Role of TRPM7 in RANKL-induced osteoclastogenesis

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Role of TRPM7 in RANKL-induced osteoclastogenesis

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This certifies that the dissertation of
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June 2013

Hwi Hoon Jung

Table of Contents

List of Figures

ABSTRACT

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The transient receptor potential melastatin type 7 (TRPM7) channel is a widely expressed non-selective cation channel with fusion to the C-terminal alpha kinase domain and regarded as a key regulator of whole body Mg^{2+} homeostasis in mammals. However, the roles of TRPM7 during osteoclastogenesis in RAW264.7 cells and bone marrow-derived monocyte/macrophage precursor cells (BMMs) are not clear. In the present study, the roles of TRPM7 in osteoclastogenesis using methods of small

interfering RNA (siRNA), RT-PCR, patch-clamp, and calcium imaging were investigated. RANKL (receptor activator of NF-κB ligand) stimulation did not affect the TRPM7 expression and TRPM7-mediated current was activated in HEK293, RAW264.7, and BMM cells by the regulation of Mg^{2+}. Knock-down of TRPM7 by siTRPM7 reduced intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) increases by 0 mM $[Mg^{2+}]_e$ in HEK293 cells and inhibited the generation of RANKL-induced Ca^{2+} oscillations in RAW264.7 cells. Finally, knock-down of TRPM7 suppressed RANKL-mediated osteoclastogenesis such as activation and translocation of NFATc1, formation of multinucleated cells, and the bone resorptive activity, sequentially. These results suggest that TRPM7 plays an essential role in the RANKL-induced $[Ca^{2+}]_i$ oscillations that triggers the late stages of osteoclastogenesis.

Key words : TRPM7, osteoclastogenesis, RANKL, calcium signaling

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Ⅰ. INTRODUCTION

Calcium (Ca^{2+}) plays a critical role in many cellular processes from differentiation to death in cells. Ca^{2+} entry into cells mediates by store-operated Ca^{2+} channels (SOCs) and transient receptor potential (TRP) channels (Berridge et al., 2003). TRP channels have been proposed to operate as SOCs. TRP channels consist of six transmembrane spanning domains (S1-6) with a pore-forming loop between S5 and S6, and include

intracellular N- and C-terminal regions (Fig. 1). TRPM7 is a non-selective cation channel that conducts both monovalent ions (eg, Na^+ and K^+) and divalent ions (eg, Ca^{2+}, Mg^{2+} and other trace metal ions) (Bae and Sun, 2011). According to the degree of amino acid homology, the TRP family can be subdivided into seven subgroups (TRPC, TRPV, TRPM, TRPA, TRPP, TRPML, and TRPN). TRPM (M stands for “melastin”) belongs to super-family of transient receptor potential (TRP) channels, which consist of eight different isoforms, TRPM1-TRPM8 (Nilius et al., 2007). Apart from other TRP channels, the distinct characteristic of TRPM does not contain N-terminal ankyrin repeat motifs but contain functional proteins in C-termini. TRPM7, for example, contain functional α-kinase segments, a type of serine/threonine-specific protein kinase (Kraft and Harteneck, 2005; McNulty and Fonfria, 2005) that is essential for modulating channel activity (Clark et al., 2008; Dorovkov and Ryazanov, 2004). Due to its structural features, TRPM7 is considered as both a kinase, which is capable of phosphorylating itself and other substrates, and a cation channel, which conducts cations (highly permeable to Ca^{2+} and Mg^{2+}) into the cell (Boesmans et al., 2011).

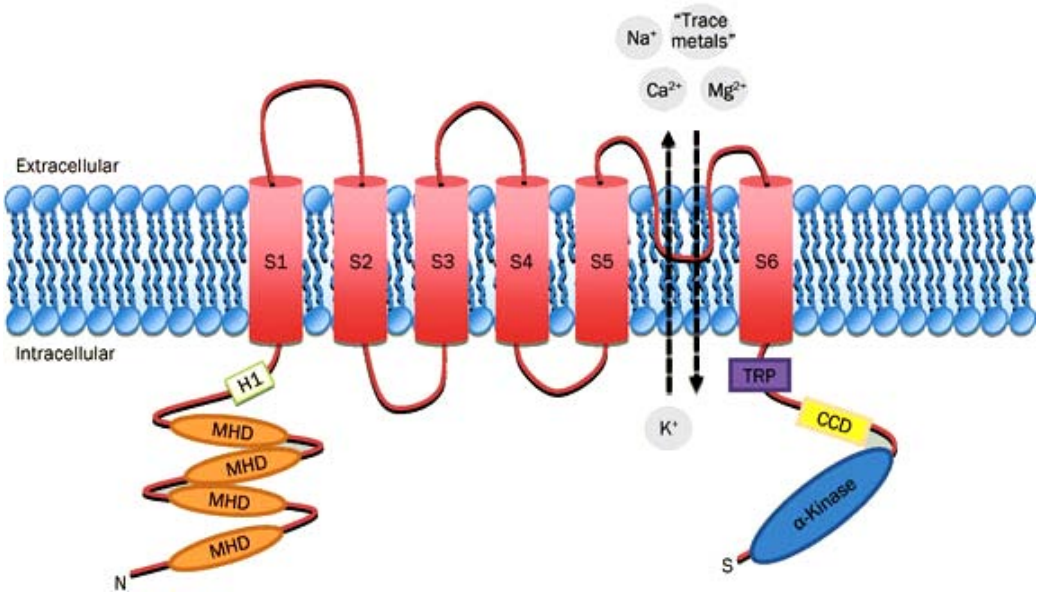


Fig. 1. Schematic diagram showing proposed transmembrane topology of TRPM7. (A) The putative membrane topology of a single subunit of TRPM7 is shown. Each subunit has six transmembrane (TM) spanning domains (S1–S6) with a re-entrant pore-forming loop between the fifth (S5) and sixth (S6) segments. The intracellularly located N-terminus has another hydrophobic region (H1) and four regions of TRPM subfamily homology domain (MHD). The intracellularly located C-terminus contains a TRP box of ~25 highly conserved residues (TRP) and a coiled-coil domain (CCD). The distal C-terminus has an atypical serine/threonine protein kinase domain.

