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Osteoblast-derived microvesicles: A novel mechanism for communication between osteoblasts and osteoclasts

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ABSTRACT

The maintenance of bone homeostasis is largely dependent upon cellular communication between osteoclasts and osteoblasts. Microvesicles (MVs) have received a good deal of attention and are increasingly considered as mediators of intercellular communication due to their capacity to merge with and transfer a repertoire of bioactive molecular content (cargo) to recipient cells, triggering a variety of biologic responses. Here, we demonstrated that MVs shed from osteoblasts contain RANKL protein and can transfer it to osteoclast precursors through receptor ligand (RANKL–RANK), leading to stimulation of RANKL–RANK signaling to facilitate osteoclast formation. Such MV-mediated intercellular communication between osteoblasts and osteoclasts may represent a novel mechanism of bone modeling and remodeling. It may be worthwhile to further explore MVs as tools to modify the biological responses of bone cells or develop an alternative drug to treat bone diseases.

Introduction

Bone is a metabolically active tissue which undergoes constant remodeling that involves resorption of mineralized bone by osteoclasts and the synthesis of bone matrix by osteoblasts. This bone remodeling process is tightly controlled and is essential for the correct function and maintenance of the skeletal system, repairing microscopic skeletal damage and replacing aged bone, with the key participant being the osteoclast [1,2]. Osteoclasts are multinucleated bone resorbing cells formed by cytoplasmic fusion of their mononuclear precursors which are derived from hematopoietic stem cells and share precursors with macrophages. Understanding of the molecular mechanisms that regulate osteoclast formation and activation has advanced rapidly since the discovery of the receptor activator of nuclear factor-κB ligand (RANKL)/RANK system. RANKL, a type II homotrimeric transmembrane protein, produced by osteoblasts as a membrane-associated factor binds to RANK, a receptor for RANKL on the surface of osteoclast precursors, and RANK–RANKL recognition induces the differentiation of the precursors into osteoclasts [1]. In this process, cell-to-cell contact (osteoblasts to osteoclast precursors) is required for RANKL/RANK signaling.

Usually, cells communicate and exchange information is ascribed to direct cell-to-cell contact (adhesion, juxtacrine interactions) or transfer by soluble mediators, which may also circulate in blood and body fluids and act in a regional or systemic manner [3]. However, recent studies have suggested that cells may also communicate by circular membrane fragments called microvesicles (MVs), which can fuse to nearby cells within their circulatory pathways [3–6]. Microvesicles are membrane-bound vesicular particles and released into the extracellular environment. MVs shed from the cell surface by most cell types can "packet" membrane components and engulf cytoplasmic contents including receptor proteins, proteolytic enzymes, signaling molecules, as well as mRNA and microRNA (miR) sequences, then transfer genetic and proteomic information to target cells, in turn to affect cell functions [7,8]. Such molecular pathways of cell-to-cell communication may play an important role in development, health and disease and has gained more and more attentions.

So far, a growing body of studies has reported that MVs are involved in cancer cell survival, invasiveness and metastases [9–12]. Tumor-derived MVs are able to participate in horizontal transfer of bioactive molecules throughout cancer cell population and to non-transformed stromal cells, endothelial cells. It has also been reported that MVs have roles in chronic inflammation, atherosclerosis, cardiovascular and renal diseases [13–16]. In all cases, MVs have been demonstrated to be important factors leading to the pathophysiology of diseases or indeed as therapeutic vehicles in possible new treatments. However, the role of MVs in the communication between bone cells was rarely reported.

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In this study, we show that osteoblast-derived microvesicles contain bone regulatory proteins and can stimulate osteoclast formation, which may represent a novel mechanism for cell-to-cell communication in the bone.

Materials and methods

Isolation of MVs from osteoblastic cell culture

The stromal/osteoblastic cell line UAMS-32P (a kind gift from CA.O'Brien) [17] was used for MVs production. Cells, cultured in α-minimum essential medium (α-MEM) containing 10% fetal bovine serum, were treated with or without PTH (0.1 μmol/L) for 12 h. Culture mediums from about 4×10^7 cells were harvested and were centrifuged at 300 ×*g* for 10 min and 2000 ×*g* for 10 min to remove cells and large debris, respectively. Supernatants were further centrifuged at 16000 ×*g* at 4 °C for 60 min. The pelleted MVs were washed in 15 mL cold PBS and centrifuged at 16000 ×*g* for another 60 min, then MVs were resuspended in PBS or serum-free α-MEM. The amount of collected MVs was estimated by flow cytometric analysis or by measuring MV-associated proteins, using Pierce® BCA Protein Assay Kit.

