Effects of TGF-β1 on the migration and morphology of RAW264.7 cells *in vitro*

MIHO UETA[1*], KAZUKI TAKAOKA[1*], MICHIYO YAMAMURA[1], HANAKO MAEDA[1], JOJI TAMAOKA[1], YOSHIORO NAKANO[2], KAZUMA NOGUCHI[1] and HIROMITSU KISHIMOTO[1]

Departments of [1]Oral and Maxillofacial Surgery, and [2]Genetics, Hyogo College of Medicine, Nishinomiya, Hyogo 663-8501, Japan

Received November 19, 2018; Accepted July 11, 2019

DOI: 10.3892/mmr.2019.10662

Abstract. Osteoclasts (OCs) differentiate from monocyte/macrophage-lineage hematopoietic precursor cells, which are known as OC precursors (OCPs). Several studies have investigated cell chemotaxis in the bone microenvironment; however, OCP migration ability in the bone microenvironment during OC differentiation is yet to be elucidated. As an initial investigation of this characteristic, the present study aimed to determine the effects of transforming growth factor (TGF)-β1 on OCP migration *in vitro*. Pre-osteoclastic RAW264.7 cells were cultured with and without TGF-β1 (2, 5 or 20 ng/ml), receptor activator of NF-κB ligand (RANKL; 50 ng/ml), and/or SB431542 (10 μM), a potent and specific inhibitor of TGF-β1 receptor kinase activity. Cell proliferation was significantly inhibited in the presence of TGF-β1 for 3 days, and the effect was reversed by SB431542. Tartrate-resistant acid phosphatase (TRAP) activity in RAW264.7 cells was significantly increased by RANKL treatment, compared with TRAP activity in control cells on day 3. The highest TRAP activity in RAW264.7 cells was induced by the combined treatment with TGF-β1 (2 ng/ml) and RANKL. When TGF-β1 signaling was inhibited by addition of SB431542 to the medium during culture, OC differentiation was notably suppressed. These findings suggest that TGF-β1 accelerates RANKL-induced OC differentiation, but does not act in a dose-dependent manner. The migration of RAW264.7 cells was promoted at 24 h, but was suppressed at 72 h, during RANKL-induced osteoclast differentiation in the presence of TGF-β1. These results were accompanied with the increased expression of small G-proteins, RhoA and Rac, at 24 h, but their expression decreased at 72 h. RAW264.7 cells treated with TGF-β1 for 24 h underwent morphological changes, from round to polygonal morphology. Furthermore, protrusions were completely lost and the cell morphology reverted from polygonal to round after TGF-β1 treatment for 72 h. Therefore, our findings indicated that OCP migration may be modified by differentiation *in vitro*.

Correspondence to: Dr Kazuki Takaoka, Department of Oral and Maxillofacial Surgery, Hyogo College of Medicine, 1-1 Mukogawa-cho, Nishinomiya, Hyogo 663-8501, Japan
E-mail: ktaka@hyo-med.ac.jp

*Contributed equally

Key words: RAW264.7 cells, osteoclast precursor, TGF-β1, *in vitro*, migration, morphology

Introduction

Bone resorption and formation are coupled to maintain skeletal mass (1); this process is tightly regulated to precisely replace any removed bone with regards to location and quantity (2). However, the process is disrupted by aging, which leads to net bone loss (2). As bone loss is a risk factor for fragility fracture, it is essential to clarify the regulation of bone remodeling at the cellular level. Osteoclasts (OCs) are formed by the fusion of precursor cells to form the monocyte/macrophage lineage (3). OC precursors (OCPs) are drawn from the bloodstream into bone by various factors released at sites undergoing resorption in the bone microenvironment, and subsequently differentiate into OCs (3). The factors involved include macrophage-colony-stimulating factor, receptor activator of NF-κB ligand (RANKL), and transforming growth factor (TGF)-β1 (4). Sphingosine-1 phosphate controls the migration of OCPs between bone tissues and the bloodstream (5,6). Several studies have investigated cell chemotaxis in the bone microenvironment (5-8); however, OCP migration in the bone microenvironment has not been analyzed. The stage at which OCPs differentiate into OCs is unclear (before, during, or after migration to bone resorption sites in the bone microenvironment).

TGF-β1 serves major roles in the proliferation, migration, differentiation and survival of a variety cell types (9). It is abundantly stored in the bone matrix and has profound biological functions, including roles in bone homeostasis (10,11). The binding of TGF-β1 to its type II receptor leads to the recruitment and phosphorylation of the type I receptor, thereby activating downstream signaling pathways, including Smad and non-Smad pathways (12). TGF-β1 consistently stimulates RANKL-induced OC differentiation in differentiation models using RAW264.7 cells, which are widely known as OCPs and are regarded as important for *in vitro* analyses (13-15).

Fox *et al* (16) reported that TGF-β directly induces the expression of nuclear factor of activated T cells c1 (NFATc1), a key regulator of OC differentiation. Yasui *et al* (17) demonstrated that TGF-β is essential for RANKL-induced OC differentiation and has a possible role in the molecular interaction between tumor necrosis factor receptor-associated factor 6 and Smad2/3 in osteoclastogenic RANKL/RANK signal transduction. Several molecules have been shown to mediate a promoting effect of TGF-β1 on RANKL-induced OC differentiation. Although the mechanisms by which monocyte/macrophage-lineage cells differentiate into OCs are well-defined, the effects of TGF-β1 on the characteristics of OCP migration *in vitro* remain unclear. Macrophage migration is affected by the duration of stimulation with TGF-β1 (18). The aim of the present study was to determine the effect of TGF-β1 on the migration of OCPs *in vitro*.

Materials and methods

Reagents. Recombinant human TGF-β1 was purchased from R&D Systems, Inc., SB431542 was purchased from Sigma-Aldrich (Merck KGaA), and recombinant human soluble RANKL was purchased from Oriental Yeast Co., Ltd. α-minimum essential medium (α-MEM) and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific, Inc.

Cell culture. RAW264.7 cells were obtained from RIKEN BioResource Center. Cells cultured in α-MEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37˚C under a humidified 5% CO_2 atmosphere were used as control cells. Other RAW264.7 cells were incubated at 37˚C under a humidified 5% CO_2 atmosphere with or without TGF-β1 (2, 5 or 20 ng/ml), SB431542 (10 μM), and/or RANKL (50 ng/ml) for the duration designated by the following experiments (Table I). Passages three to eight were used for all experiments.

Cell proliferation assay. RAW264.7 cells were plated on 96-well culture plates at $5x10^3$ cells/well. After the addition of reagents at various concentrations as aforementioned, the WST-8 reagent of a Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc.) was added to the cells (10 μl/well) and incubated at 37˚C for 2 h on days 1, 2, 3 and 4. The number of viable cells was determined by measuring the absorbance at 450 nm with a microplate reader (Benchmark Plus™ Microplate Spectrophotometer; Bio-Rad Laboratories, Inc.).

