Glycative Stress Research

Online edition : ISSN 2188-3610
Print edition : ISSN 2188-3602
Received : January 14, 2017
Accepted : February 10, 2017
Published online : March 31, 2017

Original article

RANKL regulates RAW264.7 cell osteoclastogenesis in a manner independent of M-CSF, dependent on FBS, media content and cell density.

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Abstract

***Purpose*:** The differentiation and activation of osteoclast cells are of great importance in bone metabolism as osteoclasts resorb bone and activate osteoblast cells. The receptor activator of nuclear factor kappa-B ligand (RANKL) and Macrophage Colony-Stimulating Factor (M-CSF) are the main ligands known to induce osteoclastic differentiation (osteoclastogenesis) pathways and survival related pathways, respectively. We examined whether both are require for osteoclastogenesis and survival or not.

***Methods*:** We performed Tartrate-resistant acid phosphatase (TRAP) staining and activity assay to check for multinucleated osteoclast cell formation in RAW264.7 cells. We used RANKL with and without M-CSF to induce osteoclastogenesis. Osteoclastogenic messenger RNA (mRNA) expression was measured by Real Time-Polymerase Chain Reaction (RT-PCR). Cell viability was checked using Cell Counting Kit-8 (WST-8). We also checked the effect of media content, fetal bovine serum (FBS), cell density on osteoclastogenesis. Finally, we checked for effective pathway activation using western blot.

***Results*:** We found that RANKL alone could better stimulate TRAP positive multinucleated osteoclast cell formation than in the presence of M-CSF. RANKL alone activated osteoclastogenic NF-κB, ERK, p-38 MAPK, NFATc1, and anti-apoptotic Akt in RAW264.7 cells and increased both maturation as well as activation markers such as TRAP activity, Cathepsin-K (CTSK), V-type proton ATPase (Atp6v), TRAP, Matrix metallopeptidase 9 (MMP-9) mRNA expression. FBS, media content and cell density also played crucial roles in osteoclastogenesis.

***Conclusion*:** RANKL-induced osteoclastogenesis was not dependent on M-CSF, but was instead dependent on FBS, cell density, media content. This study shows that any change among essential components can lead to inappropriate *in vitro* osteoclastogenesis in RAW264.7 cells.

***KEY WORDS*:** Osteoclastogenesis, RAW264.7 cells, RANKL, M-CSF, FBS.

Introduction

Bone remodeling (or bone homeostasis) is a lifelong process that serves to regulate bone structure to meet changing mechanical requirements and to repair micro damages or fractures in the bone matrix by maintaining plasma calcium homeostasis. Bone remodeling begins with the removal of mineralized bone through osteoclasts (a process called bone resorption) followed by the activation of bone forming osteoblast cells to recover the bone matrix that will subsequently become mineralized (ossification or new bone formation). The osteoclastic differentiation and activation are triggered mainly by osteoblast cells and vice versa. The major biological regulators of bone remodeling include parathyroid hormone (PTH) and calcitriol as well as other hormones including growth hormones, glucocorticoids, thyroid hormones, sex hormones, insulin-like growth factors (IGFs), prostaglandins, bone morphogenetic proteins (BMP), tumor growth factor-beta (TGF-β), and cytokines e.g. M-CSF, RANKL, VEGF, IL-6 [1,2]. Any imbalance between bone resorption and bone formation, can lead to many metabolic bone diseases, such as osteoporosis, rickets, osteomalacia (softening of the bones), osteogenesis imperfecta (a group of genetic disorders that mainly affect the bones that break easily), marble bone disease (osteopetrosis) and Paget's disease of the bone [3].

Osteoclast formation and functions are regulated by cytokines, primarily the receptor activator of NF-κB ligand (RANKL) and macrophage-colony-stimulating factor (M-CSF) via the activating of numerous signaling pathways, which

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are potential therapeutic targets. It is important to note that osteoclastic cells also regulate osteoblastic bone formation, both positively and negatively [4]. Aside from RANKL and M-CSF, there are several inflammatory cytokines, *e.g.* TNFα, IL-1 and IL-6, that are secreted by the macrophage under certain conditions which can also lead to osteoclastogenesis, leading to unnecessary bone resorption and thus causing inflammatory bone diseases like osteoarthritis and rheumatoid arthritis [3,5]. Therefore, not only osteoclastogenesis, but also its mediators are important as well.

M-CSF is reported to enhance mature osteoclast (OC) survival, motility and resorbing activity, possibly mediated by ERK upstream of the c-fos [6]. However, the role of M-CSF in the regulation of osteoclast differentiation remains unclear. To address whether RANKL mediated osteoclastogenesis is dependent on M-CSF or not, we analyzed *in vitro* cultures of RAW264.7 mouse monocyte/macrophage to differentiate into OCs in the presence and absence of different concentrations of M-CSF and RANKL. We found that RANKL alone induced multinucleated giant osteoclast cell formation and TRAP activity to a greater degree than the same dose of RANKL along with M-CSF. FBS is also required in in vitro cultures of macrophage cells for osteoclastogenesis. Therefore, RANKL-induced osteoclastogenic differentiation could be partially or very dependent on FBS and, if so, then the tested samples effect would be unclear as it could inhibit either RANKL or the responsible component of FBS. Therefore, we checked the effect of FBS along with the media content and initial cell number on RANKL-induced osteoclastogenesis in RAW264.7 cells. Our results demonstrated that RANKL acts as the prime osteoclastogenesis regulator independent of M-CSF. However, RANKL did exhibit dependence on FBS, media content and cell number. This study suggests that RANKL and its related downstream pathways as potential molecular targets for bone related diseases.

Materials and methods

Cell culture and reagents

The murine monocyte/macrophage RAW264.7 (ATCC® TIB-71™) cell line was purchased from American Type Culture Collection (ATCC; Manassas, VA). Cells were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St.Louis, MO) supplemented with 10% fetal bovine serum (FBS) (Nichirei Biosciences, Tokyo, Japan), penicillin 100 units/mL, streptomycin 100 μg/mL and amphotericin B 25 μg/mL (Gibco, El Paso, TX) at 37 °C under the condition of 5% CO_2 [7]. Passages 3 through 6 were used for all experiments.

In vitro osteoclastogenesis

RAW264.7 cells were seeded in multi-well plates and incubated for 24 h. Then media were exchanged with αMEM (Gibco) or DMEM, as indicated in the figures with different percentages of FBS, 0 ~ 100 ng/mL recombinant mouse RANK Ligand (rmRANKL) and 0 ~ 50 ng/mL M-CSF (R&D Systems, Minneapolis, MN) with antibiotics. After 3 days, the medium was renewed. After 5 days of cultures, cells were observed by the different assays as mentioned.

TRAP staining

Cells were fixed in a 10% formalin neutral buffer solution and stained using a TRAP staining kit (Cosmo bio co., LTD., Tokyo, Japan) according to the manufacturer's instruction. TRAP is a widely-used marker of osteoclast maturation and function [8]. Multinucleated cells having ≥ 4 nuclei were counted under a light microscope as an osteoclast cell.

TRAP activity

Cells were fixed using a cell fixation buffer (acetone : ethanol = 1 : 1) and then used to measure TRAP activity with a TRAP solution kit (Oriental Yeast Co., Tokyo, Japan) per the manufacturer's instruction. Colorimetric absorbance were taken at 405 nm using a Varioscan® Flash (Thermo scientific, Waltham, MA) microplate reader.

Evaluation of cell viability

Cell viability was evaluated using cell counting kit-8 (Dojindo, Kumamoto, Japan). RAW264.7 cells were seeded on 96-well plates at the mentioned density and were treated as regular experiments described in the results section. After 5 days, a 10% volume of WST-8 solution was added to the culture medium and the cells were incubated for 1 h. Absorbance at 450 nm was then measured as previously described using a Varioscan® Flash microplate reader [9].