Flow cytometry analysis

Labeling of MVs was performed by adding 1 μL of RANKL antibody (100 μg/mL, Santa Cruz) to 100 μL MVs suspension and incubated at 4 °C for 2 h. Supernatants were discarded after centrifugation at 16000 ×*g* at 4 °C for 60 min. Then, 1 μL of the FITC-labeled anti-goat IgG (400 μg/mL, Santa Cruz) was added to 100 μL MVs suspension and incubated at 4 °C for 1 h. After centrifugation, the supernatants were discarded and labeled MVs suspension was diluted to 1 mL with PBS, and fluorescence was analyzed by flow cytometry (BD FACSCalibur).

Western blot analysis

MVs released from about 4×10^7 UAMS-32P cells were lysed with RIPA buffer supplemented with a protease inhibitor mixture (Roche). Samples were then subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto PVDF membranes (Life Technologies, Grand Island, NY, USA). The membranes were incubated with a primary antibody, followed by incubation with HRP-conjugated secondary antibody. Immunoreactive bands were visualized with ECL western blotting luminol reagent (Santa Cruz). Densitometric analysis was performed using Image J software. Antibodies specific for RANKL, flotillin-2 and β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Transmission electron microscopy

Sample preparation, thin sectioning, and immunolabeling on section were performed essentially as described previously [18] with antibody against RANKL at 50 μg/mL and gold-coupled secondary antibody at 0.65 μg/mL. Sections were poststained with aqueous uranyl acetate/lead citrate and then examined with Hitachi HT7700 transmission electron microscopy.

Osteoclast formation assay

MVs released from about 4×10^7 UAMS-32P cells were suspended in 100 μL serum-free α-MEM medium, and 10 μL of MVs was co-cultured with RAW264.7 cells (3×10^5 cells/well) in 24-well plate (1 mL/well) for 6 days in α-MEM complete medium. On day 3, the medium was replaced with fresh medium and supplemented with 10 μL of MVs. At the end of the culture period, the cells were fixed in 10% formalin for 10 min, permeabilized with 0.1% Triton X-100 in PBS, and then stained for tartrate-resistant acid phosphatase (TRAP) by using the TRACP& ALP double-stain Kit (TaKaRa).

Generation of GFP-tagged stromal/osteoblastic cell line and fluorescence labeled MVs

Plasmid harboring retroviral vector pCAG-Mem-GFP (a kind gift from prof. Quansheng Zhou of the Soochow University), which expressed fusion protein of 20 N-terminal amino acids of neuromodulin and green fluorescent protein (GFP), was co-transfected with pCL-Ampho Retrovirus Packaging Vector into HEK293T cells using Lipofectamine 2000 (Invitrogen). Supernatants containing viral particles were collected between 48 and 72 h, filtered (0.22 μm) and then stored at −80 °C. UAMS-32P cells were infected with viral supernatants in the presence of 8 μg/mL Polybrene (Santa Cruz, CA, USA) for 72 h. The GFP-positive cells were sorted by flow cytometry.

Pelleted MVs released from GFP-tagged UAMS-32P cells with or without PTH treatment were performed in cold PBS, and total fluorescence was assayed at 488/597 nm with a spectrophotometric system (SpectraMax M5e, Molecular Devices, USA).

Fluorescence confocal microscopy

MVs released from about 4×10^7 GFP-tagged UAMS-32P cells were suspended in 100 μL cold PBS and 20 μL of MVs was co-cultured with target cells in 35 mm plates which were glass bottom. The time-lapse images for interaction between MVs and living cells were acquired with a confocal laser-scanning microscopy (Zeiss LSM 510) fitted with a ×40 objective.

