteogenic induction in order to investigate the inducible effect of OC-CM.

Results: The OC-CM significantly upregulated expressions of osteogenic genes and enhanced differentiation and biomineralization of bone stromal cells ($p < 0.05$). Pretreatment with OPG, a decoy receptor of RANKL, significantly reduced the inducible effects of OC-CM ($p < 0.05$). Similarly, the upregulated expressions and enhanced biomineralization were also significantly diminished by treatment with GW-OC-CM ($p < 0.05$).

Conclusion: Mature osteoclasts can induce osteogenic differentiation of bone stromal cells possibly via the RANK-RANKL reverse signaling.

Keywords: Bone Mineralization, Osteoblast, Osteoclast, Osteoprotegerin, RANK Ligand

Received Date: Jul 09 2024
Revised Date: Aug 30 2024
Accepted Date: Oct 09, 2024

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Introduction

In bone biology, receptor activator of nuclear factor kappa B (RANK) and its ligand (RANKL) are major regulatory molecules in bone resorption of periodontitis. RANKL released by osteoblasts directly binds to membrane-bound RANK on osteoclast precursors for osteoclastic differentiation and function. Osteoprotegerin (OPG), also secreted from osteoblasts, acts as a decoy receptor of RANKL, thereby functioning as an inhibitor of osteoclastogenesis. The equilibrium of the RANKL/RANK/OPG system regulates bone remodeling (1).

Several lines of evidence indicate that osteoclasts provide coupling signals for not just playing a role in bone resorption but also coordinating in bone formation by activation of membranous RANKL on osteoblast lineage cells (2-6). This mechanism is known as the RANK-RANKL reverse signaling (7), which stimulates differentiation of osteoblasts only through a paracrine effect, not via cell-to-cell contact (8). The key molecule for activation of osteoblastic function via RANKL is RANK, which has been previously shown to be present on the membrane of extracellular vesicles (EVs), which had been isolated from osteoclasts' conditioned medium (OC-CM) (9). Note that RANK-containing EVs are released from mature osteoclasts more than the immature ones, so the mature osteoclasts' EVs have been recognized as a potent intercellular mediator in bone biology (10). In addition, OPG, specifically binding to RANKL with high affinity, competitively blocks the inducible effect of RANK-containing EVs on osteogenic induction (2).

Although several previous studies have demonstrated osteoclast-induced osteogenic differentiation and biomineralization in mouse osteoblast cell lines (2,3), mesenchymal stem cells (4), and adipose tissue-derived mesenchymal stromal cells (5), none of these studies has investigated the inducible effects on primary bone stromal cells isolated from human mandibles. Therefore, this study aimed to determine the effect of human osteoclasts on osteogenic induction of bone stromal cells harvested from mandibular tori via the RANK-RANKL reverse signaling.

Materials and Methods

Culture of human bone stromal cells

All human cell protocols were approved by the Institutional Ethics Committee Board, Mae Fah Luang University (EC23097-22). Discarded bone specimens were obtained from four healthy patients (18-45 years old), who underwent surgical removal of their mandibular tori. Bone pieces were sequentially digested with 1 mg/ml of collagenase/dispase® (Roche, Basel, Switzerland), and the cell pellet was resuspended in complete medium, i.e., DMEM (Gibco, Gaithersburg, MD, USA), containing 10% fetal bovine serum (FBS; Gibco), 1% penicillin-streptomycin, and 1% amphotericin B (Gibco), and cultured in a humidified chamber with 5% CO_2 at 37^{o}C. The cells at the third to the fifth passages were used in subsequent experiments. To characterize their mesenchymal cell type, bone stromal cells were collected and stained with mesenchymal cell surface markers, including APC-conjugated anti-human CD73, Alexa Fluor®700-conjugated anti-human CD90, and APC/Fire™ 750-conjugated anti-human CD105 antibodies (BioLegend, San Diego, CA, USA). Expressions of these markers were analyzed by a flow cytometer (CytoFLEX SRT, BECKMAN COULTER, Brea, CA, USA). As a negative control, two markers for hematopoietic

stem cells, FITC-conjugated anti-human CD34 (Beckman Coulter Life Sciences, Marseille, France) and PC5-conjugated anti-human CD45 antibodies (Beckman Coulter Life Sciences) were utilized.