TRPM7, as a cation channel, is constitutively opened and mediates capacitative Ca^{2+} entry, which is tightly regulated by intracellular Mg^{2+} concentration such as Mg-ATP and other Mg-nucleotides (Paravicini et al., 2012). The signaling cascade for osteoclastogenesis which is the essential molecules for osteoclast differentiation and function was studied previously (Negishi-Koga and Takayanagi, 2009). M-CSF and RANKL signalings are involved in the proliferation and survival of osteoclast precursor cells. In response to macrophage colony-stimulating factor (M-CSF), hematopoietic stem cells undergo differentiation into macrophage colony-forming units (CFU-M), which are the common precursor cells of macrophages and osteoclasts. The differentiation step from CFU-M to mature osteoclasts is characterized by intracellular fusion, which is mainly induced by receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL) and its downstream molecules. Transcription factors essential for osteoclastogenesis are shown in Fig. 2. RANKL (receptor activator of nuclear factor-κB ligand) is expressed in osteoblastic/stromal cells and is critical importance for osteoclast differentiation. RANKL activation induces Ca^{2+} oscillation in the

intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$) that lead to calcineurin-mediated activation of NFATc1 which induces transcription factors following RANKL stimulation. NFATc1 plays a key role in the RANKL-induced transcriptional system during the terminal differentiation for osteoclastogenesis. Furthermore, Ca^{2+} oscillations are necessary for NFATc1 induction of osteoclastogenesis (Fig. 3). PLCγ produces inositol-1,4,5-trisphosphate (IP_3), which evokes Ca^{2+} release from ER possibly through IP_3 receptor (IP_3R) 2 and IP_3R3, and subsequently generates Ca^{2+} oscillation. Store-operated calcium entry (SOCE) may be involved in the activation of Ca^{2+} oscillation, but calcium release-activated calcium (CRAC) channels in osteoclasts have not been identified. TRPV5 channel is necessary for osteoclastic bone resorption. These channels do not contribute to Ca^{2+} oscillation at the early stage of osteoclastogenesis (Fig. 3B). Type II ryanodine receptor (RyR2) as well as Ca^{2+}-sensing receptor (CaSR) are expressed on the plasma membrane of osteoclasts and are involved in inhibition of bone resorption and induction of apoptosis of osteoclasts in response to the increase of $[Ca^{2+}]_o$.

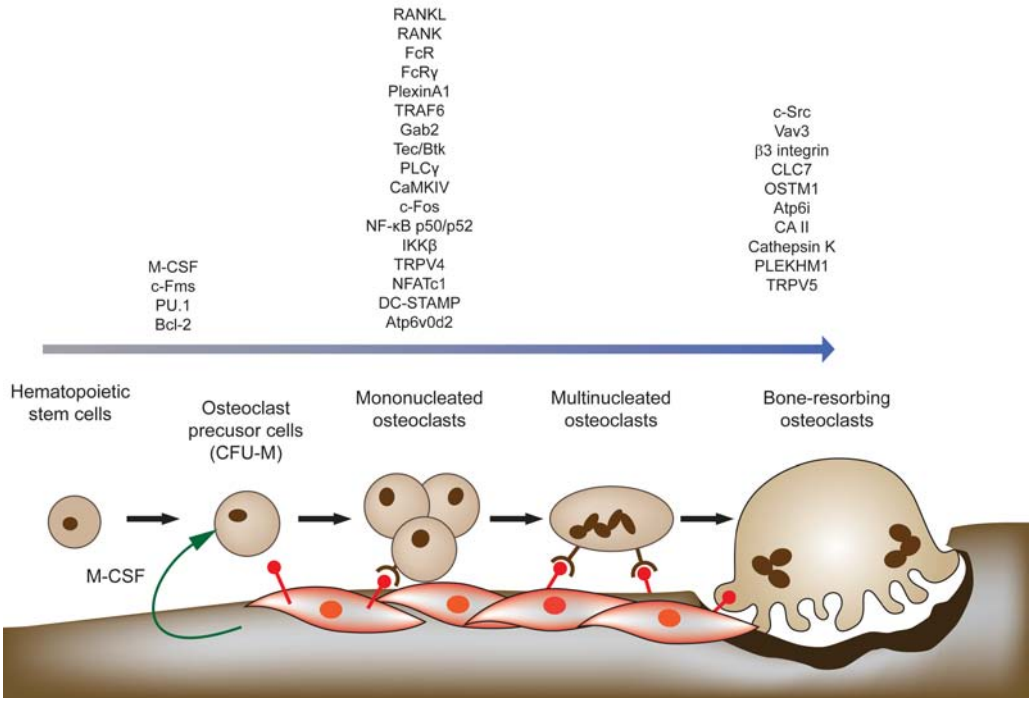


Fig. 2. Essential molecules for osteoclast differentiation and function. In the maturation stage, osteoclasts acquire bone resorbing activity, which is also dependent on M-CSF and RANKL. The molecules are depicted at the stage in which they are predominantly involved. MITF, microphthalmia-associated transcription factor; Bcl-2, B-cell leukemia ／lymphoma 2; FcRc, Fc receptor common c subunit; DAP12, DNAX-activating protein 12; TRAF6, tumor necrosis factor (TNF) receptor-associated factor 6; Gab2, growth factor receptor bound protein 2 (Grb-2)-associated binding protein 2; Btk, Bruton's tyrosine kinase; PLCγ, phospholipase C γ; CaMKIV, Ca^{2+}/calmodulin-dependent protein kinases IV; IKKβ, inhibitor of κB (IκB) kinase; TRPV4, transient receptor potential (TRP) cation channels, subfamily V, member 4; NFATc1, nuclear factor of activated T-cells c1; DCSTAMP, dendritic cell-speci fic transmembrane protein; CLC7, chloride channel 7; OSTM1, osteopetrosis associated transmembrane protein 1; CAII, carbonic anhydrase II; PLEKHM1, pleckstrin homology domain-containing family M (with RUN domain) member 1.