Tartrate-resistant acid phosphatase (TRAP) staining. RAW264.7 cells were plated on 12-well culture plates at $2x10^4$ cells/well. After the addition of reagents at various concentrations, the cultures were maintained for 7 days. The culture medium, supplemented with reagents, was replaced every 3 days. Subsequently, the cells were stained using a TRAP staining kit (Cosmobio Co., Ltd.), in accordance with the manufacturer's instructions. TRAP-positive multinucleated cells were identified as OCs at a magnification of x200 by using a light microscope (Ti-E, Nikon Instech Co., Ltd.).

TRAP activity assay. RAW264.7 cells were plated on 96-well culture plates at $2.5x10^3$ cells/well. After the addition of reagents at various concentrations, the cultures were maintained for 2, 3 or 4 days. The cells were then fixed with ethanol/acetone (1:1) as described previously (19), and evaluated for TRAP activity using a TRAP solution kit (Oriental Yeast Co. Ltd.), in accordance with the manufacturer's protocols. Briefly, 150 μl of 50 mM citrate buffer (pH 4.5) containing 5.5 mM p-nitrophenol phosphate and 10 mM sodium tartrate was added to each well. After incubation for 60 min at room temperature, 50 μl of 0.1 N NaOH was added and the absorbance at 405 nm was determined using a microplate reader (Benchmark Plus™ Microplate Spectrophotometer).

NFATc1 activation assay. To determine the role of TGF-β1-induced OC differentiation in RANKL-treated RAW264.7 cells, NFATc1 activation was assessed. RAW264.7 cells were plated at $2x10^6$ cells, pretreated at 37˚C with reagents (TGF-β1, SB431542, and/or RANKL) for 24 h in 60-mm culture plates, and prepared nuclear extracts using a Nuclear Extraction Kit (Abcam) according to the manufacturer's protocol. NFATc1 activation in the cell lysates was assessed using the NFATc1 Transcription Factor Kit (Abcam), in accordance with the manufacturer's protocols. Briefly, 100 μl of diluted primary antibody (included in the kit) was added to each well. After incubation for 60 min at room temperature, 100 μl of diluted horseradish peroxidase (HRP) antibody was added and incubated for 1 h at room temperature and the absorbance at 450 nm was determined using a microplate reader (Benchmark Plus™ Microplate Spectrophotometer).

Cell migration assay. Assays were performed with a 96-well cell migration assay kit (CytoSelect™; Cell Biolabs Inc.). Following pretreatment with reagents for 24 or 72 h, RAW264.7 cells were suspended in serum-free medium and plated at $5x10^4$ cells/well in the upper part of 96-well, 8-μm pore, cell-migration chambers, in accordance with the manufacturer's protocols. Medium containing 10% FBS was placed in the lower wells as a chemotactic stimulus. After incubation for 14 h at 37˚C, the migrated cells were dissociated from the membranes via the addition of cell detachment buffer (included in the kit) to the lower wells, lysed and incubated with CyQuant GR dye® (included in the kit) for 20 min at room temperature. Migrated cells were quantified by determining the fluorescence at 480/520 nm using a scanning fluorometer (Infinite® 200 PRO; Tecan Group, Ltd.).

Western blot analysis. RAW264.7 cells were plated at $3x10^5$ cells, pretreated with reagents for 24 or 72 h in 60-mm culture plates and lysed with RIPA buffer (cat. no. sc24948, Santa Cruz Biotechnology, Inc.) containing TBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.004% sodium azide with PMSF, sodium orthovanadate and protease inhibitor cocktail. The cell lysates were analyzed by western blotting, as described previously (20,21). Equal amounts of protein (20 μg) in each sample were separated by 10% SDS-PAGE for 30 min at a constant voltage (200 V) and transferred onto a polyvinylidene fluoride (PVDF) membrane (Bio-Rad Laboratories, Inc.). Membranes were blocked in TBS + Tween-20 (TBST; 20 mM Tris, 500 mM NaCl pH 7.5 and 0.1% Tween-20) containing 5% non-fat milk for 1 h at room temperature and then incubated

Table I. RAW264.7 cells were incubated with and without TGF-β1, SB431542, and/or RANKL.

	Group											
Treatment	Co	T2	T5	T20	T2S	T5S	R	RT2	RT5	RT20	RT2S	RT5S
TGF-β1 (ng/ml)	-	2	5	20	2	5	-	2	5	20	2	5
RANKL (50 ng/ml)	-	-	-	-	-	-	+	+	+	+	+	+
SB431542 (10 μM)	-	-	-	-	+	+	-	-	-	-	+	+

R/RANKL, receptor activator of NF-κB ligand; T/TGF-β1, transforming growth factor-β1; Co, control; S, SB431542.

overnight at 4˚C with appropriate primary antibodies. The following primary antibodies were used: Rabbit polyclonal antibodies against RhoA (1:200; cat. no. sc179; Santa Cruz Biotechnology, Inc.), Cdc42 (1:200; cat. no. sc87; Santa Cruz Biotechnology, Inc.), and Rac1/2/3 (1:1,000; cat. no. 2465; Cell Signaling Technology, Inc.), rabbit monoclonal antibody against c-Fos (1:1,000; cat. no. 2250; Cell Signaling Technology, Inc.), and goat polyclonal antibody against β-actin (1:1,000; cat. no. sc1616; Santa Cruz Biotechnology, Inc.). Subsequently, membranes were washed and incubated with secondary antibodies at room temperature for 2 h. The secondary antibodies were HRP-conjugated anti-goat IgG (1:2,000; cat. no. P0049; Dako; Agilent Technologies, Inc.), HRP-conjugated anti-mouse IgG (1:1,000; cat. no. 7076; Cell Signaling Technology, Inc.), and HRP-conjugated anti-rabbit IgG (1:10,000; cat. no. 458; MBL Co. Ltd., Nagoya, Japan). Signals were detected by chemiluminescence using a Pierce SuperSignal Western Blotting Kit (Thermo Fisher Scientific, Inc.). β-actin was evaluated as an internal control to confirm that equal amounts of total protein were present.

Cell adhesion assay. Cell adhesion assays were performed as described previously (22). RAW264.7 cells were plated at $6x10^5$ cells/well in 24-well culture plates, and pretreated with reagents for 24 or 72 h. The cells were then replated at $3x10^4$ cells/well in 96-well culture plates and incubated for 30 min at 37˚C under a humidified 5% CO_2 atmosphere. Non-attached cells were removed by three washes with PBS(-), and attached cells were evaluated with via an Cell Counting Kit-8 assay as aforementioned by measuring the absorbance at 450 nm using a microplate reader (Benchmark Plus™ Microplate Spectrophotometer; Bio-Rad).