Culture of human mature osteoclasts

Peripheral blood mononuclear cells (PBMCs) were isolated from a 20-ml volume of peripheral blood from five healthy donors. The blood was diluted with phosphate-buffered saline (PBS) at 1:1 ratio, layered on top of the Ficoll® (Cytiva Sweden AB, Sweden) density gradient medium, and centrifugated at 400g for 30 minutes at room temperature without deceleration. PBMCs were collected from the buffy coat and resuspended in complete αMEM medium (Gibco), containing 10% FBS, and 1% penicillin-streptomycin at 2 x 10^7 cells per ml. On the following day, non-adherent cells were removed by thorough washing with PBS. To obtain mature osteoclasts, PBMCs were cultured for 9 days in the complete αMEM medium, supplemented with 15 ng/ml recombinant human macrophage-colony stimulating factor (M-CSF; Stemcell Technologies, Vancouver, Canada) and 30 ng/ml recombinant human RANKL (Stemcell Technologies) with medium replacement every 3 days. To characterize the multinucleated mature osteoclasts, they were stained with tartrate-resistant acid phosphatase (TRAcP; Sigma-Aldrich, Darmstadt, Germany), 20 nM Alexa Fluor® 488-conjugated phalloidin (Invitrogen, Eugene, Oregon, US), and 1 μM DAPI (Biotium, Inc., Hayward, CA, USA). Stained cells were visualized by a fluorescence microscope (ECLIPSE Ni-E, Nikon, Tokyo, Japan).

Preparation of conditioned medium from mature osteoclasts (OC-CM)

According to the protocol of Stessuk et al. (5), OC-CM from days 9, 12, 15, and 18 was collected, pooled, centrifuged at 2,000g for 10 minutes to remove cell debris, and then concentrated by twenty-fold using centrifugation at 4,000g with the Amicon Ultra Filter Unit (100kDa Merk Millipore Ltd., Tullagreen, Carrigtwohill, Co. Cork, Ireland) for 15 minutes at 4°C. To inhibit EVs' release, mature osteoclasts on day 9 were treated with 20 μM of GW4869 for 3 days, and the conditioned medium from GW4869-treated mature osteoclasts (GW-OC-CM) were collected from days 12, 15, and 18, and then concentrated by twenty-fold following the protocol, as described above.

Osteogenic differentiation

Human bone stromal cells were seeded at 5 x 10^5 cells per well in 6-well plates. At 70-80% cell confluence, the cells were treated with concentrated OC-CM at a ratio of 1:10 (v/v) in complete medium, supplemented with 10 mM β-glycerophosphate (Sigma-Aldrich, Darmstadt, Germany), or left untreated as a negative control. This ratio, taken from a previous study (11) and tested in our pilot study, was shown to enhance biomineralization (data not shown). As a positive control for osteogenic induction, the bone stromal cells were cultured in complete medium, supplemented with 50 mg/ml ascorbic acid (Sigma-Aldrich), 10 mM β-glycerophosphate, and 100 nM dexamethasone (Sigma-Aldrich). To inhibit the RANK-RANKL signaling, the bone stromal cells were pre-treated with 1 ng/ml of recombinant human OPG (Stemcell

Technologies) for one hour before OC-CM treatment. Culture medium with or without OC-CM or OPG was refreshed every 3 days. To investigate the role of EVs from osteoclasts, the bone stromal cells were treated with concentrated GW-OC-CM at the same ratio of 1:10 (v/v) and analyzed for osteogenic gene expressions and biomineralization in comparison with those treated with OC-CM.