These receptors mediate $[Ca^{2+}]_o$-induced $[Ca^{2+}]_i$ increase through the modulating PLCγ activation, which leads to the Ca^{2+} release via IP_3 production. RyR1 and RyR3 are expressed on the ER membrane, but their role in Ca^{2+} release is not clear (Fig 3C). In our previous works, it has been reported that RANKL-induced oscillations by $[Ca^{2+}]_i$ increases is related with the extracellular Ca^{2+} influx through SERCA (sarco/endoplasmic reticulum Ca^{2+} ATPase) and SOCs, and intracellular ROS (reactive oxygen species) increases (Kim et al., 2010; Yang et al., 2009). Extracellular Ca^{2+} influx for maintaining $[Ca^{2+}]_i$ oscillations trigger the late stage in osteoclast differentiation (Takayanagi et al., 2002). However, Ca^{2+} entry pathway via the plasma membrane in osteoclastogenesis is not clearly known. The activity of TRPM7, as a Ca^{2+} permeable cation channel, is indispensable part of maintaining cell homeostasis including cell growth, proliferation and differentiation (Kraft and Harteneck, 2005). TRPM7 also can be activated by ROS and regulated intracellular Mg^{2+} level (Aarts et al., 2003; Su et al., 2010).

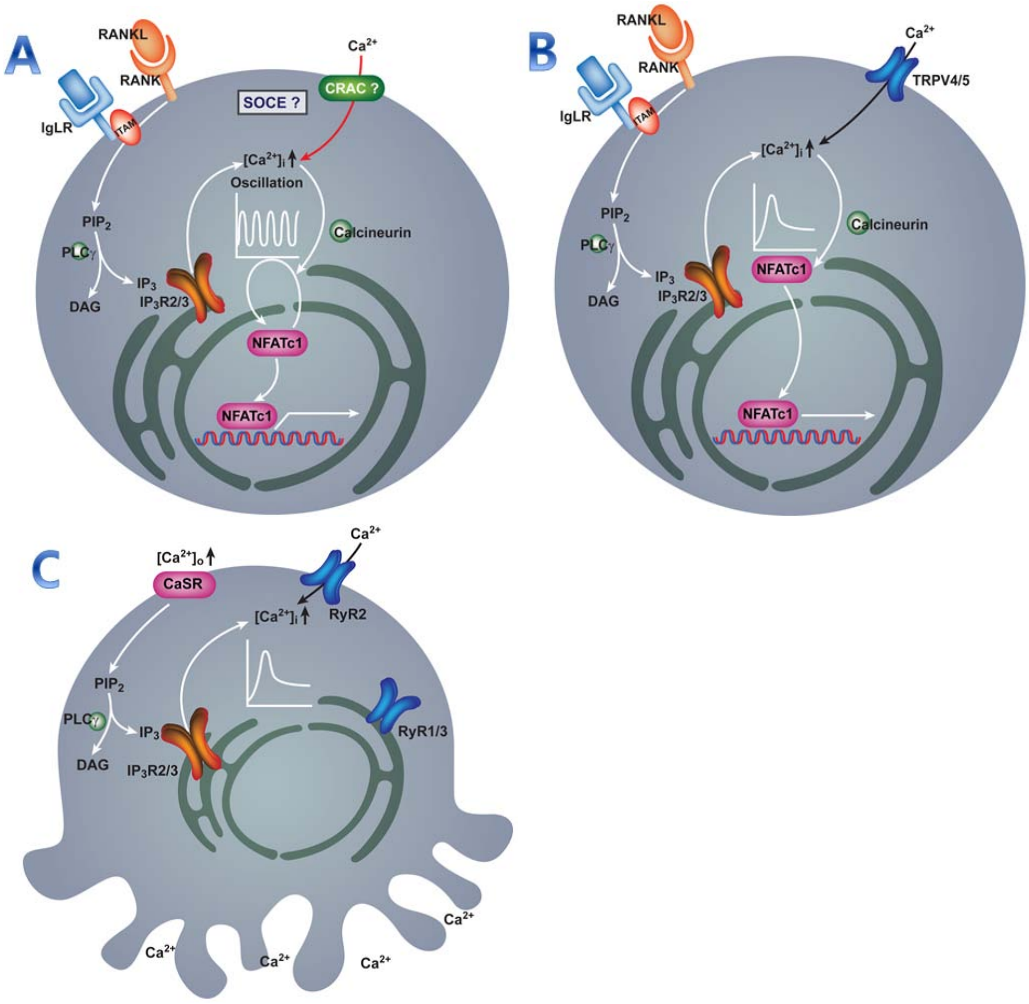


Fig. 3. Ca^{2+} signaling in osteoclasts. (A) Ca^{2+} oscillation necessary for NFATc1 induction at the early stage of osteoclastogenesis. Receptor activator of nuclear factor-κB (RANK) and immunoreceptor tyrosine-based activation motif (ITAM)-associated immunoglobulin-like receptor (IgLR) signals cooperate to activate phospholipase C γ (PLCγ). (B) The transition of Ca^{2+} signaling pattern during osteoclastogenesis. Ca^{2+} oscillation gradually disappear during differentiation, and Ca^{2+} influx from the extracellular milieu via transient receptor potential (TRP) cation channels, subfamily V, member 4 (TRPV4) becomes necessary for sustained Ca^{2+} signaling at the later stages. (C) Termination of bone resorption through sensing an increase of the $[Ca^{2+}]_o$ (Modified by (Negishi-Koga and Takayanagi, 2009).

It was suggested that TRPM7 can serve as a Ca^{2+} permeable cation channel in osteoclasts and regulate the activity of the RANKL-induced Ca^{2+} oscillations and osteoclastogenesis. In this study, the involvement of TRPM7 in RANKL-induced Ca^{2+} oscillations as a Ca^{2+} permeable channel and the role in physiological activities of osteoclasts were investigated.