Immunocytochemical analysis. After pretreatment of cells with reagents for 24 or 72 h, immunocytochemical analysis was performed. The primary antibody used was a mouse anti-vinculin antibody (1:800; cat. no. V9131; Sigma-Aldrich; Merck KGaA). Cultured cells were washed twice with PBS(-), fixed with 3.7% paraformaldehyde for 20 min at room temperature, and permeabilized with 0.1% Tween in PBS. After blocking with 2% bovine serum albumin (Roche Diagnostics) for 1 h at room temperature, the cells were treated with the primary antibody at 4˚C overnight. They were then washed and incubated with an Alexa Fluor® 488-conjugated secondary antibody (1:400, cat. no. A21206; Thermo Fisher Scientific Inc.) and rhodamine phalloidin (1:200; cat. no. PHDH1; Cytoskeleton Inc.) at room temperature in the dark for 2 h. After the cells had been washed twice with PBS(-), they were mounted with Vectashield containing DAPI (cat. no. H1500; Vector Laboratories Inc.) at room temperature for 2 h. Fluorescence images were obtained for evaluation of morphological changes, using a confocal laser microscope (LSM 780; Zeiss AG).

The total numbers of cells with polygonal or spindle morphology were counted in five non-overlapping fields at magnification, x200.

Statistical analysis. Experiments were repeated independently at least three times. All data were expressed as mean ± standard deviation. Statistical analyses were performed using one-way analysis of variance followed by a Tukey-Kramer post-hoc test for intergroup comparisons in each of the experiments using JMP statistical software, Pro14.2 (SAS Institute Inc.). $P<0.05$ was considered to indicate statistically significance difference.

Results

Effects of TGF-β1, SB431542 and RANKL on cell proliferation. RAW264.7 cells were incubated with or without TGF-β1 (2, 5 or 20 ng/ml), SB431542 (10 μM), and/or RANKL (50 ng/ml) for 4 days. Cell proliferation was observed to be significantly inhibited by TGF-β1 on days 3 and 4, and significantly inhibited by RANKL, compared with control cells on day 4. The addition of SB431542 appeared to have reversed the inhibition (Fig. 1).

Effects of TGF-β1 on OC differentiation. To investigate the effects of TGF-β1 on OC differentiation, we determined TRAP staining and activity. The level of TRAP enzymatic activity in cultured cell lysates was reported to be correlated with the relative number of OCs observed by TRAP staining (23). On day 7, RANKL induced the formation of large multinucleated (≥5 nuclei) OC-like cells. Single treatment with TGF-β1 did not induce the formation of TRAP-positive multinucleated cells, while combined treatment with TGF-β1 and RANKL increased the number of TRAP-positive multinucleated cells;

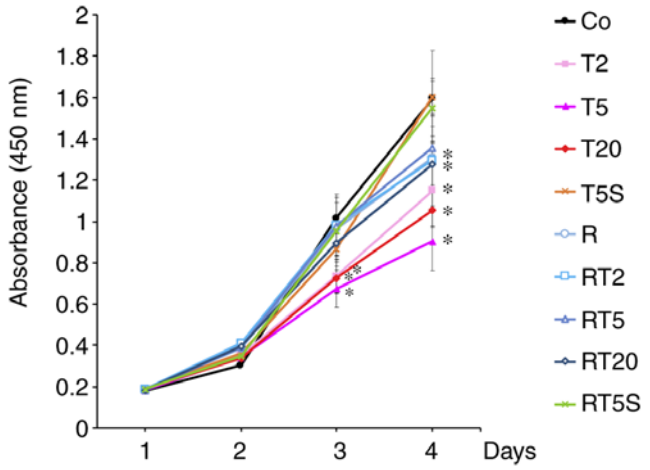

Figure 1. Cell proliferation assay. RAW264.7 cells at $5x10^3$ cells/well in 96-well culture plates were cultured with reagents at various concentrations for 1, 2, 3 or 4 days, and assessed for proliferation using the WST-8 reagent in the Cell Counting Kit-8. *P<0.05 vs. Co cells. RANKL, receptor activator of NF-κB ligand; TGF-β1, transforming growth factor-β1; Co, control group; T2, TGF-β1 (2 ng/ml); T5, TGF-β1 (5 ng/ml); T20, TGF-β1 (20 ng/ml); T5S, TGF-β1 (5 ng/ml) + SB431542; R, RANKL; RT2, RANKL + TGF-β1 (2 ng/ml); RT5, RANKL + TGF-β1 (5 ng/ml); RT20, RANKL + TGF-β1 (20 ng/ml); RT5S, RANKL + TGF-β1 (5 ng/ml) + SB431542.

the effect was reversed by addition of SB431542 (Fig. 2A). To confirm the potential effect of TGF-β1 on RANKL-induced OC differentiation, we determined TRAP enzymatic activity. TRAP activity was significantly increased in response to RANKL, and further increased by combined treatment with RANKL and TGF-β1, compared with control cells at day 3. The levels of TRAP activity in RAW264.7 cells treated with TGF-β1 (2 ng/ml) and RANKL were highest among all groups on day 4. TRAP activity after combined treatment with TGF-β1 at 5 or 20 ng/ml and RANKL was markedly lower than that after combined treatment with TGF-β1 at 2 ng/ml and RANKL. Therefore, TGF-β1 combined with RANKL did not significantly increase the level of TRAP activity in a dose-dependent manner. The level of TRAP activity in cells treated with TGF-β1 (5 ng/ml) and RANKL was significantly decreased by the addition of SB431542 compared with RT5 cells (Fig. 2B). c-Fos protein expression in RAW264.7 cells was markedly elevated following treatment with RANKL, compared with that in control cells (Fig. 2C). The level of NFATc1 in RAW264.7 cells that were treated with TGF-β1 (2 ng/ml) and RANKL was significantly increased than that in RAW264.7 cells that were treated with RANKL alone (Fig. 3).

Effects of TGF-β1 and RANKL on cell migration. To address the effect of TGF-β1 and RANKL on the migration of RAW264.7 cells, we performed a Transwell migration assay. In the presence of FBS in the lower chambers, pretreatment with TGF-β1 (2 ng/ml) for 24 h followed by RANKL significantly stimulated migration compared with control cells (Fig. 4A), while pretreatment with TGF-β1 (5 and 20 ng/ml) for 72 h significantly reduced migration (Fig. 4B). The increased migration stimulated by TGF-β1 pretreatment for 24 h was notably inhibited by the addition of SB431542 (Fig. 4A). In contrast, the suppressed migration induced by TGF-β1 for 72 h was markedly reversed by the addition of SB431542 (Fig. 4B). The effects of TGF-β1 treatment on migration were similar to the effects of combined treatment with TGF-β1 and RANKL under the same durations of treatment. These findings indicate that TGF-β1 has two different sequential effects on RAW cell migration: Stimulation, followed by inhibition. Treatment with RANKL alone did not significantly enhance the migration of RAW264.7 cells.