Results

Osteoblast-derived MVs contain RANKL protein

A previous study has reported that parathyroid hormone (PTH) can stimulate RANKL up-regulation in primary cultures of stromal/osteoblastic cells [19]. Although MVs have been reported to contain numerous proteins and lipids similar to those present in the membranes of the cells from which they originate [7,8], we were interested in observing whether MVs shed from UAMS-32P cells upon PTH triggering contain RANKL protein. Thus, we first performed western blot analysis using RANKL antibody to detect the existence of RANKL protein in the MVs released from UAMS-32P cells. As shown in Fig. 1A, RANKL increased significantly in MVs shed from PTH-treated cells, whereas the amount of the MV marker flotillin-2 [20] was nearly equivalent. Flow cytometry analysis further confirmed RANKL protein present in MVs shed from UAMS-32P cells (Fig. 1B). Additionally, visual results by immunoelectron microscopy also indicated the presence of RANKL on the surface of MVs from UAMS-32P cells (Fig. 1C).

The number of MVs present in the culture supernatants was quantified by flow cytometry. The amount of MVs released with PTH treatment was higher than without treatment (71167 ± 1465 vesicles/min vs 33417 ± 382 vesicles/min, obtained from 4×10^7 cells, Fig. 1D). Besides, it is also accompanied by a corresponding increase in the total amount of protein of MVs released from the cells with PTH treatment (168.22 ± 1.44 μg vs 62.55 ± 1.25 μg, obtained from 4×10^7 cells, Fig. 1E). These results revealed that MVs shed from osteoblasts contained osteoblast membrane proteins and PTH promoted the release of MVs from osteoblasts.

Osteoblast-derived MVs stimulate osteoclast formation

MVs are able to transfer various components of their contents into the membranes of target cells, triggering a variety of biologic activity. To examine whether osteoblast-derived MVs could transfer RANKL protein to osteoclast precursors and stimulate osteoclast formation, MVs