Isolation of total RNA and RT-PCR

RAW264.7 cells were seeded into 24-well plates at a density of 4×10^4 cells/well and incubated for 24 h. Then, media was exchanged and incubated at the indicated times. Total RNA was then extracted using Isogen II reagent (Nippon Gene, Tokyo, Japan) per the manufacturer's protocol. Five-hundred ng total RNA was reverse-transcribed with PrimeScript™ RT Master Mix (Takara Bio Inc., Shiga, Japan) using Applied Biosystems 2720 Thermal cycler.

RT-PCR was performed with a Thunderbird™ SYBR qPCR mix (Toyobo Co., Osaka, Japan) per the manufacturer's protocol [10] with gene-specific primers (Invitrogen Tokyo, Japan). The primers were used are as follows: NFATc1, 5′-GGA GCG GAG AAA CTT TGC G-3′ (forward), 5′-GTG ACA CTA GGG GAC ACA TAA CT-3′ (reverse); MMP-9, 5′-CTG GAC AGC CAG ACA CTA AAG-3′ (forward), 5′-CTC GCG GCA AGT CTT CAG AG-3′ (reverse); CTSK, 5′-GAA GAA GAC TCA CCA GAA GCA G-3′(forward), 5′-TCC AGG TTA TGG GCA GAG ATT-3′ (reverse); glyceraldehyde dehydrogenase (GAPDH), 5′-AGG TCG GTG TGA ACG GAT TTG-3′(forward), 5′-TGT AGA CCA TGT AGT TGA GGT CA-3′(reverse) [11]; TRAP, 5′-GCG ACC ATT GTT AGC CAC ATA CG-3′ (forward), 5′-CGT TGA TGT CGC ACA GAG GGA T-3′(reverse); Atp6v0d2, 5′-ACG GTG ATG TCA CAG CAG ACG T-3′ (forward), 5′-CCT CTG GAT AGA GCC TGC CGC A-3′ (reverse) [8]. GAPDH was used as an internal control.

Protein Extraction and Western Blot Analysis

RAW264.7 cells were seeded in 6-well plate at a density of 2×10^5 cells/well and incubated for 24 h. They were then treated with αMEM supplemented with 10%

FBS, antibiotics and 100 ng/mL RANKL for up to 60 min. The cells were then lysed with RIPA buffer containing 50 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, 1% Triton ×-100 with complete protease inhibitor (Wako, Osaka, Japan) and phosphatase inhibitor (Roche Applied Science, Penzberg, Germany). Equal amounts of cell lysates (5 μg) were electrophoresed by SDS-PAGE (10% polyacrylamide), followed by the transferring of proteins on polyvinylidene difluoride (PVDF) membrane which were blocked with a 5% skim milk solution in TBS-T. Then membranes were immunoblotted with each primary antibody. The antibody against GAPDH was from Abcam (Cambridge, MA), while remaining antibodies were from Cell Signaling Technology (Danvers, MA). The antigen–antibody complexes were visualized with the appropriate secondary antibodies (Santa Cruz Biotechnology, Dallas, TX) and chemiluminescence horseradish peroxidase (HRP) substrate along with detection system as recommended by the manufacturer. The results illustrated in each figure are representative of three independent experiments. Image J was used to measure the optical density of protein bands.

Statistical analysis

Data were expressed as mean ± the standard error of the mean (SEM). All statistical analyses were performed using the Tukey-Kramer test for intergroup comparison in each of the experiments. Results were considered significant at a significance level of 5%.

Results

Effect of RANKL and M-CSF on multinucleated TRAP positive osteoclast cell formation

RAW264.7 cells were treated with 0 ~ 100 ng/mL RANKL with and without 10 ng/mL M-CSF and then incubated for 5 days. After which, TRAP positive multinucleated osteoclast cells (≥ 4 nuclei and ≥ 10 nuclei) were counted under a light microscope. RANKL significantly increased multinucleated osteoclast (≥ 4 nuclei) cell formation up to 50 ng/mL RANKL, but the amount for 100 ng/mL did not differ from 50 ng/mL. However, giant multinucleated osteoclast (≥ 10 nuclei) formation was increased by RANKL dose dependently (***Fig. 1-A~C***). Osteoclastogenesis was unchanged in the presence of 10 ng/mL M-CSF with differing concentrations of RANKL (***Fig. 1-B, C***).

To further investigate on whether M-CSF has some effect on RANKL induced osteoclastogenesis, we used RANKL 100 ng/mL along with 0 ~ 50 ng/mL M-CSF. RANKL 100 ng/mL in the presence of M-CSF slightly increased small multinucleated osteoclast (≥ 4 nuclei) cell formation (***Fig. 1-D, E***), but significantly decreased large multinucleated osteoclast (≥ 10 nuclei) cell formation (***Fig. 1-D, F***). Showing that M-CSF can either inhibit small osteoclast cell's fusion, or stimulate cell multiplication rather than differentiate them. To confirm the effect of M-CSF on RANKL-induced osteoclastogenesis, we also checked TRAP enzymatic activity. TRAP activity was significantly increased dose dependently by RANKL (***Fig. 2-A***), while cell proliferation fell, suggesting a shift to differentiation (***Fig. 2-B***). , M-CSF alone did not increase TRAP activity. RANKL 100 ng/mL along with M-CSF did not increase TRAP activity, moreover 50 ng/mL M-CSF decreased TRAP activity 20% compared to RANKL alone (***Fig. 2-C***) shows that RANKL can regulate osteoclastogenesis independent of M-CSF. Cell proliferation was not altered by M-CSF in the presence or absence of RANKL (***Fig. 2-D***), showing that RAW264.7 cells can grow and differentiate without M-CSF. RANKL treated cells compared to no-RANKL groups shows that cell multiplication falls due to differentiation by RANKL (***Fig. 2-D***).

FBS plays crucial role in RANKL-induced osteoclastogenesis

FBS is a widely used supplement for culture media, therefore, it is also important to know if there any effects of FBS on RANKL-induced osteoclastogenesis. To address this, RAW264.7 cells were treated with αMEM containing 0 ~ 10% FBS, with and without 100 ng/mL RANKL, for 5 days. TRAP activity was not significantly changed by FBS alone in the absence of RANKL (***Fig. 3-A***). RANKL 100 ng/mL failed to induce osteoclastogenic TRAP activity in the absence of FBS (***Fig. 3-A***). However, in the presence of 2.5% or 10% FBS, osteoclastic TRAP activity was significantly induced. Showing that FBS plays a vital role in RANKL-induced osteoclastogenesis. Cell proliferation was also significantly increased by FBS regardless of RANKL concentration. Without FBS, cells grown showed almost 80% mitochondrial activity (WST-8 assay) (***Fig. 3-B***), but osteoclastogenesis could not be induced.

Effect of initial cell number on RANKL-induced osteoclastogenesis

RAW264.7 cells underwent multiplication first upon RANKL stimulation. The new cells then fused together and formed small-multinucleated transparent osteoclast cells on day 2 (***Fig. 4-A***). After this, the small osteoclast cells fused together and formed giant multinucleated (≥ 10 nuclei) transparent osteoclast cells (day 3, 4). On day 5, these giant osteoclast cells matured and lost their transparency, possibly due to alternate protein synthesis. Without RANKL, cells simply multiplied without fusing. As we found, RANKL treatment induced multiplication first, so the low cell number may be a possible cause. Therefore, we checked whether a higher initial cell number could induce osteoclastogenesis more or not next. Cells were seeded at 1×10^4 cells/well and 2×10^4 cells/well in 96-well plate. TRAP activity was significantly reduced by the greater cell number (***Fig. 4-B***) and cell proliferation was slightly increased (***Fig. 4-C***) showing that proper osteoclastogenesis requires a certain initial cell density and RANKL-treated cells are programmed to multiply first, then new cells fuse together.