Effects of TGF-β1 and RANKL on expression of Rho GTPases. Rho GTPases play key roles in the regulation of cellular responses required for cell migration (24). We investigated whether RAW264.7 cell migration in response to TGF-β1 requires Rho GTPases. When RAW264.7 cells were treated with TGF-β1 (2 and 5 ng/ml), or TGF-β1 (2 and 5 ng/ml) and RANKL for 24 h, RhoA and Rac protein expression levels were markedly increased (Fig. 5). Conversely, when RAW264.7 cells were treated with TGF-β1 with or without RANKL for 72 h, RhoA and Rac protein expression levels were significantly decreased, while that of Cdc42 was slightly decreased (Fig. 5).

Effects of TGF-β1 and RANKL on cell adhesion. Pretreatment with TGF-β1 (2 and 5 ng/ml) for 24 h markedly reduced cell adhesion; this effect was reversed by addition of SB431542 (Fig. 6A). Extending the pretreatment with TGF-β1 (2 and 5 ng/ml) from 24 to 72 h induced a marked recovery in cell adhesion (Fig. 6B). Treatment with both TGF-β1 and RANKL had an effect on cell adhesion similar to that of pretreatment with TGF-β1 alone.

Effects of TGF-β1 and RANKL on morphological changes, the actin cytoskeleton and focal adhesions of RAW264.7 cells. The actin cytoskeleton and focal adhesion formation were evaluated by antibody staining (Fig. 7A and B). RAW264.7 cells treated with TGF-β1 for 24 h exhibited morphological changes from round to polygonal or spindle morphology (Fig. 7C). TGF-β1 treatment induced protrusions that were positive for actin and vinculin (Fig. 7A). The formation of protrusions was notably abolished in response to TGF-β1 treatment for 72 h; RANKL treatment induced the formation of multinucleated cells (Fig. 7B).

Discussion

OC differentiation is a key regulatory point for bone disease therapy. It is important that the mechanism underlying OC differentiation in the bone microenvironment is elucidated. Since the discovery of the RANK signaling pathway, several studies have focused on clarifying the involvement of this pathway in OC differentiation and function (13,14), and have provided insight into the mechanisms for OC differentiation and activation in bone resorption (25). OCP migration ability during the OC differentiation stage requires further investigation; thus, we explored the link between OCP migration and differentiation over time.

TGF-β inhibits the proliferation of epithelial, endothelial and hematopoietic cell lineages (26-28). The roles of TGF-β in tumorigenesis are complex and paradoxical. Specifically, TGF-β has a tumor-suppressive role in normal tissues and early-stage cancers, but switches to a tumor-promotive role

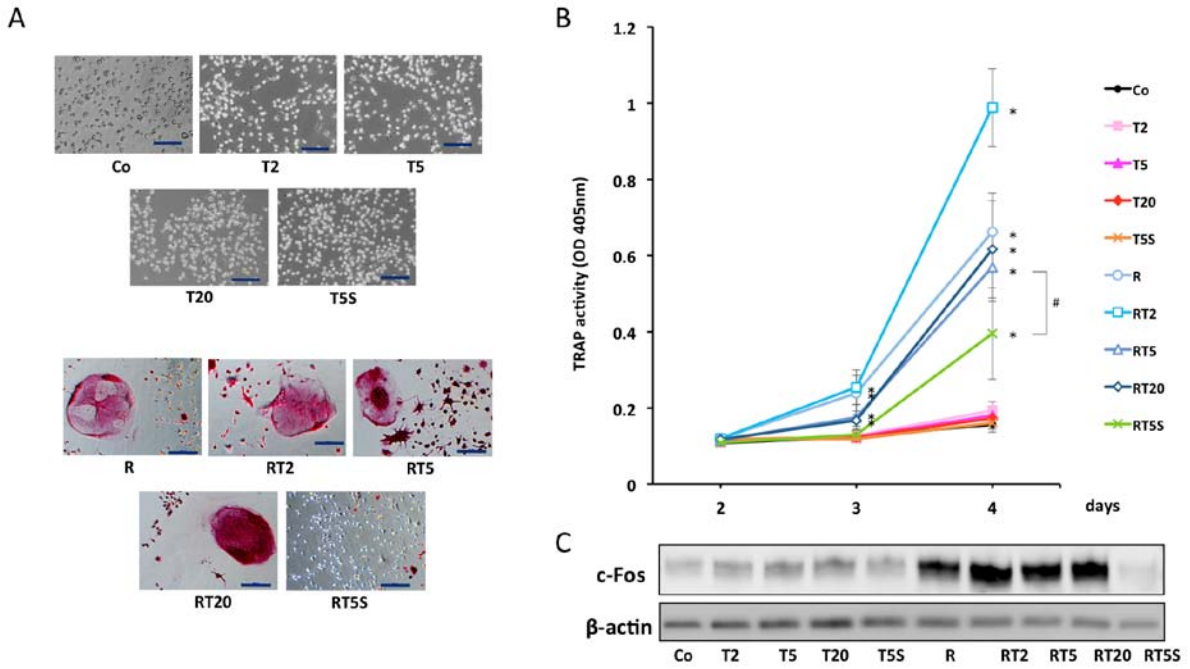

Figure 2. Effect of TGF-β1 on differentiation of RAW264.7 cells. (A) TRAP staining. After 7 days of culture with reagents at various concentrations, RAW264.7 cells were stained for TRAP. TRAP-positive multinucleated cells were observed under a light microscope as osteoclasts. Scale bars, 100 μm. (B) TRAP activity. RAW264.7 cells at $5x10^3$ cells/well in 96-well culture plates were cultured with reagents at various concentrations for 2, 3 or 4 days, and analyzed for their TRAP activity. *P<0.05 vs. compared with control cells. #P<0.05. (C) Expression levels of c-Fos protein evaluated by western blot analysis. RAW264.7 cells ($3x10^5$ cells) were pretreated with reagents for 24 h in 60-mm culture plates and lysed. The cell lysates were subjected to western blot analysis. RANKL, receptor activator of NF-κB ligand; TGF-β1, transforming growth factor-β1; T2, TGF-β1 (2 ng/ml); T5, TGF-β1 (5 ng/ml); T20, TGF-β1 (20 ng/ml); T5S, TGF-β1 (5 ng/ml) + SB431542; R, RANKL; RT2, RANKL + TGF-β1 (2 ng/ml); RT5, RANKL + TGF-β1 (5 ng/ml); RT20, RANKL + TGF-β1 (20 ng/ml); RT5S, RANKL + TGF-β1 (5 ng/ml) + SB431542; OD, optical density; TRAP, tartrate-resistant acid phosphatase.

in late-stage cancers (29). The switch in TGF-β function is known as the 'TGF-β paradox' (29). Understanding of the underlying mechanisms that determine when and how TGF-β switches from a tumor suppressor to a tumor promoter remains a great challenge in the research field. Our study demonstrated the presence of a TGF-β switch for OCPs *in vitro*.