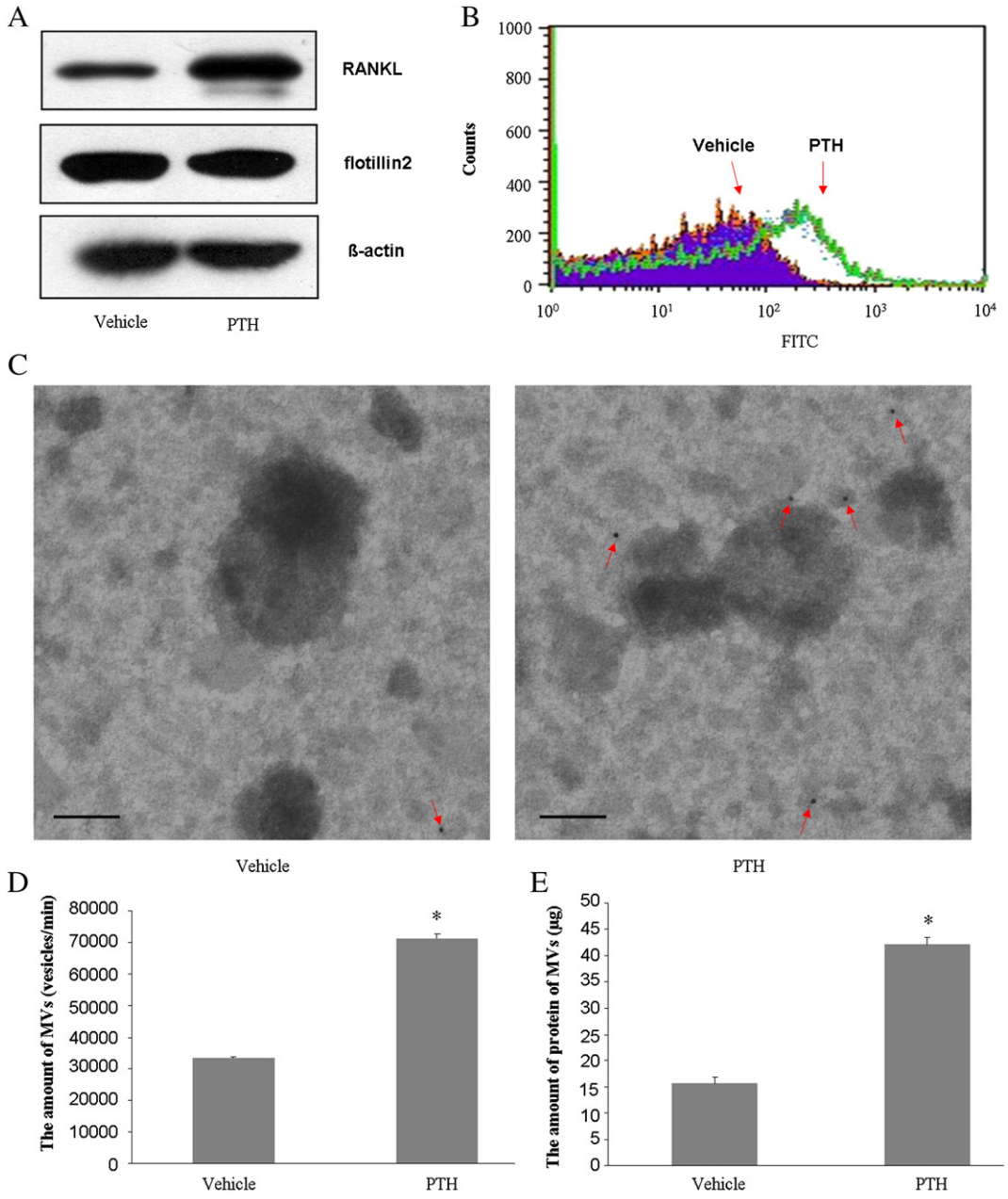

Fig. 1. Biochemical characterization of the MVs shed from UAMS-32P cells. (A) Lysates of MVs from UAMS-32P cells with or without PTH treatment were prepared and subjected to western blot analysis for RANKL, flotillin-2 and β-actin (30 μg/lane). (B) FITC-labeled RANKL on surface of MVs shed from UAMS-32P cells with or without PTH treatment was determined by FACS analysis. (C) Ultrastructural localization of RANKL (arrows) on MVs shed from UAMS-32P cells with or without PTH treatment. Bar: 200 nm. (D) The amount of MVs shed from UAMS-32P cells with or without PTH treatment was quantified by flow cytometry (obtained from 4×10^7 cells). (E) The total amount of protein of MVs shed from UAMS-32P cells with or without PTH treatment (obtained from 4×10^7 cells). Each bar represents the mean ± STDEV (n = 3). *, $P < 0.05$ by Student's t test. Vehicle: without PTH treatment; PTH: with PTH treatment.

shed from UAMS-32P cells after treatment with PTH for 12 h were co-cultured with RAW264.7 cells, the macrophage cell line which can differentiate to osteoclast after RANKL stimulation. TRAP staining showed that MVs prepared from UAMS-32P cells with PTH treatment significantly facilitated osteoclast precursor differentiation, and this effect of MVs could be blocked by adding RANKL antibody or osteoprotegerin (OPG), which is a soluble decoy receptor for RANKL that blocks ligand binding to RANK, thereby preventing the signaling required for osteoclast differentiation and activation (Fig. 2). To further demonstrate that MVs stimulate RANKL–RANK signaling, we investigated the translocation of nuclear factor of activated T cell (NFATc1), which is the master regulator of osteoclastogenesis in response to RANKL. Results showed that MVs released from PTH-treated UAMS-32P cells activated NFATc1 nuclear translocation (Supplemental Fig. 1). These results indicated that MV-mediated transfer of RANKL protein from osteoblasts to osteoclast precursors was indeed capable of inducing osteoclast formation. Therefore, the osteoblast-MV delivery system, which affords fusion of MVs to the plasma membrane of osteoclasts, is a reasonable transfer model for RANKL.