RANKL induced Osteoclastogenic mRNA expression independent of M-CSF

Since RANKL 100 ng/mL with 10% FBS containing αMEM significantly induced osteoclastogenic TRAP activity and multinucleated TRAP positive cell (≥ 10 nuclei) formation in the absence of M-CSF, we checked whether RANKL alone could induce other osteoclastogenic gene expression using RT-PCR. Cells were seeded in 24-well

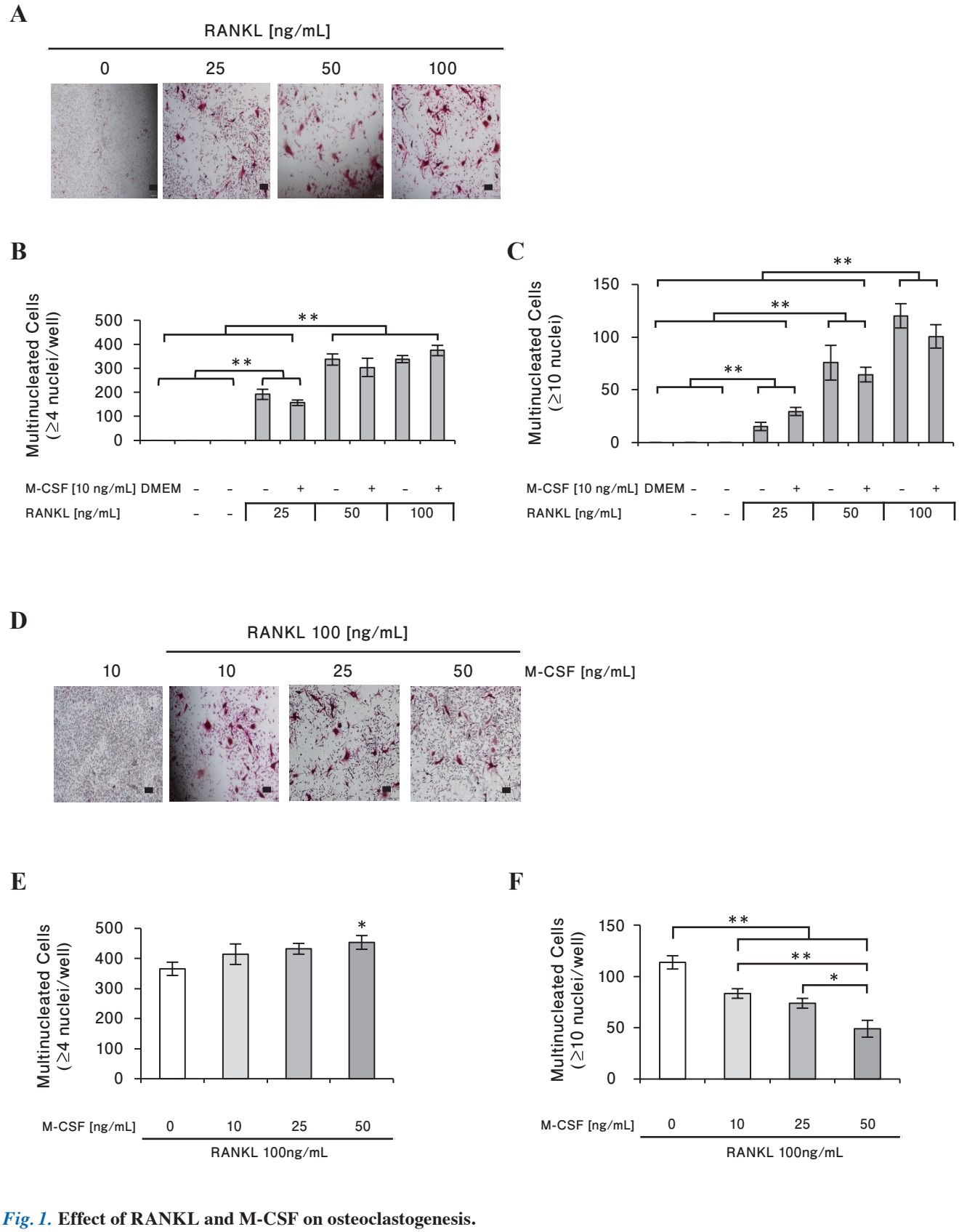

Fig. 1. **Effect of RANKL and M-CSF on osteoclastogenesis.**

RAW264.7 cells were treated with αMEM containing 10% FBS with the mentioned concentrations of RANKL with or without differing doses of M-CSF for 5 days. Microscopic observation of TRAP stained cells **A, D)** 100 x magnification, the bar in each figure represents 100 μm. **B, E)** Multinucleated cells having ≥ 4 nuclei; **C, F)** ≥ 10 nuclei. All data are shown as means ± SEM; n = 6, Tukey-Kramer test, * $p < 0.05$, ** $p < 0.01$.

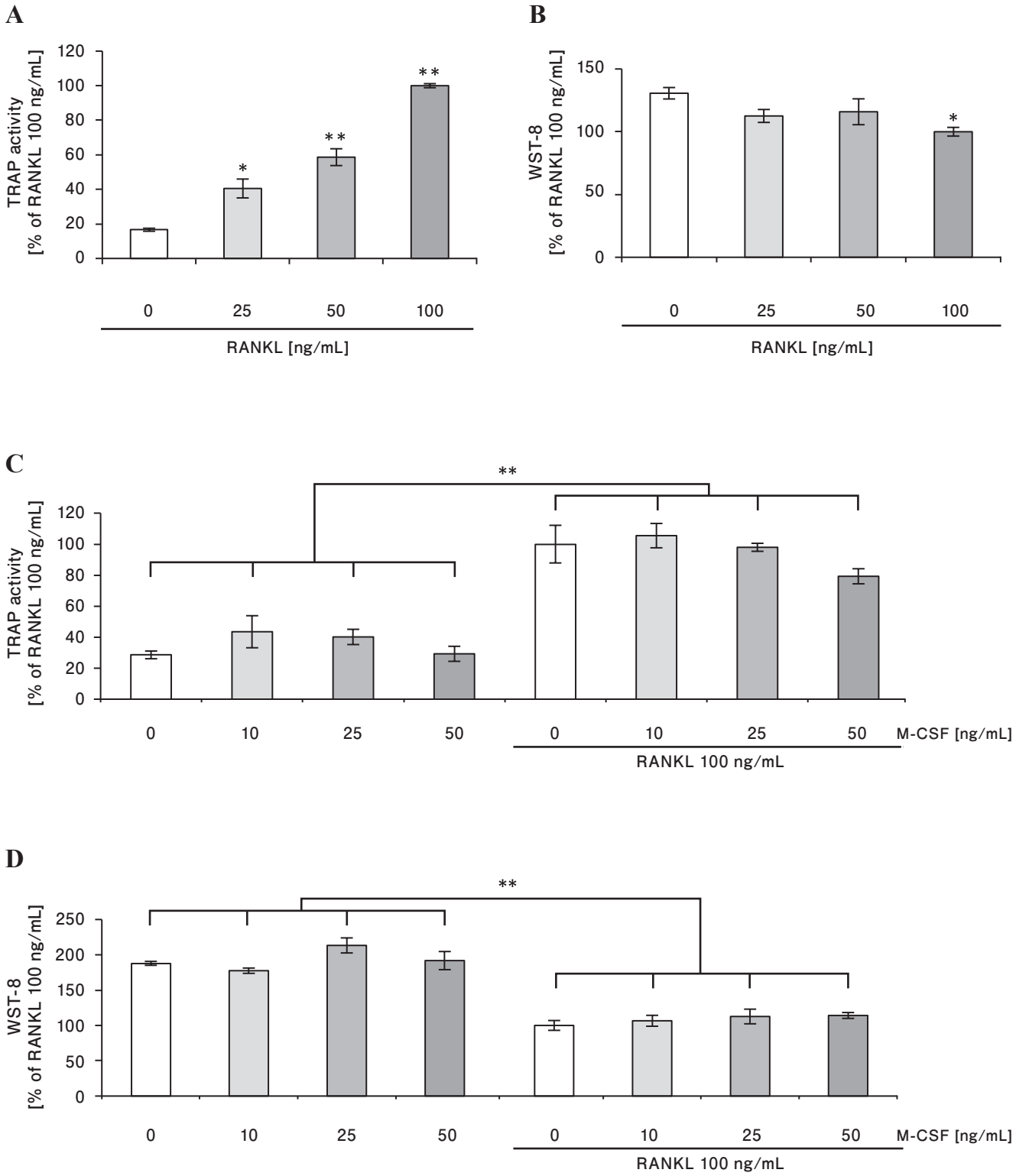

Fig. 2. **Effect of RANKL and M-CSF on TRAP activity and cell viability.**

A) Effect of RANKL (0 to 100 ng/mL) on TRAP activity. **B)** WST-8 assay at day 5. **C)** Effect of M-CSF (0 to 50 ng/mL) with or without 100 ng/mL RANKL on TRAP activity. **D)** WST-8 assay. Data are shown as mean ± SEM, n = 3.