Analyses of osteogenic differentiation, biomineralization, and gene expressions

Alkaline phosphatase (ALP) staining (Abcam, Cambridge, UK) was performed on day 7, while biomineralization was examined by Alizarin red staining (Sigma-Aldrich) and von Kossa staining (Abcam) on day 14. ALP and von Kossa staining intensities were analyzed by ImageJ program (National Institutes of Health, Bethesda, MD, USA). The Alizarin red staining dye was dissolved in 10% cetylpyridinium chloride solution (Sigma-Aldrich), and its amounts were quantified by absorbance at a 570-nm wavelength.

Expressions of *RUNX2,* Osterix *(OSX),* and Collagen type I alpha 1 *(COL1A1)* mRNA were determined at 24 h by reverse transcription (RT)-quantitative polymerase chain reaction (qPCR). In brief, total RNA was extracted and quantified by NanoDrop One (Thermo Fisher Scientific, Carlsbad, CA, USA). Complementary DNA (cDNA) was synthesized from a 200ng amount of each RNA sample using the High-Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific). PCR was performed using CFX Opus 96 Real-Time PCR System (Bio-Rad Laboratories, Inc, Berkeley, CA, USA). The sequences of oligonucleotide primers are summarized in Table 1.

Table 1. Oligonucleotide primers used in this study, (F = Forward, R = Reverse).

Gene		Primer sequences
GAPDH	F	5'-TCA TGG GTG TGA ACC ATG AGA A-3'
	R	5'-GGC ATG GAC TGT GGT CAT GAG-3'
RUNX2	F	5'-ATG ATG ACA CTG CCA CCT CTG A-3'
	R	5'-GGC TGG ATA GTG CAT TCG TG-3'
OSX	F	5'-GCC AGA AGC TGT GAA ACC TC-3'
	R	5'-GCT GCA AGC TCT CCA TAA CC-3'
COL1A1	F	5'-GTG CTA AAG GTG CCA ATG GT-3'
	R	5'-ACC AGG TTC ACC GCT GTT AC-3'

Statistical analysis

All experiments were performed in triplicate. Shapiro-Wilk test was used to check the normality of data distribution. One-way analysis of variance followed by Student–Newman–Keuls post hoc test was used to determine the significant difference between groups at $p < 0.05$. The statistical analysis was performed using SPSS software version 26.0 for Windows.

Results

Characterization of human bone stromal cells and mature osteoclasts

The harvested bone stromal cells showed a spindle-shaped morphology (Fig 1A), were negative for expression of the hematopoietic cell marker, CD34 (Fig 1B) or CD45 (Fig 1C), but were positive for expressions of mesenchymal cell markers, CD73 (Fig 1D), CD90 (Fig 1E), and CD105 (Fig 1F). The mature osteoclasts were positive for TRAcP staining (Fig 1G), contained multiple nuclei (Fig 1H), and showed actin ring formation by immunofluorescence (Fig 1I). Treatment of mature osteoclasts with 20 μM of GW4869 for 3 days showed entrapped vesicles within the cells (Fig 1J).

Inhibition of enhanced osteogenic differentiation by pretreatment of bone stromal cells with osteoprotegerin or by treatment of mature osteoclasts with GW4869

Treatment of the bone stromal cells with OC-CM significantly increased ALP staining and biomineralization by Alizarin red and von Kossa staining, compared with a negative untreated control ($p < 0.05$; Fig 2A-D). Enhanced ALP staining and biomineralization were significantly decreased by pretreatment of bone stromal cells with OPG ($p < 0.05$; Fig 2A-D). Likewise, the degrees of *RUNX2, OSX,* and *COL1A1* mRNA expressions were upregulated by OC-CM treatment ($p < 0.05$; Fig 2E-G), whereas these upregulated expressions were significantly inhibited by pretreatment of bone stromal cells with OPG ($p < 0.05$; Fig 2E-G).