Fluorescence labeling of MVs

Fluorescent proteins fused with MV membrane protein can be useful MV tracers. For direct visualization of transfer of MVs from cell to cell in live culture, we generated GFP-tagged UAMS-32P cells which stably express green fluorescent protein (GFP) in plasma membrane using a lentivector system (Fig. 3A). As shown in Fig. 3B, the majority of cells were positive (97.3% for GFP-tagged). As green fluorescent protein

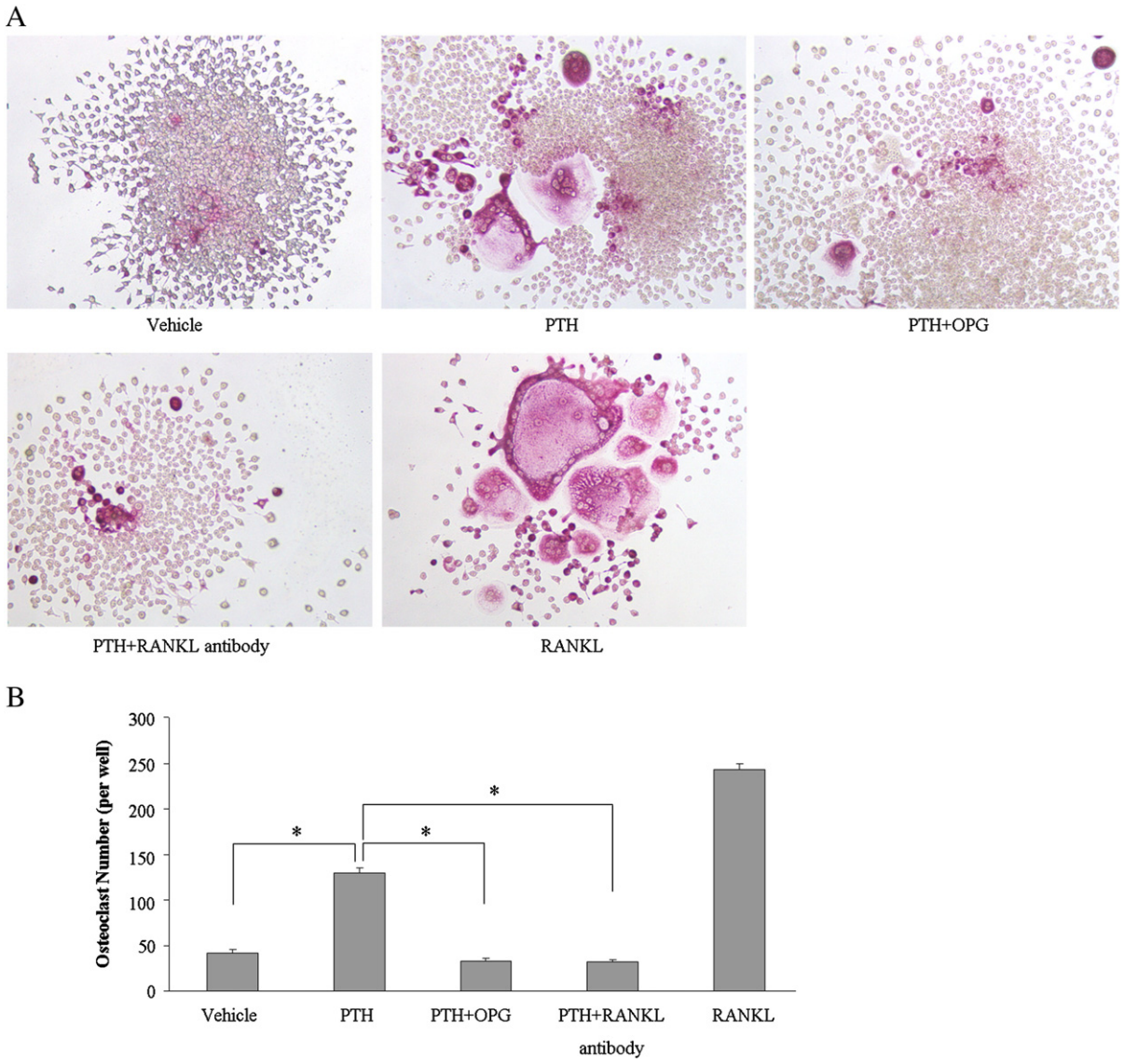

Fig. 2. Osteoblast-derived MVs stimulate osteoclast formation. (A) RAW264.7 cells and MVs shed from UAMS-32P cells without PTH treatment (Vehicle group) or with (PTH group) were co-cultured. OPG (100 ng/mL) and RANKL antibody (100 μg/mL) were preincubated with MVs for 30 min to block the effect of MVs (PTH + OPG, PTH + RANKL antibody group). Soluble RANKL (10 ng/mL) was added as positive control (RANKL group). Cells were fixed and stained for ALP activity (200× magnification). (B) The numbers of TRAP staining positive cells. Each bar represents the mean ± STDEV in 24 well-plate ($n = 3$). *, $P < 0.05$ by one-way ANOVA.