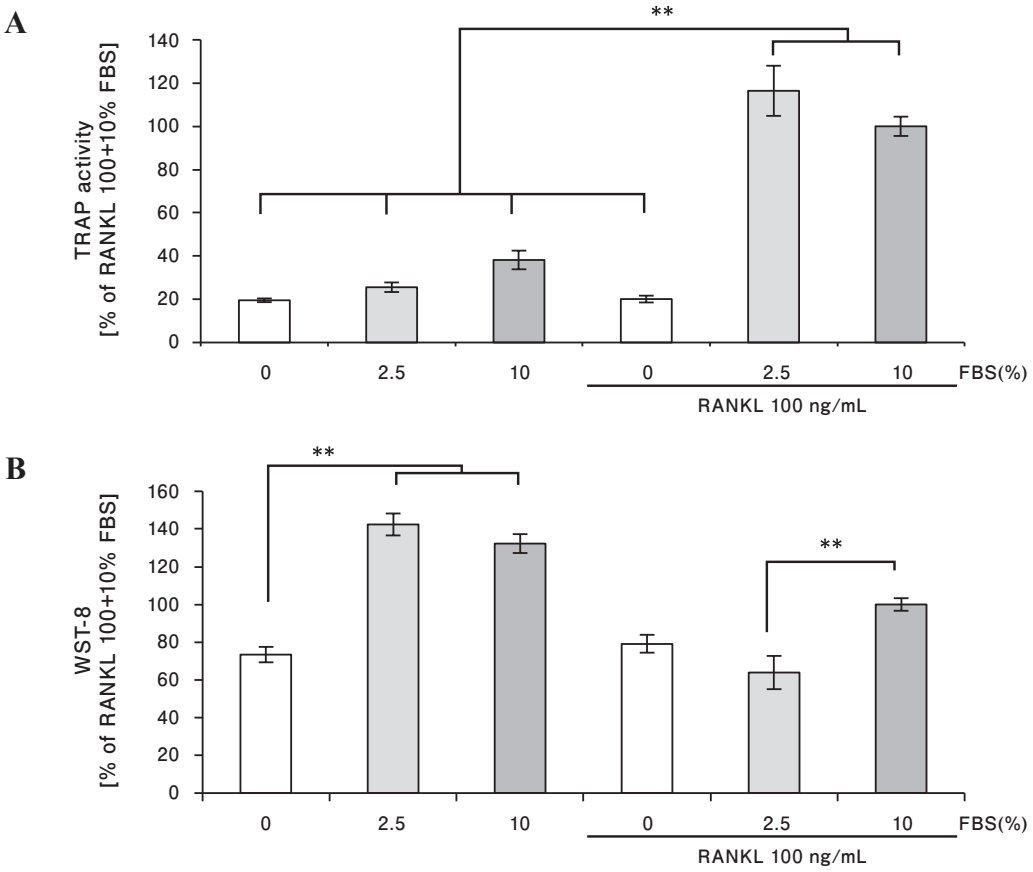

Fig. 3. **Effect of FBS on TRAP activity and cell viability.**

A) Effect of FBS on TRAP activity. **B)** WST-8 assay at day 5. All data are as mean ± SEM, n = 4, Tukey-Kramer test, * $p < 0.05$, ** $p < 0.01$.

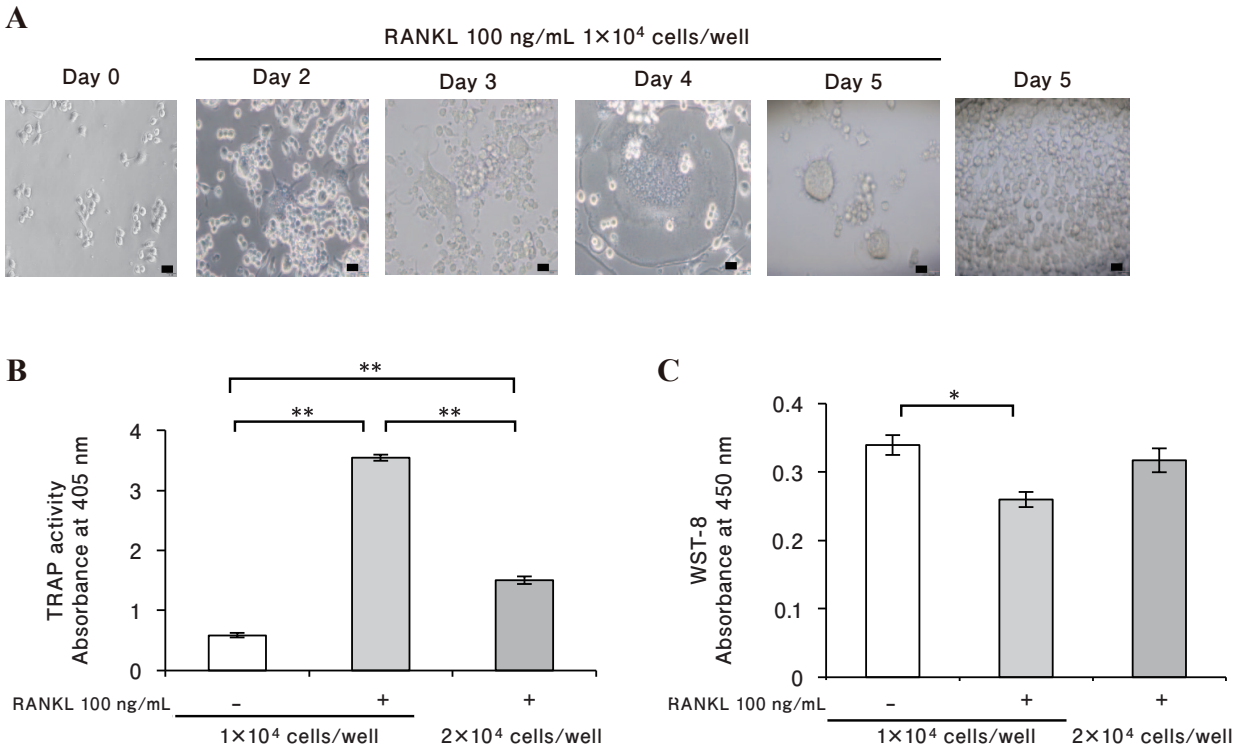

Fig. 4. **Effect of RAW264.7 cell number on osteoclastogenesis.**

A) RAW264.7 cells treated with RANKL 100 ng/mL was photographed using light microscope each day. The bar in each figure represents 20 μm. **B)** TRAP activity. **C)** WST-8 assay at different cell density at day 5. All data are as mean ± SEM, n = 3, Tukey-Kramer test, * $p < 0.05$, ** $p < 0.01$.