II. MATERIALS AND METHODS

1. Cell culture and reagents

RAW264.7 (Korean Cell Line Bank, South Korea) and primary cultured BMMs were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA) and minimum essential medium alpha (α-MEM) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and incubated in 5% CO2 incubator. M-CSF and RANKL were treated at 50 ng/ml concentration in α-MEM. RANKL and M-CSF were purchased from KOMA Biotech (Seoul, Korea). HEK293 cells were cultured in DMEM containing 10% FBS, and 100 units/ml penicillin and streptomycin. Fura-2/AM was purchased from Teflabs (Austin, TX, USA). Gadolinium chloride (Gd^{3+}) and adenosine triphosphate (ATP) were from Sigma Aldrich (St Louis, MO, USA). Monoclonal antibody (mAbs) for NFATc1 and polyclonal antibody for TRPM7 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2. Preparation of BMMs

The femur and tibia were isolated from 4-6 weeks old mice as described previously (Son et al., 2012). Whole cells derived from bone marrow of femur and tibia was collected and cultured in α-MEM medium containing 10% FBS and 10 ng/ml M-CSF. The following day, non-adherent cells in media were collected and seeded on adequate plates and treated with M-CSF (50 ng/ml). After 2 days non-adherent cells were washed out and adherent cells were used as BMMs.

3. RT-PCR (Reverse transcription polymerase chain reaction)

Total RNA was isolated from each cell using Trizol reagents (Invitrogen). Total isolated RNA was amplified according to the manufature's protocol using AccuPower® RT PreMix (BIONEER, Daejeon, Korea). cDNA was amplified by PCR with HiPi™ Thermostable DNA polymerase (Elpis, Seoul, Korea). The primer sequences of genes were as follows: TRPM7 (531 bp), 5'-AGG AGA ATG TCC CAG AAA TCC-3' (forward) and 5'-TCC TCC AGT TAA AAT CCA AGC-3' (reverse); β-

actin (514 bp), 5'-TGT GAT GGT GGG AAT GGG TCA G-3' (forward) and 5'-TTT GAT GTC ACG CAC GAT TTC C-3' (reverse).

4. siRNA transfection

Small interfering (si) RNA for TRPM7, sense (5'-ACC GAG CTG GTC GCA CAA TTA TTT CAA GAG AAT AAT TGT GCG ACC AGC TCT TTT TC-3') and anti-sense (5'-TGC AGA AAA AGA GCT GGT CGC ACA ATT ATT CTC TTG AAA TAA TTG TGC GAC CAG CT-3'), was annealed with T4 DNA ligase and inserted into 50 ng/μl psi-STRIKE vector (Promega Corporation, Madison, WI, USA) by following the manufacture's procedure. Cells were seeded on 35 mm dish at a density of 1-5 × 10^5 cells in antibiotics free media. After 24 hrs, cells were re-plated in adequate dish. Each plasmid and Lipofectamine 2000 (Invitrogen) was diluted in 250 μl Opti-MEM respectively and then mixed. The mixture was incubated for 20 min at room temperature before adding the cell media. Cells were assorted with pEGFP expression.

5. Western blot

Whole cell lysates were prepared using RIPA lysis buffer (20 mM Tris, pH 7.4, 250 mM NaCl, 2 mM EDTA, pH 8.0, 0.1% Triton-X100, 0.01 mg/ml aprotinin, 5 μg/ml leupeptin, 0.4 mM PMSF, and 4 mM NaVO4), and then spun at 12,000 rpm for 10 min to remove insoluble material. Proteins (50-100 μg/well) were subjected to 6-12% SDS-PAGE respectively, and then were separated by size. Separated proteins were electro-transferred to a nitrocellulose membrane, blocked with 6% skimmed milk, and probed with Abs against TRPM7 (1:1000) and NFATc1 (1:3000). Thereafter, blots were washed, exposed to horseradish peroxidase-conjugated secondary antibodies for 1 hr, and finally detected by chemiluminescence (Amersham Pharmacia Biotech, Alington Heights, IL, USA).

6. $[Ca^{2+}]_i$ measurement

Cells were seeded on cover glass in 35-mm dishes (5 X 10^4 cells). After 24 h, cells were stimulated with RANKL (50 ng/ml) for indicated time. Cells in physiological salt solution (140 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 10 mM HEPES, 10 mM glucose, 310 mOsm, pH 7.4) were incubated with 5 μM Fura-2/AM 0.05% Pluronic F-127 for 30 min at room temperature and washed out with bath solution. The adherent cells on cover glass were placed on the bottom of a perfusion chamber, and bath solution was perfused continuously. Fura-2 fluorescence intensity was measured using excitation wavelengths of 340 and 380 nm, and emitted fluorescence 510 nm (Ratio = F340/F380) was collected and monitored at 2 s intervals using a CCD camera (Universal Imaging Co., Downingtown, PA, USA) as described previously (Park et al., 2011). Images were digitized and analyzed through MetaFluor software (Universal Imaging).

7. Electrophysiology

Whole-cell voltage-clamp recordings were made using the perforated patch-clamp method at room temperature. Currents were recorded using a MultiClamp 700B amplifier (Axon Instruments, Union City, CA, USA), subsequently digitized with a sampling rate of 10 kHz, and analyzed using pCLAMP10 software (Axon Instruments). The pipette resistance varied between 3-5 MΩ. Whole-cell currents were elicited by voltage ramps from –100 mV to +100 mV (50-ms duration) applied every 2 s from a holding potential of 0 mV. Pipettes for recordings of TRPM7 currents were filled with an internal solution containing (in mM): 140 CsCl, 8 NaCl, 10 EGTA, 10 HEPES, adjusted to pH 7.2 with CsOH (Demeuse et al., 2006). Nystatin was diluted to a final concentration of 250 μg/ml in the internal solution. The external solution containing (in mM): 140 NaCl, 3 KCl, 1 CaCl2, 2 MgCl2, 10 HEPES, 10 glucose, adjusted to pH 7.2 with NaOH, and the external solution was replaced by 3 mM CaCl2 to change a Mg^{2+}-free external solution.