was fused with membrane protein, MVs would be also GFP-tagged when directly budded from the cell plasma membrane. As shown in Fig. 3C, fluorescence of MVs, collected from GFP-tagged UAMS-32P cells with or without PTH treatment, was quantified by spectrophotometric analysis. Results showed that MVs from GFP-tagged UAMS-32P cells with PTH treatment had stronger fluorescence, indicating that PTH facilitated MVs release from osteoblasts.

MVs interact with target cells through specific receptor ligands

In order to investigate intercellular location of RANKL delivered to target cells via MVs, we directly visualize the transfer by imaging GFP-tagged MVs with confocal laser scanning microscopy. As shown in Fig. 4, GFP-tagged MVs shed from PTH-treated UAMS-32P cells (RANKL on surface) were bound to the surface of RAW264.7 cells (RANK on surface) after co-cultured from 4 to 72 h (Fig. 4A). They were also bound to the surface of RANK-expressing NIH3T3 cells, whereas they were transferred into cytosol of NIH3T3 cells because of receptor ligand absence (Fig. 4B). Additionally, preincubated with RANKL antibody or OPG, MVs were also transferred into cytosol after co-cultured with RAW264.7 cells (Fig. 4C, D). These results implicate that MVs interact only with target cells that they specifically recognize rather than just with any cell present in the microenvironment.

Discussion

The maintenance of bone homeostasis is largely dependent on cellular communication between osteoblasts and osteoclasts. Usually, there are three modes of osteoblast–osteoclast communication. Osteoblasts and osteoclasts can make direct contact, allowing membrane-bound ligands and receptors to interact and initiate intracellular signaling. They can also form gap junctions allowing passage of small water-soluble molecules between the two cell types. Communication can also occur through diffusible paracrine factors, such as growth factors, cytokines, chemokines and other small molecules secreted by either cell type and acting on the other via diffusion [21]. Here, we found that microvesicles, the membrane-bound vesicular particles, released from osteoblasts can facilitate osteoclast formation, which may represent a novel mechanism for osteoblasts and osteoclasts communication.