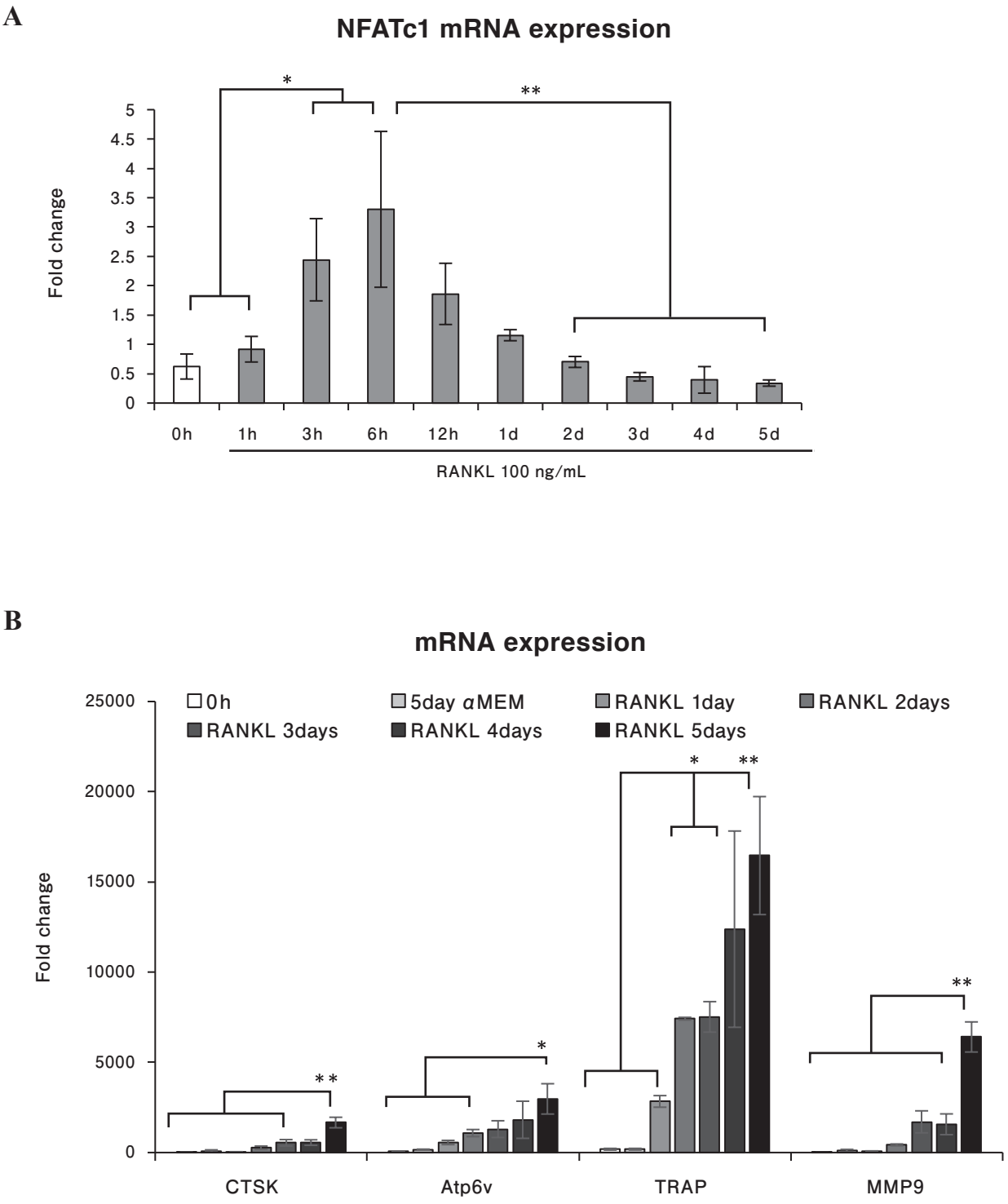

Fig. 5. **RANKL induced osteoclastogenic mRNA expression without M-CSF.**

RAW264.7 cells were plated in 24-well plates at 4 × 10^4 cells/well, and the next day cells were treated with αMEM containing 10% FBS without or with 100 ng/mL RANKL. After 3 days, the media was renewed. The treated cells were collected after the indicated times and mRNA was extracted. These were then used for cDNA synthesis and checked by RT-PCR. Relative mRNA expression, data was normalized by GAPDH and showed as fold change. **A)** NFATc1, **B)** CTSK, Atp6v, TRAP, MMP9. All data are shown as mean ± SEM, n = 3, Tukey-Kramer test, * $p < 0.05$, ** $p < 0.01$.

plates at a density of 4×10^4 cells/well and incubated for 24 h. The cells were then treated with αMEM supplemented with 10% FBS and antibiotics with and without 100 ng/mL RANKL. mRNA expression of the nuclear factor of activated T cells (NFATc1), the master transcription factor for osteoclast differentiation, was significantly induced by RANKL 100 ng/mL, peaking after 6 h of treatment before declining (***Fig. 5-A***). Other osteoclast maturation and activation marker genes *i.e.* CTSK, Atp6v, TRAP, MMP-9 mRNA expression was also significantly induced by RANKL and peaked after 5 days of treatment (***Fig. 5-B***). This data shows that RANKL alone can induce proper osteoclastogenesis in the absence of M-CSF.

Effect of media content on RANKL induced osteoclastogenesis

RAW264.7 cells were treated with RANKL 100 ng/mL in αMEM or DMEM to check whether the medium itself has effect on osteoclastogenesis or not. TRAP activity was not changed by media content (***Fig. 6-A***), but TRAP, MMP9, CTSK, Atp6v mRNA expression was significantly reduced in DMEM after 5 days of treatment (***Fig. 6-C***). Cell proliferation was significantly increased in DMEM compared to αMEM (***Fig. 6-B***). A possible cause being the downregulation of osteoclastogenic mRNA expression as we normalized the data using GAPDH. DMEM may induce cell proliferation along with differentiation in the presence of RANKL.

RANKL activated both osteoclastic and survival related pathways

To check the effect of RANKL on osteoclastogenic and survival related pathway activation, RAW264.7 cells were seeded in a 6-well plate at a density of 2×10^5 cells/well and incubated for 24 h. Followed by treatment with αMEM supplemented with 10% FBS, antibiotics and 100 ng/mL RANKL for up to 60 min. The cell lysates were then used for western blot against mentioned proteins. RANKL treatment activated phosphorylation of osteoclastogenic NF-κB, ERK, p38 MAPK and survival related Akt (Thr308) pathways, but not pAkt (Ser473) (***Fig. 7-A, B***) in the absence of M-CSF showing that RANKL itself can trigger both the survival and differentiation of RAW264.7 cells.

Discussion

In this study, we analyzed *in vitro* cultures of RAW264.7 mouse monocyte/macrophage differentiation into osteoclasts in the presence and absence of different concentrations of M-CSF and RANKL. We also checked the effect of FBS, media content and initial cell number on osteoclastogenesis in RAW264.7 cells. We observed that RANKL could regulate osteoclastogenesis and osteoclast survival independent of M-CSF. RANKL alone stimulated the expression of all major osteoclast maturation and activation markers by activating NF-κB, ERK, p-38 MAPK, NFATc1, and anti-apoptotic Akt. We also found that the initial cell number of RAW264.7 plays crucial role in osteoclastogenesis. Besides these, FBS plays key role in in vitro osteoclastogenesis as well as RAW264.7 cell proliferation.

In the previous report, osteoclasts were completely absent in CSF-1 mutant mice with osteopetrosis, demonstrating the critical role of the macrophage-colony stimulating factor (M-CSF) in osteoclast differentiation from hematopoietic precursors [6,12-14]. Osteoclasts are differentiated cells of monocyte/macrophage lineage, originating from hematopoietic precursors. Thus, mutation in the CSF-1 gene may block either the differentiation of monocyte/macrophage from hematopoietic stem cells or directly block the osteoclastogenic differentiation of monocyte/macrophage. In our study, we found that RAW264.7 cells' osteoclastgenesis is regulated solely by RANKL alone. M-CSF significantly inhibited large osteoclast formation, increased small osteoclast formation and decreased overall TRAP activity by 20% (***Fig. 2-C***). This shows that RANKL-induced osteoclastogenesis and osteoclast activation are not dependent on M-CSF in RAW 264.7 cells. RANKL functions as a key factor for osteoclast differentiation, M-CSF did not induce osteoclastogenesis in either the absence or presence of RANKL. The reason for this could be either that M-CSF inhibits small osteoclast cell's fusion or that it stimulates cell proliferation rather than differentiation. However, M-CSF plays an important role in resorption by mature human osteoclast. M-CSF 10 ~ 25 ng/mL effectively augments RANKL-induced resorption, not by enhancing survival, but instead due to an increased activation of resorption in OCs by potentiating RANKL-induced c-fos activation and extracellular signal-regulated kinase (ERK) 1/2 phosphorylation in mature OCs [6].