8. Immunocytochemistry

Cells were seeded on coverslips (22 X 22) and treated with 50 ng/ml RANKL for 48 h. After fixation in 4% paraformaldehyde (PFA) for 5 min, cells were sequentially incubated in blocking solution (0.1% gelatin, 1% BSA, 0.01% sodium azide, 5% goat serum) for 30 min, overnight in blocking solution containing Abs against NFATc1 (1:100), and finally were treated with Alexa 488-labeled antimouse IgG antibody (Molecular Probes) in blocking solution for 1 h. Nuclei was separately stained with DAPI.

9. TRAP staining

The method of TRAP staining has been detailed previously (Yang et al., 2009). Briefly, cells were seeded on 48 well-plate at a density of 2 X 10^4 cells/well and cultured in α-MEM containing 10% FBS with 50 ng/ml RANKL and M-CSF. After 6 days, TRAP (tartrate-resistant acid phosphatase) staining was performed to determine the extent of differentiation. Cytochemical staining of TRAP+ cells were performed

using the Leukocyte Acid Phosphate Assay Kit (Sigma Aldrich) by following the manufacturer's procedure. TRAP+ multinucleated (3 nuclei) cells were counted.

10. Pit assay

Cells were seeded on bone-slice covered plates (OAAS) and maintained with 50 ng/ml M-CSF and sRANKL for 15 days. After that, cells were washed with sodium hypochlorite solution for 1 h at room temperature. Bone slices were imaged, and pits were calculated using MetaMorph software (Molecular Devices).

11. Statistics

All data were expressed as the mean ± SEM. Statistical significance was determined by using a paired or unpaired Student’s t-test. Statistical significance was set at $p < 0.05$ level.

Ⅲ. RESULTS

1. Expression and actions of endogenous TRPM7 channels

TRPM7 has been identified as spontaneously activated Ca^{2+}- and Mg^{2+}-entry channels and plays a key role for osteoblasts proliferation (Abed and Moreau, 2007). In this study, whether RANKL stimulation affects on the expression of TRPM7 was examined. In order to confirm the expression of TRPM7, RANKL was respectively treated for 6 days that is predicted time to be differentiated into osteoclast. As shown in Fig. 4, the expression of TRPM7 in RAW264.7 cells was not altered in the presence of RANKL. mRNA expressions of TRPM7 were reduced in siTRPM7-tranfected cells (Fig. 5A). In whole-cell patch-clamp experiments, the removal of Mg^{2+} in extracellular solutions led to activation of TRPM7-mediated currents that exhibit a highly nonlinear current-voltage (I-V) relationship with noticeable outward rectification at positive potentials in eGFP-transfected cells but not showed these effects in siTRPM7-transfected cells (Fig. 5B and 5C). A strongly out- ward-rectifying I-V relationship of TRPM7 is very similar features to other previous reports and these results show that TRPM7 is expressed regardless of RANKL stimulation and activated by low-Mg^{2+} concentration (Kim et al., 2005; Park et al., 2011; Son et al., 2012).

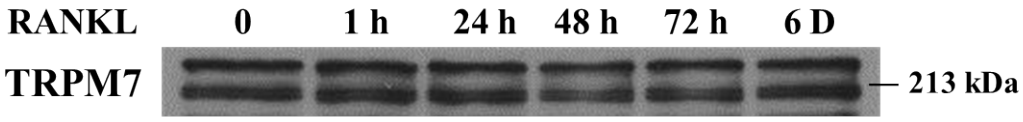


Fig. 4. Expression of endogenous TRPM7 channels by RANKL stimulation in RAW264.7 cells. Whole cells lysates were collected from cells stimulated with RANKL for indicated time. TRPM7 was blotted with its antibody.