Moreover, the enhanced staining intensities of ALP, Alizarin red, and von Kossa were significantly decreased by treatment of bone stromal cells with GW-OC-CM ($p < 0.05$; Fig 3A-D). The upregulated expression of *RUNX2* and *OSX* were significantly inhibited by treatment of bone stromal cells with GW-OC-CM ($p < 0.05$; Fig 3E-G). As a positive control for osteogenic induction, increased ALP and biomineralization as well as upregulated osteogenic gene expressions were observed in bone stromal cells treated with osteogenic medium (Fig 2,3).

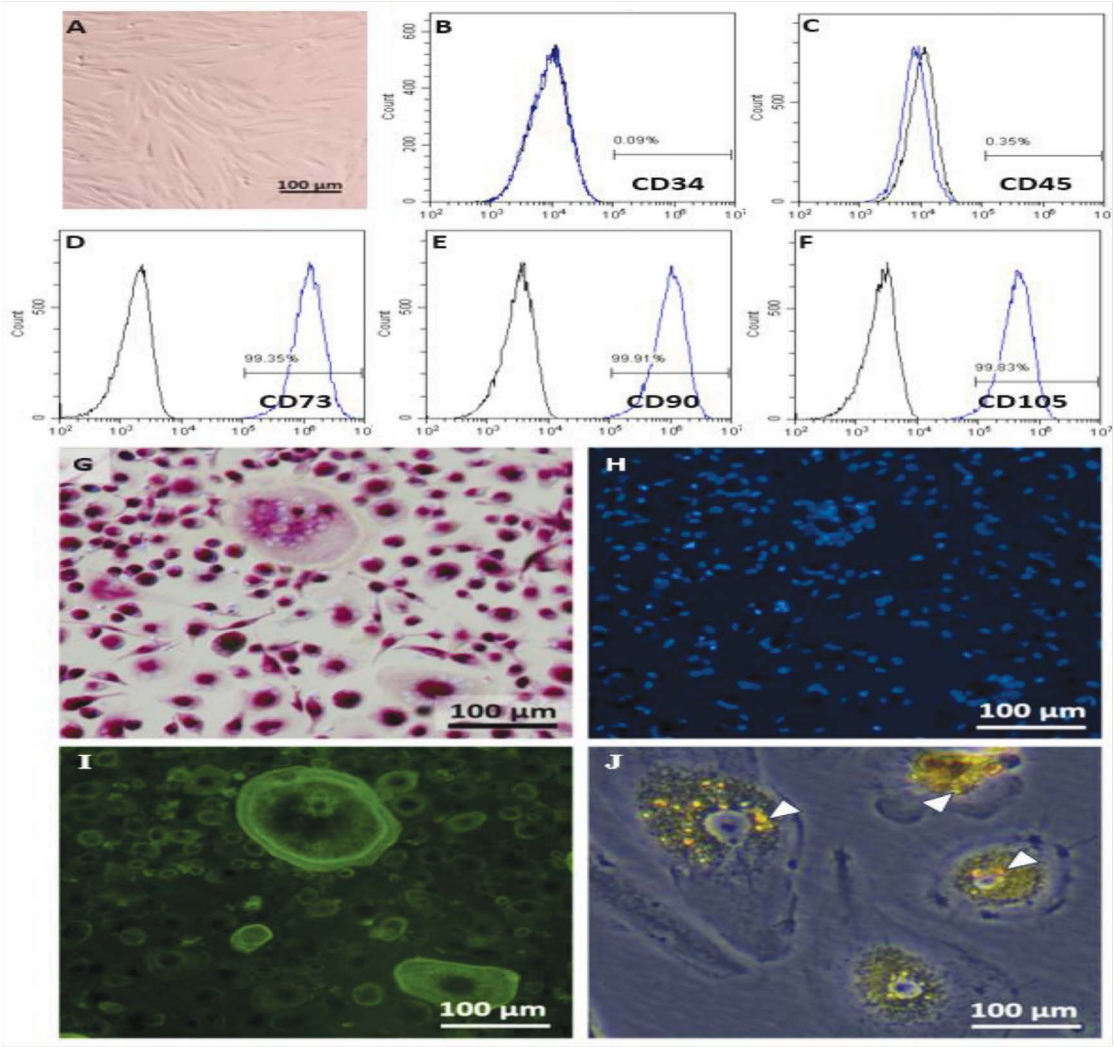

Fig.1 Characterization of bone stromal cells (A-F) and multinucleated mature osteoclasts that were positive for