As RAW264.7 cell *in vitro* culture requires FBS, we checked whether FBS has any effect on osteoclastogenesis or not. FBS alone (10%) induces TRAP activity twice as much than with no FBS. RANKL 100 ng/mL was unsuccessful in inducing osteoclastogenesis in the absence of FBS (***Fig. 3-A***). FBS 2.5% ~ 10% significantly increased TRAP activity as well as cell proliferation, showing that FBS is essential for both RAW264.7 cell proliferation and differentiation. In a study by Wang *et al*., it was presented that FBS promoted osteoclastogenesis in suitable concentrations by regulating the migration of osteoclast precursors and expressions of TRAP and CTSK [15]. FBS contains most of the factors required for cell attachment, growth, proliferation and differentiation and is thus used as an almost universal cell culture supplement for most types of human and animal cells. Although FBS has been in use for over 50 years, it remain uncharacterized. Recent proteomic and metabolomic studies revealed approx. 1,800 proteins and more than 4,000 metabolites present in the serum [16]. As RANKL cannot induce osteoclastogenesis in the absence of FBS, it shows that RANKL-induced osteoclastogenesis is very dependent on FBS. However, it is unclear which component(s) are playing a key role due to its very complex nature. As FBS is required for *in vitro* experiments, the possibility for false positive results in cases of RANKL inhibition studies is present if the samples inhibit the responsible component(s) of FBS; osteoclastogenesis be reduced without inhibiting RANKL-induced pathways.

RANKL treatment induced RAW264.7 cells to multiply first and new cells then fused together and formed giant osteoclast cells (***Fig. 4-A***). We used a high cell number to

check whether RANKL-treated cells can fuse together and increase TRAP activity or not. An increased cell number did not increase osteoclastogenesis (***Fig. 4-B***), showing that fusion is happening between RANKL-induced new daughter cells which are proliferated to differentiate, not by the fusion between RANKL-induced parental cells. Our results suggest that osteoclastic fusion requires new daughter cells originated from a RANKL-induced parental cell and that they are already programmed to fuse.

The nuclear factor of activated T cells (NFATc1) is known as the master transcription factor for osteoclast differentiation. This factor was induced by RANKL 100 ng/mL in the absence of M-CSF and reached its peak after 6 h of treatment before declining in RAW264.7 cells (***Fig. 5-A***). Other osteoclast maturation and activation marker gene, *i.e.* CTSK, Atp6v, TRAP, and MMP-9 mRNA, expression was also significantly induced by RANKL 100 ng/mL and reached their peaks after 5 days of treatment (***Fig. 5-B***). This data shows that RANKL alone can induce proper osteoclastogenesis by inducing osteoclastic gene expression in the absence of M-CSF.

Next, we considered the difference in the culture medium content. DMEM contains approximately four times as much of the vitamins and amino acids compared to the αMEM and two to four times as much glucose [17]. Osteoclastogenesis is coupled by several other cells such as MSC, osteoblast, osteocytes etc. Therefore, the co-culture of the macrophage with them is also important in bone research, and many times it requires DMEM. RAW264.7 cells were treated with RANKL 100 ng/mL in αMEM and DMEM to check whether DMEM can support osteoclastogenesis or not. TRAP activity was not changed (***Fig. 6-A***), but TRAP, MMP9, CTSK and Atp6v mRNA expression was significantly reduced in DMEM after 5 days of treatment (***Fig. 6-C***). Cell proliferation was increased in DMEM regardless of RANKL concentration, but decreased in αMEM with RANKL due to differentiation (***Fig. 6-B***). TRAP activity was induced in DMEM, but mRNA expression was reduced. One possible reason could be that DMEM supports both proliferation and differentiation. Seeing as we need to use a housekeeping gene to normalize any data, a high number of cell proliferation may lower the comparative mRNA expression in our results. For *in vitro* osteoclastogenesis in co-culture, we need to investigate more on the media in which it is conducted so that it can support both osteoblast and osteoclast precursors along with their differentiations.

Osteoblast lineage regulates osteoclast differentiation and survival by synthesizing M-CSF and RANKL upon certain physiological conditions. Osteoclast differentiation *in vitro* depends on exogenous M-CSF [18], and M-CSF removal from purified osteoclast cultures from bone marrow results in apoptosis by activating caspase and MST1 kinase [19]. In another report, M-CSF was found to activate phosphoinositide 3-kinase (PI3K) and anti-apoptotic Akt kinase in osteoclast cells [20]. Akt activity is essential for cell survival. Akt target the apoptotic machinery like BAD, caspase-9, glycogen-synthase kinase etc. [21, 22]. RANKL and TNFα act primarily via NF-κB activation leading to the transcription and *de novo* synthesis of anti-apoptotic proteins [23]. However, some studies suggest that M-CSF, RANKL and TNFα, three cytokines with different functions, can stimulate the Akt pathway [3, 24-26]. In our study, we found that RAW264.7 cells treated with 100 ng/mL RANKL without M-CSF in αMEM supplemented with 10% FBS and antibiotics activated osteoclastogenic NF-κB, ERK, p38 MAPK, along with anti-apoptotic Akt (Thr308) (***Fig. 7-A, B***) within 60 min of treatment, showing that the RANKL activation of these osteoclastic and survival pathways are independent of M-CSF (***Fig. 8***).

Conclusion

In this study, we found that RANKL-induced osteoclastic differentiation is independent on M-CSF, but is dependent on FBS, media content and initial cell density. This study will help to discover new targets for inhibiting or stimulating osteoclast differentiation, maturation and activation and thereby prevent unusual bone loss or excess bone mineral density related diseases.

Acknowledgements

This work was partially supported by the Japanese Council for Science, Technology and Innovation, SIP (Project ID 14533567), “Technologies for creating next-generation agriculture, forestry and fisheries” (funding agency: Bio-oriented Technology Research Advancement Institution, NARO).

Conflict of interest

The authors claim no conflict of interest in this study.