In the present study, cell proliferation was significantly inhibited in the presence of TGF-β1 after 3 days, and this effect was prevented by the addition of SB431542. Cell proliferation was also significantly inhibited by RANKL treatment for 4 days. The TRAP activity in RAW264.7 cells was significantly increased by RANKL treatment compared with control cells on day 4. As RAW264.7 cells fused together and form large multinucleated transparent cells from day 4, RANKL was proposed to inhibit RAW264.7 cell proliferation to stimulate the fusion of these cells and the subsequent formation of giant multinucleated transparent cells. Park *et al* (30) reported in a minireview that osteoclasts are bone-resorbing cells derived from hematopoietic precursors, and require macrophage-colony-stimulating factor and RANKL for their survival, proliferation, differentiation, and activation. As RANKL promotes cell proliferation, it enables partial recovery from the inhibition of cell proliferation by TGF-β1, as demonstrated on day 4. OC differentiation of RAW264.7 cells was significantly induced *in vitro* by treatment with TGF-β1 and RANKL. TRAP activity on day 4 in RAW264.7 cells treated with both TGF-β1 (2 ng/ml) and RANKL was the highest among the obtained data. When TGF-β1 signaling was inhibited by the addition of SB431542 to the medium during culture, OC differentiation was suppressed.

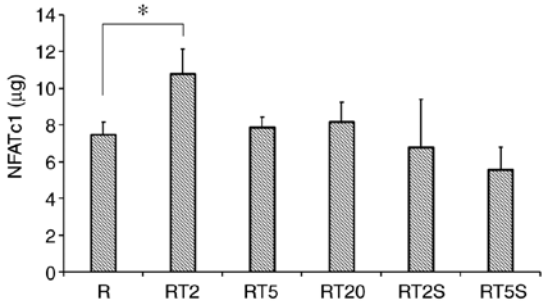

Figure 3. NFATc1 activation assay. RAW264.7 cells were plated at $2x10^6$ cells per 60-mm culture plate, pretreated with reagents at various concentrations for 24 h, and lysed. NFATc1 activation was quantified in cell lysates. The absorbance at 450 nm was determined using a microplate reader. *P<0.05. RANKL, receptor activator of NF-κB ligand; TGF-β1, transforming growth factor-β1; R, RANKL; RT2, RANKL + TGF-β1 (2 ng/ml); RT5, RANKL + TGF-β1 (5 ng/ml); RT20, RANKL + TGF-β1 (20 ng/ml); RT5S, RANKL + TGF-β1 (5 ng/ml) + SB431542. NFATc1, nuclear factor of activated T cells 1.

Furthermore, we reported a significant enhancement of OC differentiation in RANKL-treated RAW264.7 cells that were exposed to TGF-β1, through the quantitative measurement of NFATc1 activation. These findings suggest that TGF-β1 accelerates RANKL-induced OC differentiation, but does not act in a dose-dependent manner. TGF-β1 is known to induce apoptosis in mature osteoclasts; Houde *et al* (31) reported that this effect of TGF-β1 is mediated by upregulation of Bim. TGF-β1 can also exhibit the opposite effect, differentiation. Therefore, TGF-β1 may influence OC differentiation when

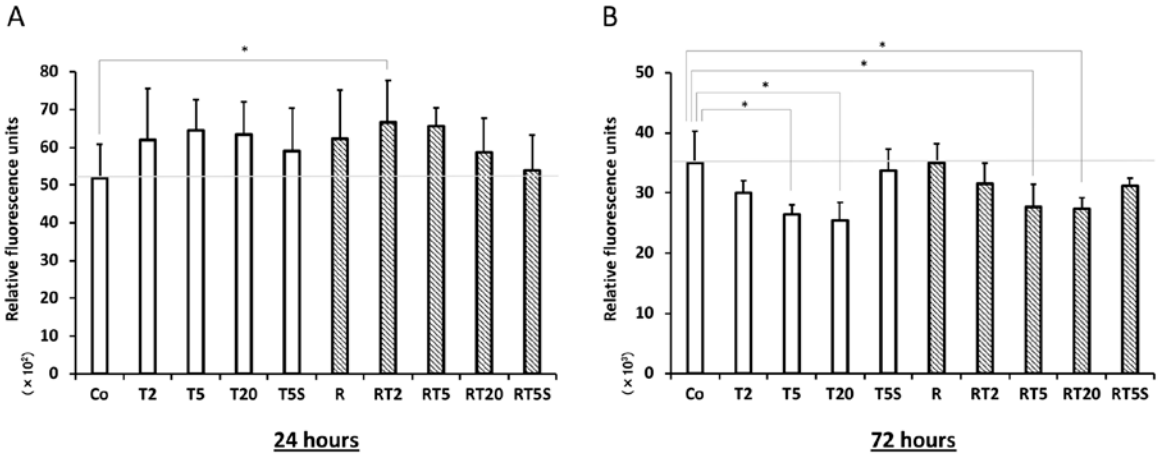

Figure 4. Cell migration assay. Following pretreatment with reagents at various concentrations for (A) 24 or (B) 72 h, cells suspended in serum-free medium were plated in the upper part of 96-well, 8-μm pore, cell-migration chambers. Medium containing 10% fetal bovine serum was placed in the lower wells as a chemotactic stimulus. After 14 h, migrated cells were detached from the membranes and quantified by measuring the fluorescence at 480/520 nm using a scanning fluorometer. *P<0.05 vs. Co cells. RANKL, receptor activator of NF-κB ligand; TGF-β1, transforming growth factor-β1; Co, control group; T2, TGF-β1 (2 ng/ml); T5, TGF-β1 (5 ng/ml); T20, TGF-β1 (20 ng/ml); T5S, TGF-β1 (5 ng/ml) + SB431542; R, RANKL; RT2, RANKL + TGF-β1 (2 ng/ml); RT5, RANKL + TGF-β1 (5 ng/ml); RT20, RANKL + TGF-β1 (20 ng/ml); RT5S, RANKL + TGF-β1 (5 ng/ml) + SB431542.