TRAcP staining (G), contained multiple nuclei (H), and showed actin ring formation by immunofluorescence (I). By inverted phase-contrast microscopy, treatment of mature osteoclasts with 20 µM of GW4869 for 3 days showed entrapped vesicles within the cells (arrowheads in J).

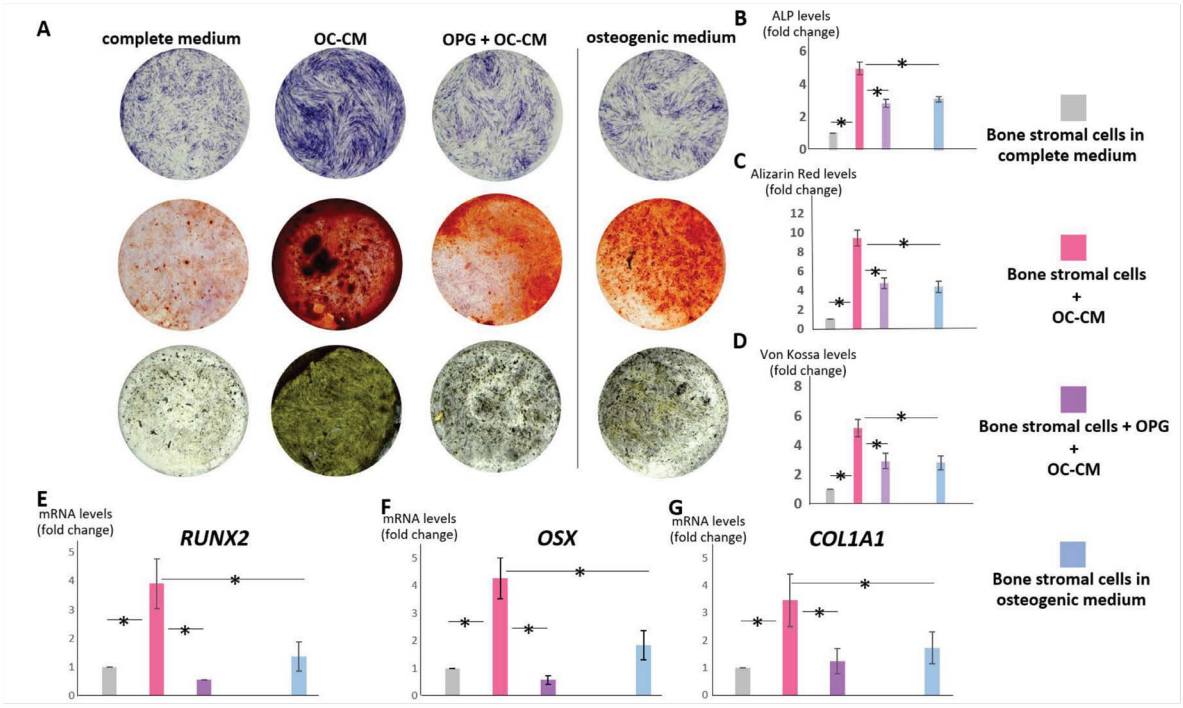

Fig.2 Enhanced ALP staining and biomineralization staining (A-D), and expressions of osteogenic genes, *RUNX2* (E), *OSX* (F), and *COL1A1* (G), of bone stromal cells by treatment with conditioned medium of mature osteoclasts (OC-CM) were significantly inhibited by pretreatment of bone stromal cells with osteoprotegerin (OPG).
Error bars in B-G = standard deviation; *p < 0.05.