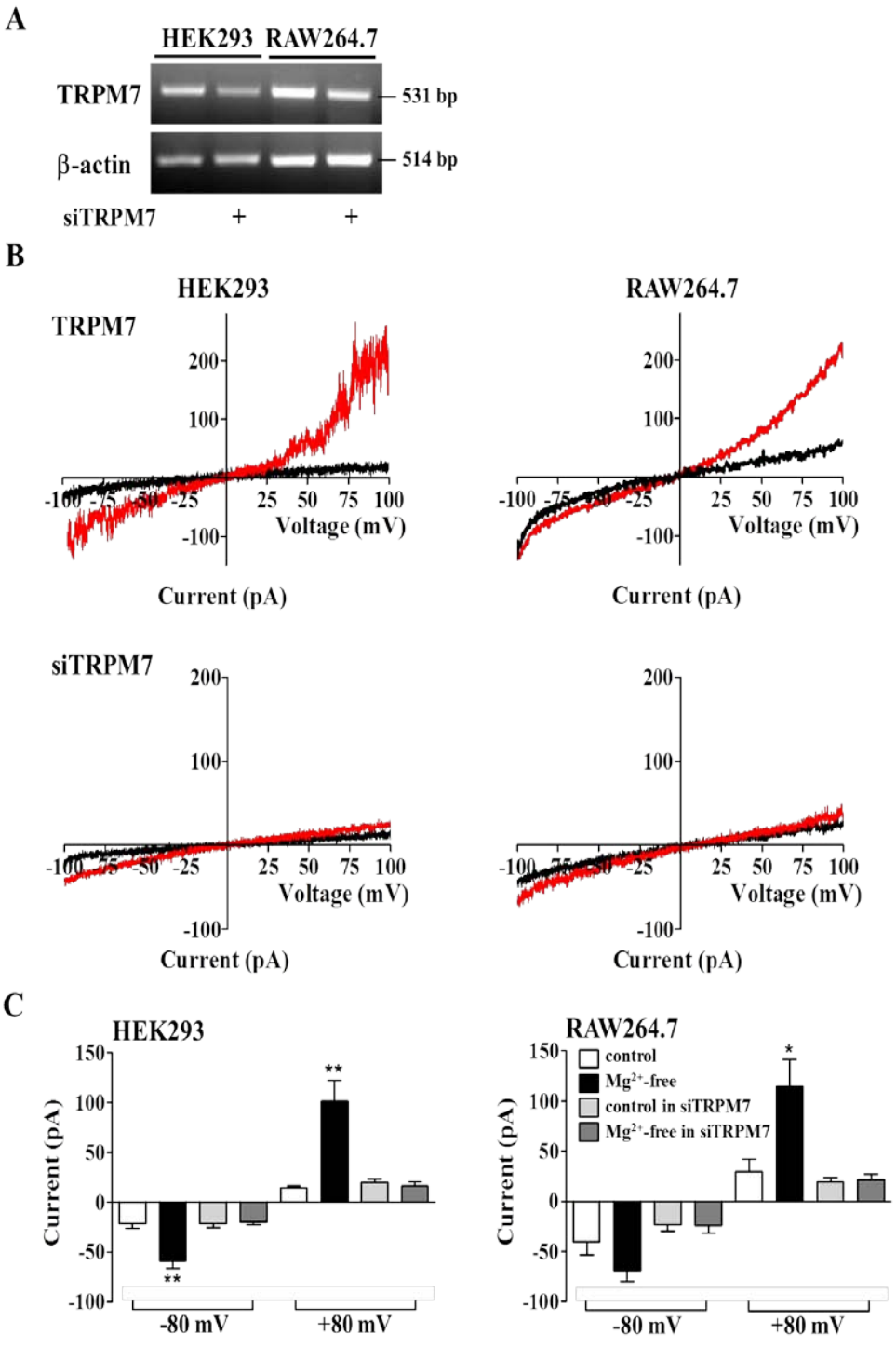

A
HEK293
RAW264.7
TRPM7
531 bp
β-actin
514 bp
siTRPM7
+
+
B
HEK293
RAW264.7
TRPM7
200
100
-100
-75
-50
-25
25
50
75
100
Voltage (mV)
Current (pA)
siTRPM7
C
HEK293
RAW264.7
Current (pA)
150
100
50
0
-50
-100
**
*
control
Mg2+-free
control in siTRPM7
Mg2+-free in siTRPM7
-80 mV
+80 mV

Fig. 5. Expression and activation of endogenous TRPM7 channels in HEK293 and RAW264.7 cells. (A) mRNA expression of TRPM7 were decreased after transfection of siTRPM7 in HEK293 and RAW264.7 cells. (B) Activation of endogenous TRPM7-mediated currents by a voltage ramp (-100 mV to +100mV in 50-ms intervals, Vh = 0 mV), used to determine current-voltage relations in cells. TRPM7 currents were activated by 0 mM $[Mg^{2+}]_e$ and these effects diminished in TRPM7 knock-down cells. (C) The amplitude of endogenous TRPM7-mediated currents at -80 and +80 mV in HEK293 and RAW264.7 cells. Data were expressed as the mean ± SEM. **$p < 0.01$, *$p < 0.05$ compared with control.

2. Effects of deletion of TRPM7 on RANKL-induced Ca^{2+} signaling

RANKL-induced Ca^{2+} increase, which is composed of internal Ca^{2+} release and extracellular Ca^{2+} influx, is essential step for triggering late-stage of osteoclastogenesis by activating NFATc1 (Kim et al., 2010; Yang et al., 2009). To confirm whether TRPM7 is involved in the induction of RANKL-induced Ca^{2+} oscillations as a Ca^{2+} permeable channel, we examined the effects of deletion of TRPM7 on Ca^{2+} responses in the presence or absence of RANKL. Reduced mRNA expression of TRPM7 by siTRPM7 treatment (Fig. 6A) resulted in disappearance of Ca^{2+} oscillations in response to RANKL stimulation (Fig. 6B), which indicates that activation of TRPM7 is related with the induction of Ca^{2+} oscillations in osteoclastogenesis. Furthermore, whether siTRPM7 inhibited Ca^{2+} signaling by 0 mM $[Mg^{2+}]_e$ in the presence or absence of RANKL of RAW264.7 cells were observed (Fig, 7B and 7C). The generation of Ca^{2+} oscillations are related with Ca^{2+} entry and Ca^{2+} release from intracellular IP_3-sensitive Ca^{2+} stores, and Ca^{2+} entry is mediated through the Mg^{2+}-sensitive pathway in polarized cells (Jans et al., 2002; Yu and Sokabe, 1997).