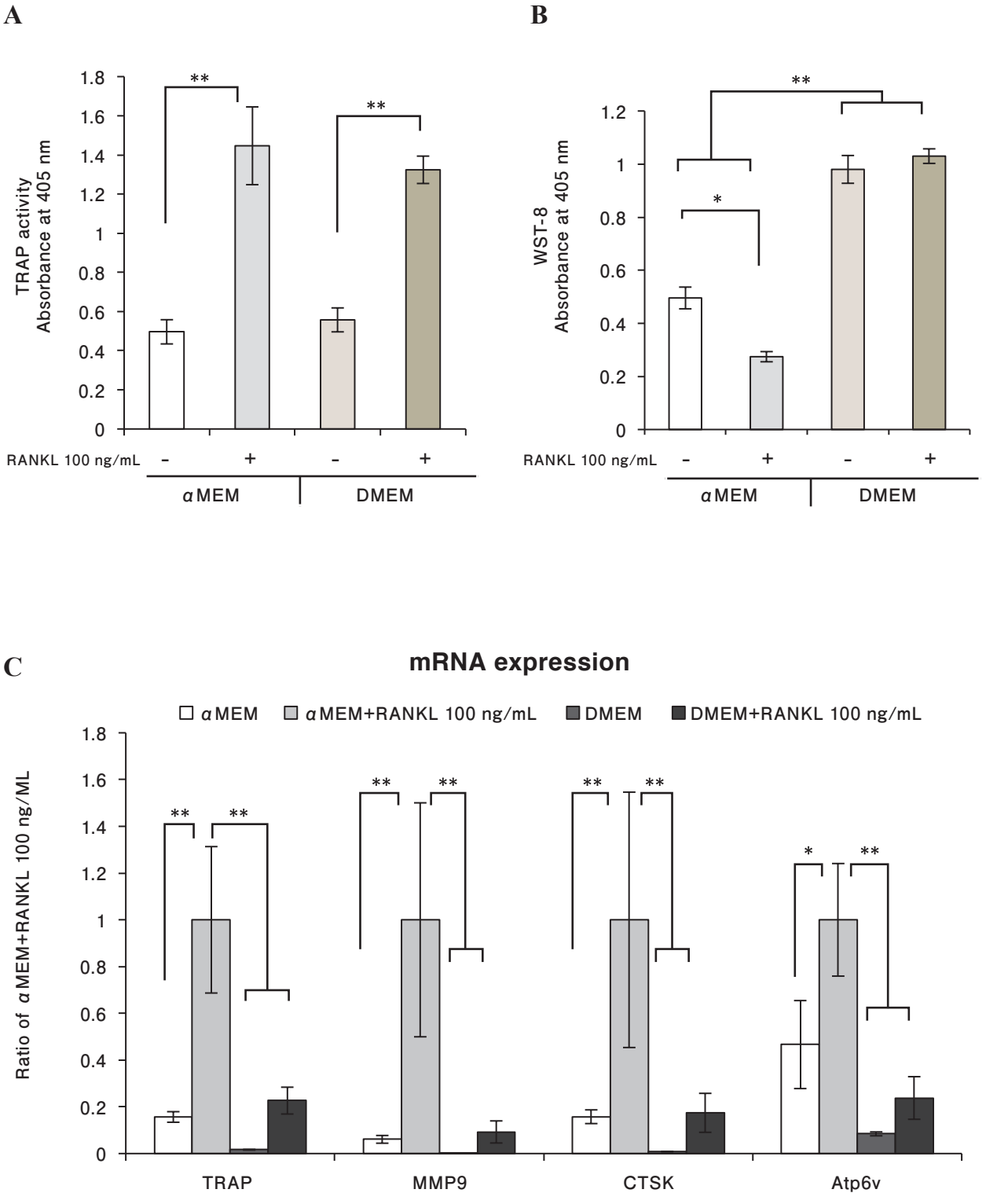

Fig. 6. **Effect of media on RANKL-induced osteoclastogenesis.**

RAW264.7 cells were plated in 24-well plates at 4×10^4 cells/well, and the next day cells were treated with αMEM or DMEM containing 10% FBS with or without 100 ng/mL RANKL. After 3 days, the media was renewed. At day 5, cells were used for **A)** Osteoclastogenic TRAP activity assay, **B)** WST-8 assay, and **C)** RT-PCR analyses. Relative TRAP, MMP9, CTSK, Atp6v mRNA expression, data was normalized by GAPDH and shown as a fold change. All data are as mean ± SEM, n = 3, Tukey-Kramer test, * $p < 0.05$, ** $p < 0.01$.

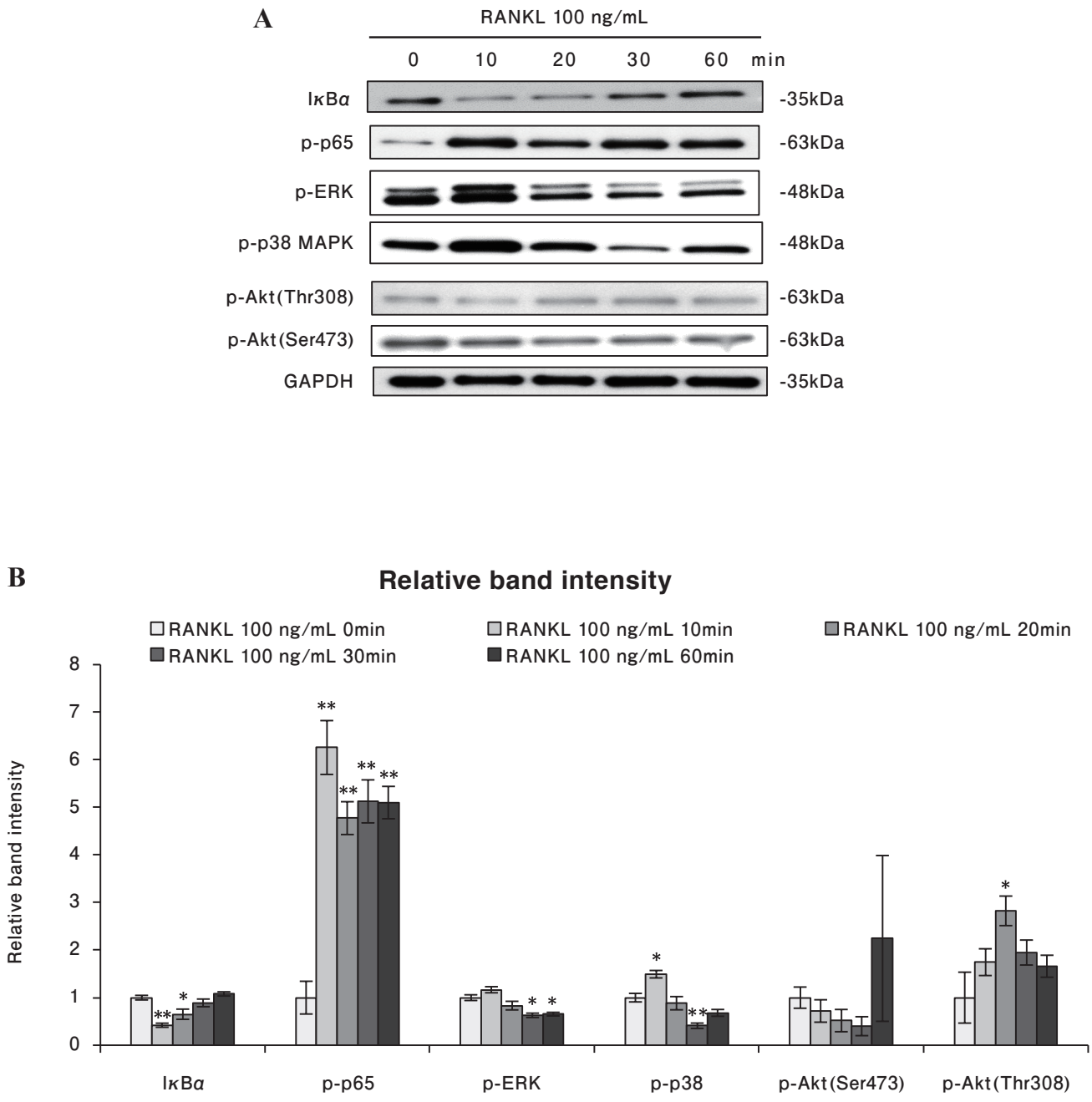

Fig. 7. **RANKL alone activated both osteoclastogenic and survival related pathways.**

A) RAW264.7 cells were plated in a 6-well plate at 2×10^5 cells/well, a followed by treatment with αMEM containing 10% FBS with 100 ng/mL RANKL the following day. After 0~60 min, the cells were collected and the cell lysates were prepared using RIPA buffer. Then 5 μg protein samples were used for western blot analysis using antibodies against the indicated proteins. **B)** ImageJ analysis of protein bands. Data were normalized by GAPDH and expressed as mean ± SEM, n = 3, Tukey-Kramer test, * $p < 0.05$, ** $p < 0.01$.