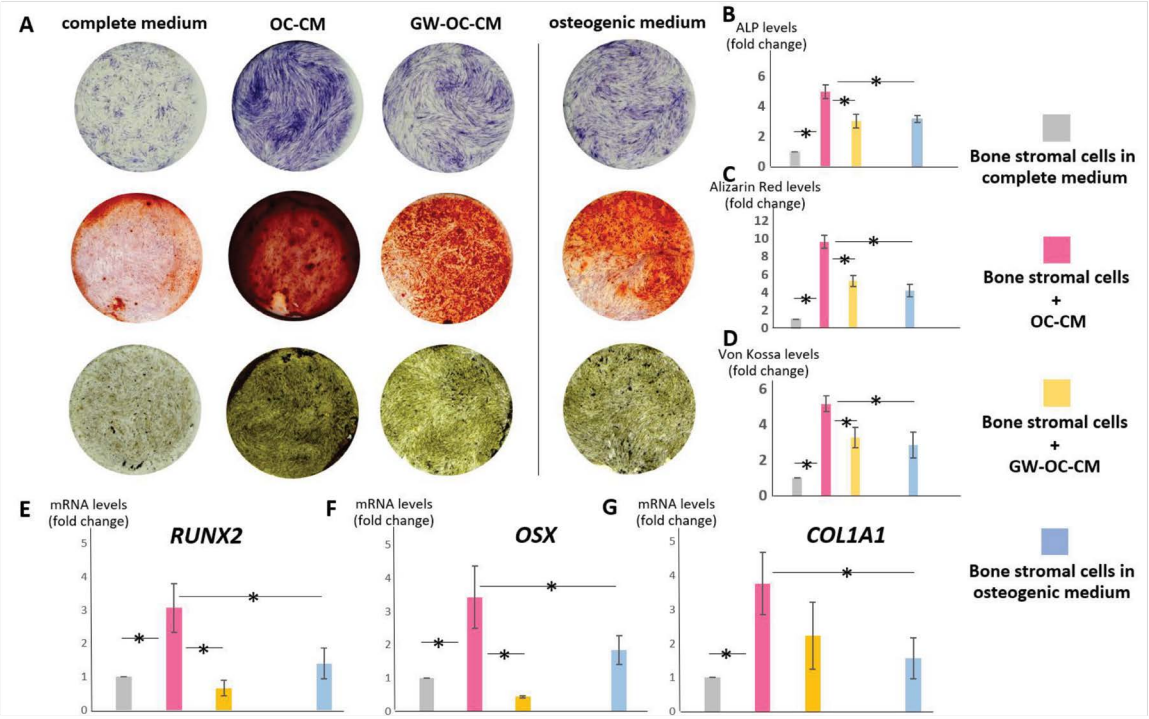

Fig 3. Enhanced ALP staining and biomineralization staining (A-D), and expressions of osteogenic genes, *RUNX2* (E), *OSX* (F), and *COL1A1* (G), of bone stromal cells by treatment with conditioned medium of mature osteoclasts (OC-CM) were inhibited by treatment with conditioned medium of mature osteoclasts treated with GW4869 (GW-OC-CM). Error bars in B-G = standard deviation; *p < 0.05.