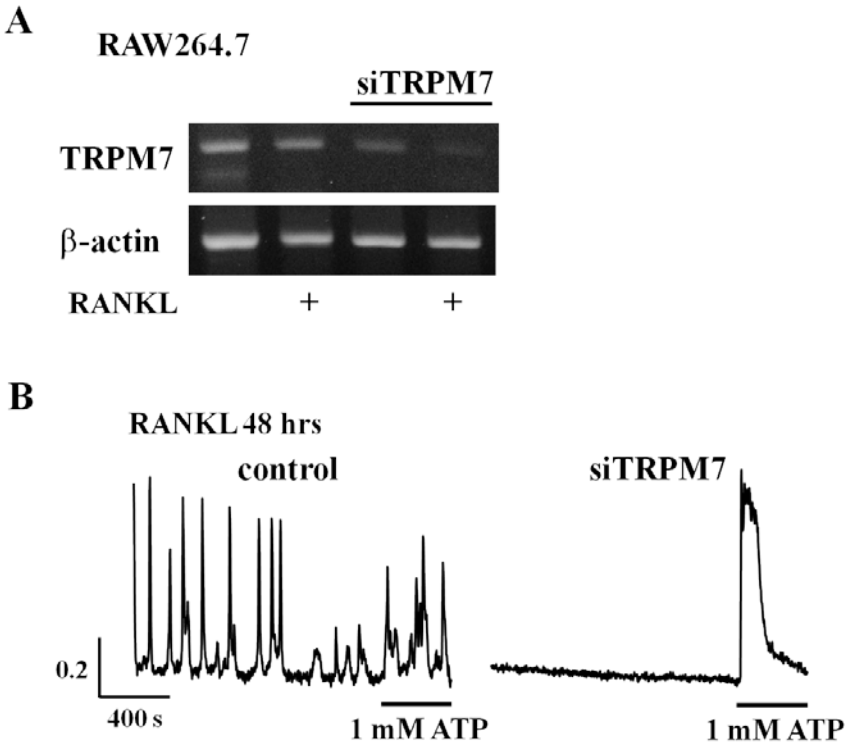
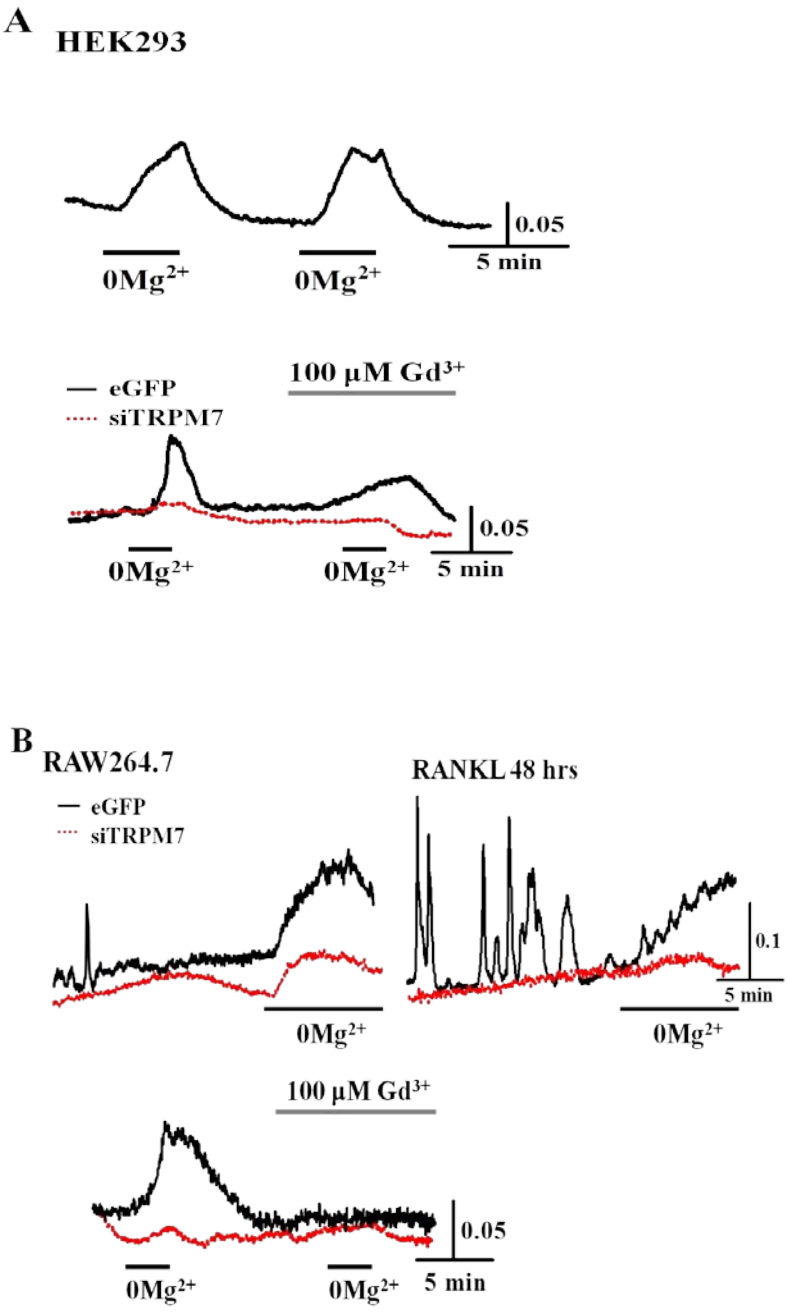


Fig. 6. Effects of deletion of TRPM7 on RANKL-induced $[Ca^{2+}]_i$ increases .

(A) Cells were transfected with siTRPM7 using lipofectamine2000. After 72 h of transfection, whole mRNA was collected using Trizol method. Beta-actin is used as loading control. (B) Control and siTRPM7 treated cells were treated with RANKL. After 48 h of RANKL stimulation, $[Ca^{2+}]_i$ in single cell was measured using Fura-2 fluorescence dye. To confirm cell's viability, 1mM ATP was used.


A
HEK293
0.05
5 min
0Mg2+
0Mg2+
100 μM Gd3+
eGFP
siTRPM7
0.05
0Mg2+
0Mg2+
5 min
B
RAW264.7
RANKL 48 hrs
eGFP
siTRPM7
0.1
5 min
0Mg2+
0Mg2+
100 μM Gd3+
0.05
0Mg2+
0Mg2+
5 min