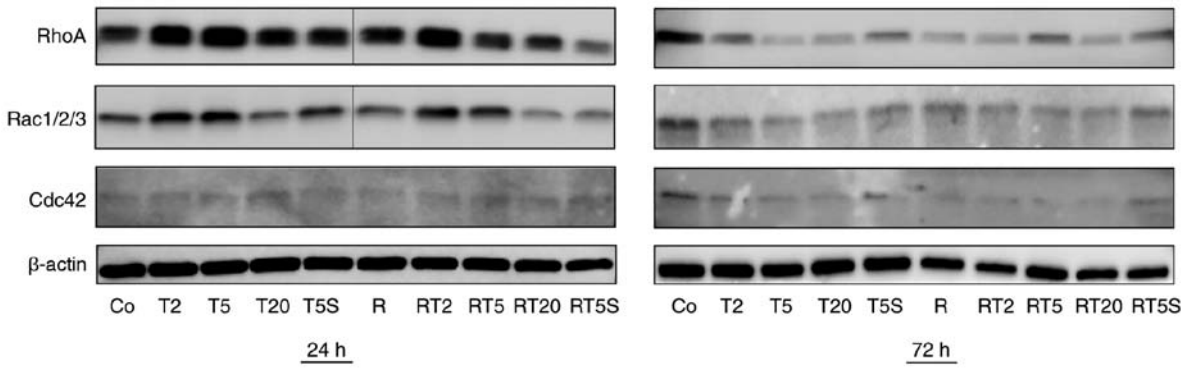

Figure 5. Effect of TGF-β1 and RANKL on expression of Rho GTPases. Expression levels of RhoA, Rac1/2/3, and Cdc42 proteins were evaluated by western blot analysis. RAW264.7 cells ($3x10^5$ cells) were pretreated with reagents at various concentrations for 24 or 72 h in 60-mm culture plates, and lysed. The cell lysates were subjected to western blot analysis. RhoA and Rac1/2/3 data (pretreatment with reagents for 24 h) were obtained from the same gel. Cdc42, cell division cycle 42; RANKL, receptor activator of NF-κB ligand; TGF-β1, transforming growth factor-β1; Co, control group; T2, TGF-β1 (2 ng/ml); T5, TGF-β1 (5 ng/ml); T20, TGF-β1 (20 ng/ml); T5S, TGF-β1 (5 ng/ml) + SB431542; R, RANKL; RT2, RANKL + TGF-β1 (2 ng/ml); RT5, RANKL + TGF-β1 (5 ng/ml); RT20, RANKL + TGF-β1 (20 ng/ml); RT5S, RANKL + TGF-β1 (5 ng/ml) + SB431542.

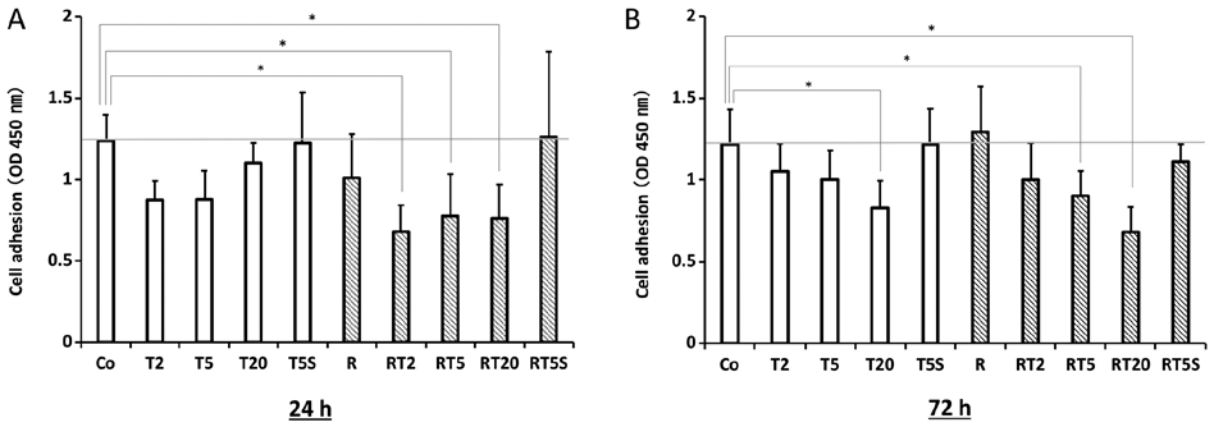

Figure 6. Cell adhesion assay. RAW264.7 cells ($6x10^5$ cells/well) were pretreated with reagents at various concentrations for (A) 24 or (B) 72 h in 24-well culture plates, re-plated in 96-well culture plates at $3x10^4$ cells/well, and incubated for 30 min. Non-attached cells were removed by three washes with PBS(-). Attached cells were assessed using the Cell Counting Kit-8 by measuring the absorbance at 450 nm with a microplate reader. *P<0.05. RANKL, receptor activator of NF-κB ligand; TGF-β1, transforming growth factor-β1; Co, control group; T2, TGF-β1 (2 ng/ml); T5, TGF-β1 (5 ng/ml); T20, TGF-β1 (20 ng/ml); T5S, TGF-β1 (5 ng/ml) + SB431542; R, RANKL; RT2, RANKL + TGF-β1 (2 ng/ml); RT5, RANKL + TGF-β1 (5 ng/ml); RT20, RANKL + TGF-β1 (20 ng/ml); RT5S, RANKL + TGF-β1 (5 ng/ml) + SB431542.