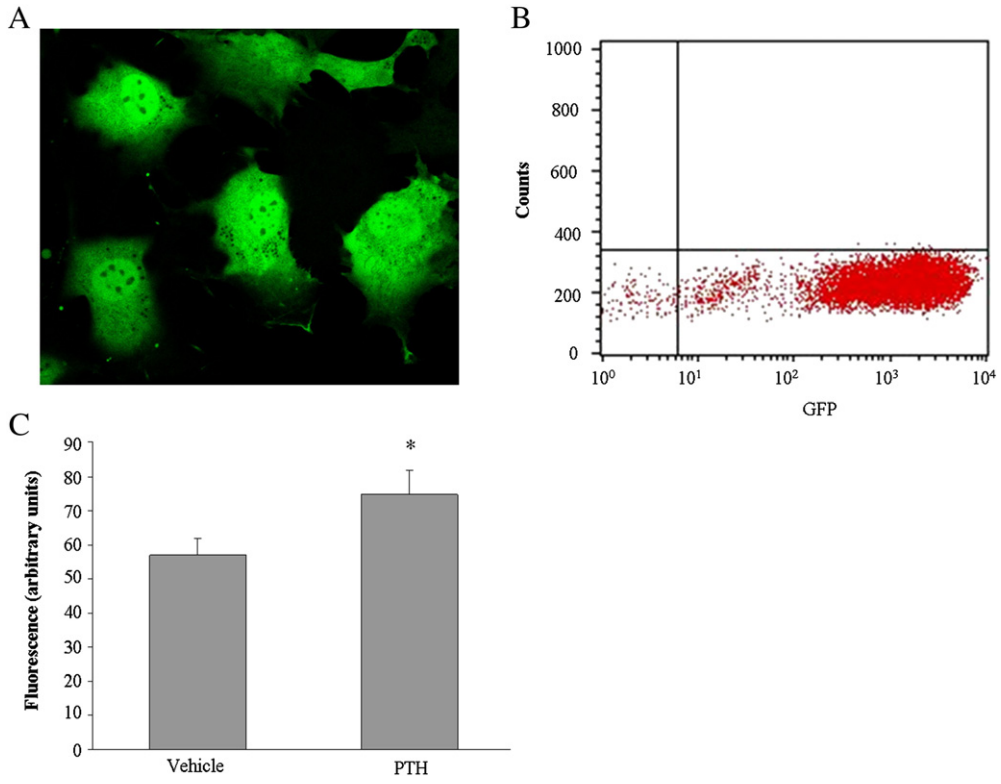

Fig. 3. Identification of GFP-tagged UAMS-32P cells and fluorescence labeled MVs. (A) The image of GFP-tagged UAMS-32P cells acquired by fluorescence microscope. (B) FACS analysis of GFP-tagged UAMS-32P cells. (C) Spectrophotometric analysis of fluorescent MVs released from GFP-tagged UAMS-32P cells without (vehicle group) or with PTH treatment (PTH group). Each bar represents the mean ± STDEV in 6 well-plate ($n = 3$). *, $P < 0.05$ by Student's t test.