Discussion

The current study demonstrated that mature osteoclasts, derived from human PBMCs, released mediators into their culture medium, which influenced the osteogenic differentiation of primary bone stromal cells harvested from human mandibular tori, as evidenced by increased ALP staining and biomineralization upon Alizarin red staining and von Kossa staining as well as upregulation of *RUNX2, OSX,* and *COL1A1* mRNA expressions. These results are consistent with the findings from previous studies (4-6), in which enhanced osteogenic differentiation of mesenchymal stem cells was observed. However, to further verify changes in the degrees of mRNA expressions as aforementioned, analysis of protein expressions for *RUNX2, OSX,* and *COL1A1* will be required in a future study, as with previous studies (12,13). Pretreatment of the bone stromal cells with OPG significantly decreased this osteogenic differentiation, implying that the binding between OPG and RANKL on the membrane of bone stromal cells prevents the RANK-RANKL reverse signaling from mature osteoclasts, indicating that RANKL present on the membrane of bone stromal cells plays a crucial role in this signaling pathway.

Furthermore, the significance of the RANK-RANKL reverse signaling from mature osteoclasts is verified by treatment of mature osteoclasts with GW4869, an inhibitor of EV release. The entrapment of EVs within mature osteoclasts implied that EVs' release was prohibited by treatment with this inhibitor (Fig 1J). This treatment resulted in significant suppression of ALP staining, biomineralization, and mRNA expressions of *RUNX2* and *OSX* in the bone stromal cells, corresponding with the depletion of EVs from OC-CM that significantly reduced their ability to induce osteogenic differentiation (6). EVs released into the culture medium may thus be a critical messenger for cell-to-cell communication. Indeed, it was confirmed by this study that a substantial part of the osteogenic inductive mechanism from OC-CM was mediated by EVs. However, the upregulated mRNA expression of *COL1A1* was not significantly inhibited by treatment with GW-OC-CM. This may be explained by the inducible effect of other mediators, found within OC-CM but not present on EVs' membrane, which can also exert the osteo-inductive action. Particularly, a previous study (14) has shown an ability of apoptotic bodies released from osteoclasts to activate the RANK-RANKL reverse signaling, as with EVs. In that study, even though 68% of the induced apoptotic osteoclasts are demonstrated to release the apoptotic bodies, a few non-induced live cells (4%) can still release these bodies. Therefore, it is of interest to further investigate the role of apoptotic bodies released from mature osteoclasts in upregulation of *COL1A1* gene expression.

Under an appropriate culture condition, bone marrow–derived mesenchymal cells are multipotent, meaning that they can give rise to various mesodermal cell types, including adipocytes, chondrocytes, and osteoblasts (15). The ability of osteoclasts-derived EVs has been shown, with an emphasis on RANK as a key molecule to enhance osteogenic induction of osteoblastic cell lines (2,3), human mesenchymal cells (4), and adipose tissue-derived mesenchymal cells (5). Besides RANK-containing EVs, microRNAs (miRNAs) in EVs are essential for regulating diverse osteoblastic functions in osteogenesis (16). For example, miR-324 in osteoclasts' EVs promotes the osteogenic differentiation of mesenchymal stem cells (17), whereas osteoclasts' EVs, containing miR-214-3p, inhibit osteoblastic bone formation (18). Particularly, miR-214-3p has been shown to suppress osteogenic differentiation of myoblast cells by targeting *OSX* (19). Consequently, it is worthwhile to explore the combined effects of both RANK and miRNAs, which are associated with osteoclasts' EVs, on regulation of osteogenic differentiation. Note that the bone stromal cells of the osteoblast lineage vary in their differentiation stages and osteogenic potencies, which result in differences in bone cell stemness, proliferation, differentiation, and bone homeostasis (20). For instance, the bone stromal cells harvested from cancellous bone of human mandibular tori are mostly osteoblast progenitors, which are by default limited to differentiation into osteoblasts (21).

In summary, our findings demonstrate the involvement of RANKL on human bone stromal cells' membrane and RANK in osteoclast conditioned medium for the reverse signaling of regulatory osteogenic differentiation of human bone stromal cells.

Acknowledgements

Financial support from Intramural Endowment fund, Mae Fah Luang University, to S.S. and S.K. is gratefully acknowledged.

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