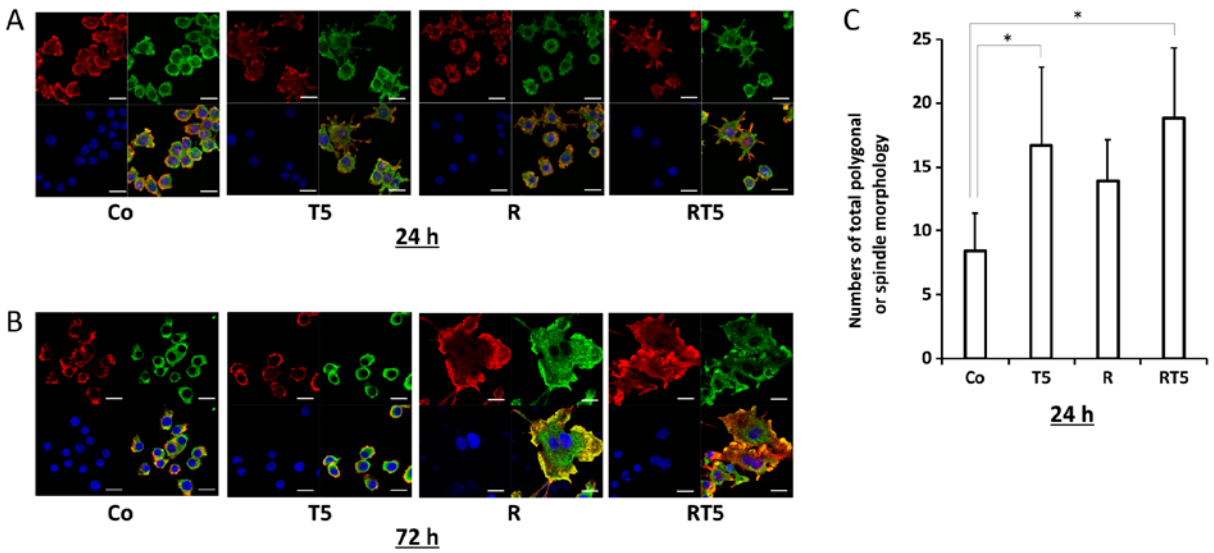

Figure 7. Immunohistochemical staining of actin and vinculin in cells. (A and B) Cells pretreated with reagents at various concentrations for (A) 24 or (B) 72 h were evaluated by immunohistochemical staining. Rhodamine phalloidin-labeled F-actin (red), Alexa Fluor 488-labeled vinculin (green), DAPI-stained nuclei (blue), and overlaid fluorescent images of immunostained cellular components (merged) are shown. Scale bars, 20 μm. (C) Treatment with TGF-β1 resulted in morphological changes from round to polygonal cellular morphology. The total numbers of cells with polygonal or spindle morphology were counted in five non-overlapping fields at magnification, x200. *P<0.05. RANKL, receptor activator of NF-κB ligand; TGF-β1, transforming growth factor-β1; Co, control group; T5, TGF-β1 (5 ng/ml); R, RANKL; RT5, RANKL + TGF-β1 (5 ng/ml).

present throughout differentiation; lower doses may promote differentiation, while higher doses inhibit differentiation.

We analyzed the migration ability of RAW264.7 cells using a Transwell chamber assay. Cell migration was increased by TGF-β1 treatment at 24 h, but was decreased at 72 h. We found that OCP migration was enhanced by TGF-β1 at the early stages of OC differentiation. At later stages, the differentiated cells exhibited suppressed migration. Kim *et al* (18) reported that treatment with TGF-β1 stimulated the migration of macrophages, while long-term exposure decreased their migration. In the present study, TGF-β1 activated RhoA at early stages, followed by inactivation, suggesting that inactivation of RhoA may be the cause of the reduced cell migration in response to TGF-β1 at later stages. RhoA and Rac expression levels were increased after 24 h of TGF-β1 treatment, but were notably reduced after 72 h of treatment. Cell migration was decreased at 72 h following treatment with both TGF-β1 and RANKL compared with control cells; TRAP activity had increased 72 h after treatment with TGF-β1 and RANKL. Therefore, changes in cell migration may be caused by OC differentiation. Prior to OC differentiation, the expression levels of RhoA and Rac proteins, which play key roles in regulating the cellular responses required for cell migration (24), may increase in response to TGF-β1, though these levels may be decreased at the onset of differentiation. In contrast, cell adhesion was significantly decreased after 24 h of TGF-β1 treatment, but increased after 72 h of treatment, except in cultures treated with high-dose TGF-β1 (T20 and RT20 cells). Therefore, adhesion ability was reduced by TGF-β1 prior to OC differentiation, but was recovered by the onset of differentiation.

RhoA activity has opposing effects, by decreasing cell migration through adhesion while increasing migration through cell-body contraction (24). High levels of Rho activity are correlated with the potential of attachment to the extracellular matrix, and also inhibit cell migration (24,32). However, cell-body contraction of moving cells was determined to be regulated by RhoA (33). In less adherent cells that lack focal adhesions, such as macrophages and neutrophils, RhoA does not affect adhesion, but does induce cell-body contraction (18). OCPs are monocyte/macrophage-lineage hematopoietic precursor cells (3). In macrophages, Allen *et al* (34) reported that Rho and Rac are required for the cell migration process, while Cdc42 is not essential.

We analyzed the morphological changes of RAW264.7 cells during differentiation by immunofluorescence staining. RAW264.7 cells treated with TGF-β1 for 24 h displayed morphological changes from round to polygonal cellular morphology. Furthermore, TGF-β1 induced protrusions that were positive for actin and vinculin. These changes in cells with a motile phenotype were confirmed by Transwell chamber assays. The protrusions were completely lost and the cell morphology changed from polygonal to round after TGF-β1 treatment for 72 h.

Murphy-Ullrich (35) reported that the cell adhesion process involved a transition from a state of weak adherence, characterized by the attachment of a round cell to a substrate, to a strongly-adherent state, which is characterized by focal adhesions and stress fibers. The strongly-adherent state is exhibited by differentiated and quiescent cells. There is an intermediate state, which is characterized by a spread cell shape and increased motility (35).

In conclusion, we proposed that when RAW264.7 cells receive signals for OC differentiation, including TGF-β1 and RANKL signals, TGF-β1 enhances the migration and alters the morphology of RAW264.7 cells by inducing the production of RhoA and Rac prior to differentiation. Subsequently, cells starting to differentiate show reduced migration through decreases in RhoA and Rac production, and the recovery of adhesion ability. Therefore, OCP migration may be modified

by differentiation in the bone microenvironment; further studies are required to determine the factors that control these phenomena. It is possible that clarification of the association between migration and differentiation of OCPs could improve understanding of bone-resorptive disorders mediated by OCs.

Acknowledgements

The authors would like to thank Professor Takashi Daimon (Department of Medical Informatics, Hyogo College of Medicine, Nishinomiya, Japan) for assistance with the statistical analyses and Shinobu Osawa M.Sc. for technical assistance. The authors also thank Dr Alison Sherwin, and Dr Ryan Chastain-Gross for editing a draft of this manuscript.