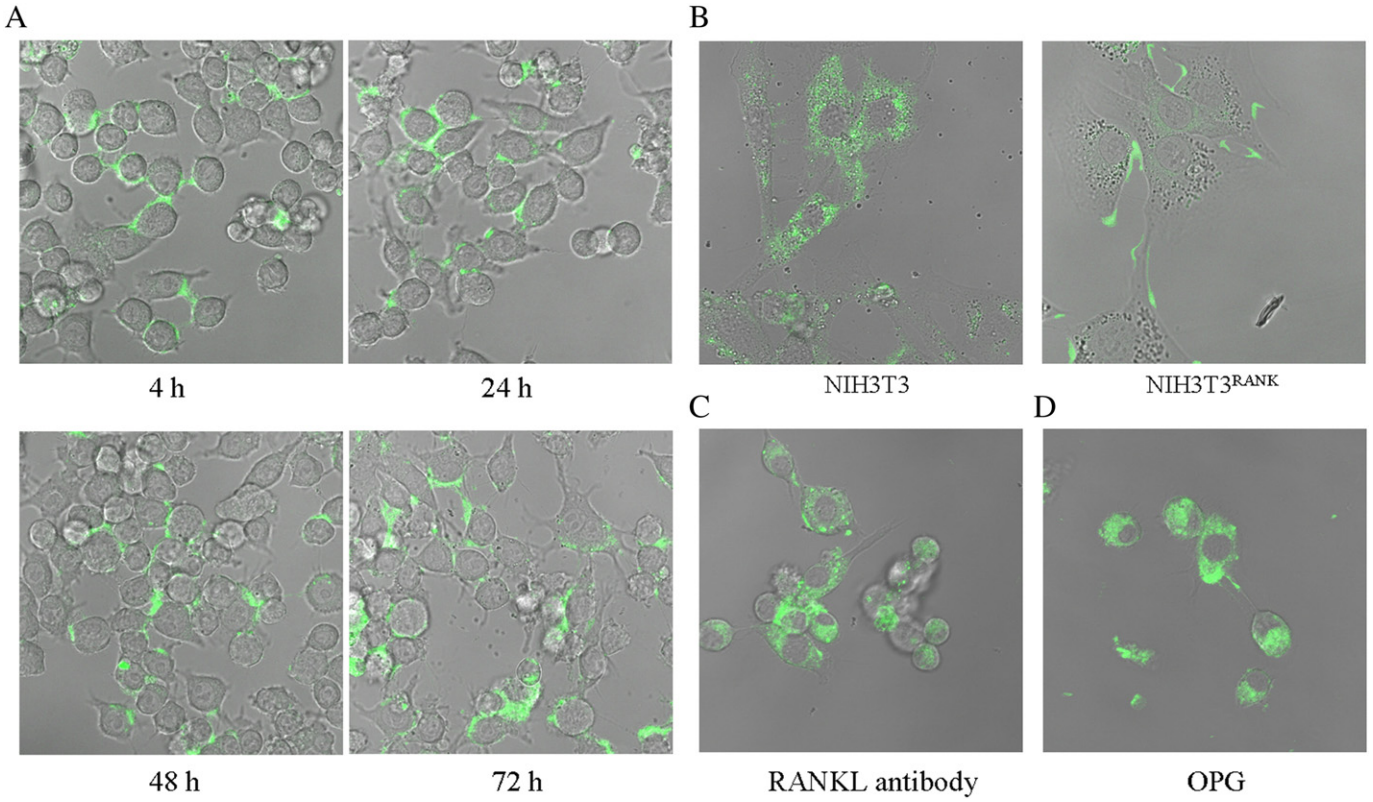

Fig. 4. Intracellular localization of MVs in target cells. (A) MVs released from UAMS-32P cells with PTH treatment were bound to the surface of RAW264.7 cells at different times (4, 24,48, 72 h). (B) MVs released from UAMS-32P cells with PTH treatment were transferred into cytosol of NIH3T3 cells or bound to the surface of RANK-expressing NIH3T3 cells ($NIH3T3^{RANK}$ cells) after incubation for 4 h. (C) MVs pre-incubated with RANKL antibody (100 ng/mL) for 15 min were transferred into cytosol after co-cultured with RAW264.7 cells for 4 h. (D) MVs pre-incubated with OPG (100 ng/mL) for 15 min were transferred into cytosol after co-cultured with RAW264.7 cells for 4 h.

Microvesicles are created by direct budding from the cell plasma membrane into the extracellular environment. They contain numerous proteins and lipids similar to those present in the membranes of the cells from which they originate and are able to transfer various components of their contents into the membranes of target cells, triggering a variety of biologic responses. In this study, we confirmed that osteoblast-derived MVs contain RANKL, a member of the tumor necrosis factor (TNF) family of cytokines, produced by stromal/osteoblasts as a membrane-bound protein; when MVs co-cultured with osteoclast precursors, they interacted with target cells and stimulated the osteoclast precursor differentiation to osteoclasts with osteoblasts absent.

There are different mechanisms by which MVs may interact with target cells. MVs may act as signaling complexes by direct stimulation of target cells; may act by transferring surface receptors between cells; may deliver proteins, mRNA and bioactive lipids into the target cells; and may deliver infectious agents into cells. In the present study, we found that osteoblast-derived MVs interacted with target cells (RAW264.7 cells; osteoclast precursors) that specifically recognized through receptor ligand (RANKL–RANK); other cells without specific receptor were not recognized. This interaction was also limited to a receptor-mediated binding to the surface of target cells leading to cell signaling. OPG addition blocked MV-derived RANKL binding to RANK, resulting in the prevention of the signaling required for osteoclast differentiation. This observation implicated that MVs released from a given cell type may interact through specific receptor ligands with other cells, leading to target cell stimulation by transferring surface receptors.

An interesting and important finding was that MVs were internalized when the specific recognition could not be achieved between osteoblast-derived MVs and target cells. Once internalized, MVs may remain segregated and be transferred to lysosomes or dismissed by the cells following the fusion with the plasma membrane, thus leading to a process of transcytosis; the detail studies on this should be performed in the future. Besides, this study was focused on RANKL pathway on osteoblast-derived MVs, whether other marker proteins such as E11, sclerostin, and FGF23 will present in the MVs and how they affect osteoclast formation should be explored in future studies. Furthermore, efficient strategies that will allow us to modulate MVs secretion should be developed, thus we can employ MVs as tools to modify the biological responses of bone cells or develop an alternative drug to treat bone diseases.

Collectively, RANKL-containing MVs released from osteoblasts were transferred to osteoclast precursors, which resulted in the stimulation of RANKL–RANK signaling to facilitate osteoclast formation. Such MV-mediated intercellular communication between osteoblasts and osteoclasts may represent a novel mechanism of bone modeling and remodeling.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bone.2015.05.022.

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