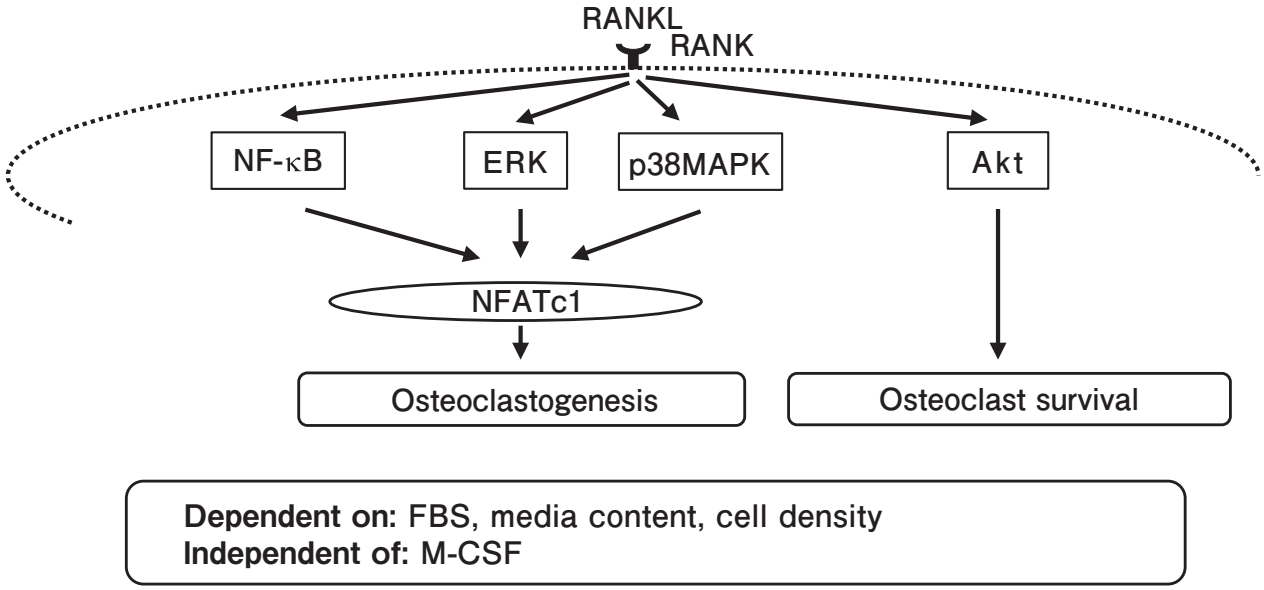

Fig. 8. **Schematic representation of RANKL induced osteoclastogenesis in RAW264.7 cells.**

References

1) Hadjidakis DJ, Androulakis II. Bone remodeling. Ann NY Acad Sci. 2006; 1092: 385-396.
2) Miyamoto T. Regulation of osteoclast differentiation and bone homeostasis. Anti-Aging Med. 2012; 9: 161-164.
3) Glantschnig H, Fisher JE, Wesolowski G, et al. M-CSF, TNFα and RANK ligand promote osteoclast survival by signaling through mTOR/S6 kinase. Cell Death Differ. 2003; 10: 1165-1177.
4) Boyce BF, Rosenberg E, de Papp AE, et al. The osteoclast, bone remodeling and treatment of metabolic bone disease. Eur J Clin Invest. 2012; 42: 1332-1341.
5) Steeve KT, Marc P, Sandrine T, et al. IL-6, RANKL, TNF-α/IL-1: Interrelations in bone resorption pathophysiology. Cytokine Growth Factor Rev. 2004; 15: 49-60.
6) Jason MH, Fiona MC, Nathan JP, et al. M-CSF potently augments RANKL-induced resorption activation in mature human osteoclasts. PLoS One. 2011; 6: e21462.
7) Sato K, Yagi M, Umehara H, et al. Establishment of a model for evaluating tumor necrosis factor-α production by cultured RAW264.7 in response to glycation stress. Glycative Stress Res. 2014; 1: 1-7.
8) Arriero M del M, Ramis JM, Perelló J, et al. Inositol hexakisphosphate inhibits osteoclastogenesis on RAW 264.7 cells and human primary osteoclasts. PLoS One. 2012; 7: e43187.
9) Sato K, Yagi M, Takabe W, et al. Inhibitory effect of plant extract on tumor necrosis factor-α formation from carboxymethyllysine stimulated macrophages. Glycative Stress Res. 2015; 2: 191-196.
10) Mamun-Or-Rashid ANM, Takabe W, Yonei Y. Melatonin has no direct effect on inflammatory gene expression in CML-HSA stimulated RAW264.7 cells. Glycative Stress Res. 2016; 3: 141-151.
11) Ghayor C, Correro RM, Lange K, et al. Inhibition of osteoclast differentiation and bone resorption by N-methylpyrrolidone. J Biol Chem. 2011; 286: 24458-24466.
12) Yoshida H, Hayashi S-I, Kunisada T, et al. The murine mutation osteopetrosis is in the coding region of the macrophage colony stimulating factor gene. Nature. 1990; 345: 442-444.
13) Wiktor-Jedrzejczak W, Bartocci A, Ferrante AW, et al. Total absence of colony-stimulating factor 1 in the macrophage-deficient osteopetrotic (op/op) mouse. Proc Natl Acad Sci USA. 1990; 87: 4828-4832.
14) Tanaka S, Takahashi N, Udagawa N, et al. Macrophage colony-stimulating factor is indispensable for both proliferation and differentiation of osteoclast progenitors. J Clin Invest. 1993; 91: 257-263.

15) Wang Y, Wang B, Fu L, et al. Effect of fetal bovine serum on osteoclast formation *in vitro*. J Hard Tissue Biol. 2014; 23: 303-308.
16) Gstraunthaler G, Lindl T, van der Valk J. A plea to reduce or replace fetal bovine serum in cell culture media. Cytotechnology. 2013; 65: 791-793.
17) Pombinho AR, Laize V, Molha DM. Development of two bone-derived cell lines from the marine teleost *Sparus aurata* : Evidence for extracellular matrix mineralization and cell-type-specific expression of matrix Gla protein and osteocalcin. Cell Tissue Res. 2004; 315: 393-406.
18) Fuller K, Owens JM, Jagger CJ. Macrophage colony-stimulating factor stimulates survival and chemotactic behavior in isolated osteoclasts. J Exp Med. 1993; 178: 1733-1744.
19) Reszka AA, Halasy-Nagy JM, Masarachia PJ, et al. Bisphosphonates act directly on the osteoclast to induce caspase cleavage of mst1 kinase during apoptosis. A link between inhibition of the mevalonate pathway and regulation of an apoptosis-promoting kinase. J Biol Chem. 1999; 274: 34967-34973.
20) Nakamura I, Lipfert L, Rodan GA, et al. Convergence of alpha(v)beta(3) integrin- and macrophage colony stimulating factor-mediated signals on phospholipase Cγ in prefusion osteoclasts. J Cell Biol. 2001; 152: 361-373.
21) Datta SR, Brunet A, Greenberg ME. Cellular survival: A play in three Akts. Genes Dev. 1999; 13: 2905-2927.
22) Kelley TW, Graham MM, Doseff AI, et al. Macrophage colony-stimulating factor promotes cell survival through Akt/protein kinase B. J Biol Chem. 1999; 274: 26393-26398.
23) Barkett M, Gilmore TD. Control of apoptosis by Rel/NF-κB transcription factors. Oncogene. 1999; 18: 6910-6924.
24) Wong BR, Besser D, Kim N, et al. TRANCE, a TNF family member, activates Akt/PKB through a signaling complex involving TRAF6 and c-Src. Mol Cell. 1999; 4: 1041-1049.
25) Lee SE, Chung WJ, Kwak HB, et al. Tumor necrosis factor-alpha supports the survival of osteoclasts through the activation of Akt and ERK. J Biol Chem. 2001; 276: 49343-49349.
26) Xing L, Venegas AM, Chen A, et al. Genetic evidence for a role for Src family kinases in TNF family receptor signaling and cell survival. Genes Dev. 2001; 15: 241-253.