Funding

The present study was supported by JSPS KAKENHI (grant nos. 24593069, 15K11332, and 18K09825 to KT, 26861761 to MY, and 19K19182 to MU) and by a Grant-in-Aid for Researchers, Hyogo College of Medicine, 2016 (to KT).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MU, KT, and HK conceived and designed the study. MU, KT, MY, HM, JT, and YN performed the experiments. MU, KT, and KN analyzed the data. MU, KT, KN, and HK wrote the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Rodan GA and Martin TJ: Therapeutic approaches to bone diseases. Science 289: 1508-1514, 2000.
2. Ota K, Quint P, Weivoda MM, Ruan M, Pederson L, Westendorf JJ, Khosla S and Oursler MJ: Transforming growth factor beta 1 induces CXCL16 and leukemia inhibitory factor expression in osteoclasts to modulate migration of osteoblast progenitors. Bone 57: 68-75, 2013.
3. Boyce BF, Zuscik MJ and Xing L: Biology of bone and cartilage. In: Genetics of bone biology and skeletal disease. Thakker RV, Eisman J, Igarashi T and Whyte MP (eds.) Elsevier, London, UK, pp3-24, 2012.
4. Boyce BF: Advances in the regulation of osteoclasts and osteoclast functions. J Dent Res 92: 860-867, 2013.
5. Kikuta J, Iwai K, Saeki Y and Ishii M: S1P-targeted therapy for elderly rheumatoid arthritis patients with osteoporosis. Rheumatol Int 31: 967-969, 2011.
6. Ishii M and Kikuta J: Sphingosine-1-phosphate signaling controlling osteoclasts and bone homeostasis. Biochim Biophys Acta 1831: 223-227, 2013.
7. Yu X, Huang Y, Collin-Osdoby P and Osdoby P: Stromal cell-derived factor-1 (SDF-1) recruits osteoclast precursors by inducing chemotaxis, matrix metalloproteinase-9 (MMP-9) activity, and collagen transmigration. J Bone Miner Res 18: 1404-1418, 2003.
8. Wright LM, Maloney W, Yu X, Kindle L, Collin-Osdoby P and Osdoby P: Stromal cell-derived factor-1 binding to its chemokine receptor CXCR4 on precursor cells promotes the chemotactic recruitment, development and survival of human osteoclasts. Bone 36: 840-853, 2005.
9. Rahimi RA and Leof EB: TGF-beta Signaling: A tale of two responses. J Cell Biochem 102: 593-608, 2007.
10. Janssens K, ten Dijke P, Janssens S and Van Hul W: Transforming growth factor-beta1 to the bone. Endocr Rev 26: 743-774, 2005.
11. Tang SY and Alliston T: Regulation of postnatal bone homeostasis by TGFβ. Bonekey Rep 9: 255, 2013.
12. Ikushima H and Miyazono K: TGF-β signal transduction spreading to a wider field: A broad variety of mechanisms for context-dependent effects of TGF-β. Cell Tissue Res 347: 37-49, 2012.
13. Koseki T, Gao Y, Okahashi N, Murase Y, Tsujisawa T, Sato T, Yamato K and Nishirara T: Role of TGF-beta family in osteoclastogenesis induced by RANKL. Cell Signal 14: 31-36, 2002.
14. Shui C, Riggs BL and Khosla S: The immunosuppressant rapamycin, alone or with transforming growth factor-beta, enhances osteoclast differentiation of RAW264.7 monocyte-macrophage cells in the presence of RANK-ligand. Calcif Tissue Int 71: 437-446, 2002.
15. Chin SL, Johnson SA, Quinn J, Mirosavljevic D, Price JT, Dudley AC and Thomas DM: A role for alphaV integrinsubunitin TGF-beta-stimulated osteoclastogenesis. Biochem Biophys Res Commun 307: 1051-1058, 2003.
16. Fox SW, Evans KE and Lovibond AC: Transforming growth factor-beta enables NFATc1 expression during osteoclastogenesis. Biochem Biophys Res Commun 366: 123-128, 2008.
17. Yasui T, Kadono Y, Nakamura M, Oshima Y, Matsumoto T, Masuda H, Hirose J, Omata Y, Yasuda H, Imamura T, *et al*: Regulation of RANKL-induced osteoclastogenesis by TGF-β through molecular interaction between Smad3 and Traf6. J Bone Miner Res 26: 1447-1456, 2011.
18. Kim JS, Kim JG, Moon MY, Jeon CY, Won HY, Kim HJ, Jeon YJ, Seo JY, Kim JI, Kim J, *et al*: Transforming growth factor-1 regulates macrophage migration via RhoA. Blood 108: 1821-1829, 2006.
19. Takahashi N, Yamana H, Yoshiki S, Roodman GD, Mundy GR, Jones SJ, Boyde A and Suda T: Osteoclast-like cell formation and its regulation by osteotropic hormones in mouse bone marrow cultures. Endocrinology 122: 1373-1382, 1988.
20. Hashitani S, Urade M, Nishimura N, Maeda T, Takaoka K, Noguchi K and Sakurai K: Apoptosis induction and enhancement of cytotoxicity of anticancer drugs by celecoxib, a selective cyclooxygenase-2 inhibitor, in human head and neck carcinoma cell lines. Int J Oncol 23: 665-672, 2003.
21. Hiromoto T, Noguchi K, Yamamura M, Zushi Y, Segawa E, Takaoka K, Moridera K, Kishimoto H and Urade M: Up-regulation of neurophil gelatinase-associated lipocalin in oral squamous cell carcinoma: Relation to cell differentiation. Oncol Rep 26: 1415-1421, 2013.
22. Baksh D, Song L and Tuan RS: Adult mesenchymal stem cells: Characterization, differentiation, and application in cell and gene therapy. J Cell Mol Med 8: 301-316, 2004.
23. Simonet WS, Lacey DL, Dunstan CR, Kelley M, Chang MS, Lüthy R, Nguyen HQ, Wooden S, Bennett L, Boone T, *et al*: Osteoprotegerin: A novel secreted protein involved in the regulation of bone density. Cell 89: 309-319, 1997.
24. Ridley AJ: Rho GTPases and cell migration. J Cell Sci 114: 2713-2722, 2001.
25. Boyle WJ, Simonet WS and Lacey DL: Osteoclast differentiation and activation. Nature 423: 337-342, 2003.
26. Blobe GC, Schiemann WP and Lodish HF: Role of transforming growth factor-beta in human disease. N Engl J Med 342: 1350-1358, 2000.
27. Massague J: TGFbeta in cancer. Cell 134: 215-230, 2008.
28. Tian M, Neil JR and Schiemann WP: Transforming growth factor-β and the hallmarks of cancer. Cell Signal 23: 951-962, 2011.

29. Morrison CD, Parvani JG and Schiemann WP: The relevance of the TGF-β paradox to EMT-MET programs. Cancer Lett 341: 30-40, 2013.
30. Park JH, Lee NK and Lee SY: Current understanding of RANK signaling in osteoclast differentiation and maturation. Mol Cells 40: 706-713, 2017.
31. Houde N, Chamoux E, Bisson M and Roux S: Transforming growth factor-beta1 (TGF-beta1) induces human osteoclast apoptosis by up-regulating Bim. J Biol Chem 284: 23397-23404, 2009
32. Cox EA, Sastry SK and Huttenlocher A: Integrin-mediated adhesion regulates cell polarity and membrane protrusion through the Rho family of GTPases. Mol Biol Cell 12: 265-277, 2001.
33. Mitchison TJ and Cramer LP: Actin-based cell motility and cell locomotion. Cell 84: 371-379, 1996.
34. Allen WE, Zicha D, Ridley AJ and Jones GE: A role for Cdc42 in macrophage chemotaxis. J Cell Biol 141: 1147-1157, 1998.
35. Murphy-Ulirich JE: The de-adhesive activity of matricellular proteins: Is intermediate cell adhesion an adaptive state? J Clin Invest 107: 785-790, 2001.