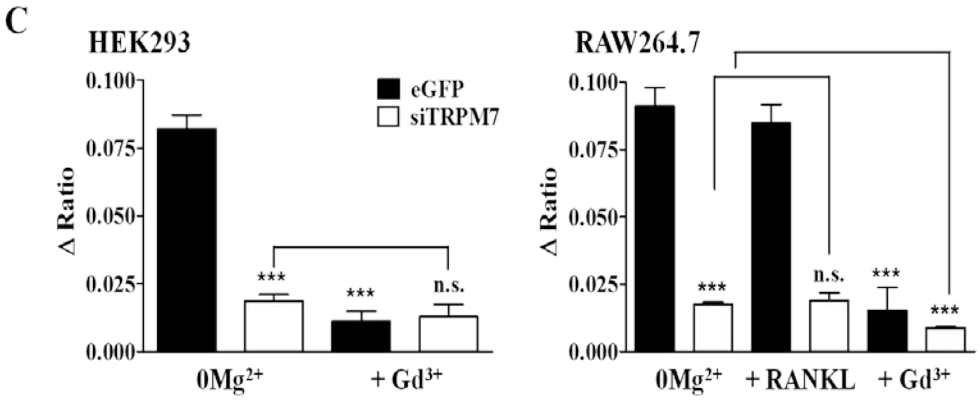


Fig. 7. Effects of deletion of TRPM7 on $[Ca^{2+}]_i$ increases mediated by removal of extracellular Mg^{2+}. (A) Application of 0 mM $[Mg^{2+}]_e$ induced $[Ca^{2+}]_i$ increases and it was repeated by the second application of 0 mM $[Mg^{2+}]_e$ in HEK293 cells. $[Ca^{2+}]_i$ increases also inhibited by 100 μM Gd^{3+} and transfected siTRPM7. (B) Application of 0 mM $[Mg^{2+}]_e$ induced $[Ca^{2+}]_i$ increases in the absence or presence of RANKL and it was inhibited by 100 μM Gd^{3+} and transfected siTRPM7 in RAW264.7 cells. (C) The degree of $[Ca^{2+}]_i$ increases by 0 mM $[Mg^{2+}]_e$ in HEK293 and RAW264.7 cells. Data were expressed as the mean ± SEM. ***$p < 0.001$ compared with 0 mM $[Mg^{2+}]_e$ treated control. n.s., not significant.

In HEK293 cells, Ca^{2+} signaling by 0 mM $[Mg^{2+}]_e$ was completely blocked by 100 μM Gd^{3+}, a known blocker of Ca^{2+} release activated Ca^{2+} entry (Fig. 7A and 7C). Interestingly, the $[Ca^{2+}]_i$ increases by Mg^{2+} free solution were not observed in TRPM7 knock-down cells. These results suggest that TRPM7 is playing an essential role in RANKL-induced Ca^{2+} oscillations as mediating an extracellular Ca^{2+} entry which is regulated through the Mg^{2+}-sensitive pathway.

3. Effects of deletion of TRPM7 in late-stage of osteoclastogenesis

It has been known that RANKL-induced Ca^{2+} oscillations is a key factor to determine the late-stage of osteoclastogenesis by sequentially activating calmodulin, calcineurin, and NFATc1 (Takayanagi et al., 2002). Activated NFATc1 gets translocated into the nucleus and function as a transcription factor, which induces the expression of proteins such as TRAP, Src kinase, INF-γ, calcitonin, etc (Boyle et al., 2003). Over these series of protein expression, precursor cells get fused into multi-nucleated cells, which have abilities of mineralizing the bone. According to previous results, it has

been hypothesized that abolished Ca^{2+} oscillations by deletion of TRPM7 would affect on the activities of bone resorption. To confirm this hypothesis, the expression and translocation of NFATc1 in response to deletion of TRPM7 was examined in RAW264.7 cells and BMMs. As a result, lack of TRPM7 diminished RANKL-induced NFATc1 translocation into nucleus and reduced NFATc1 expression by 60% in RAW264.7 cells (Fig. 8A) and BMMs (Fig. 8B). Furthermore, deletion of TRPM7 significantly reduced RANKL-induced responses, formation of multi-nucleated cells (MNCs) in BMMs (Fig. 9) and bone resorptive activity in RAW264.7 cells (Fig. 10). Taken together, these results suggest that abolished Ca^{2+} oscillations by deletion of TRPM7 affected on NFATc1 activities, fusion into MNCs, and bone resorption.

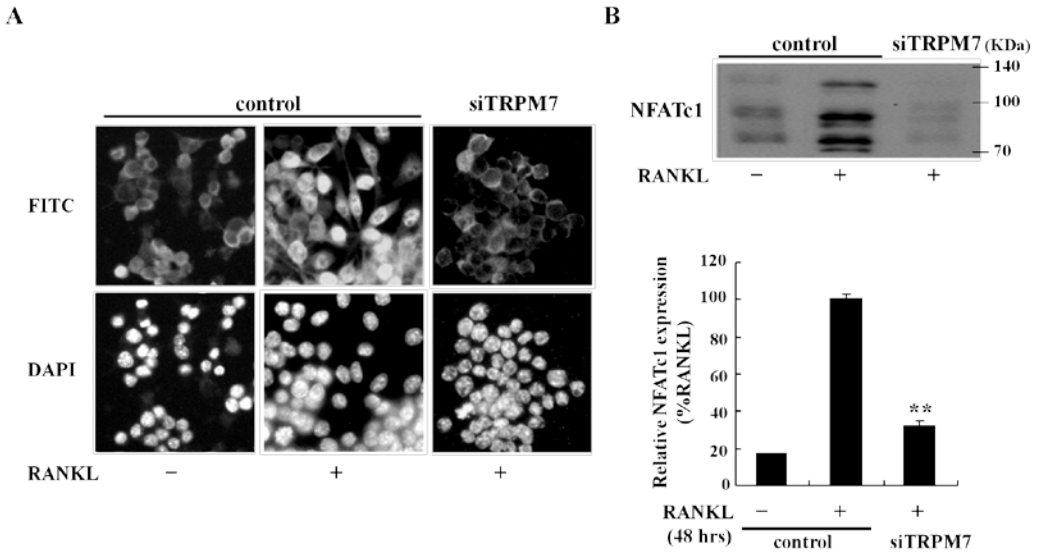


Fig. 8. Effects of deletion of TRPM7 on NFATc1 expression. (A-B) Cells were transfected with sicontrol and siTRPM7 in the presence or absence of RANKL for 48 h. To confirm the localization and expression of NFATc1, cells were fixed with 4% paraformaldehyde (PFA) and whole lysates were collected using RIPA lysis buffer respectively. Localization inside the cell and NFATc1 expression in whole lysates was confirmed with antibody for NFATc1.

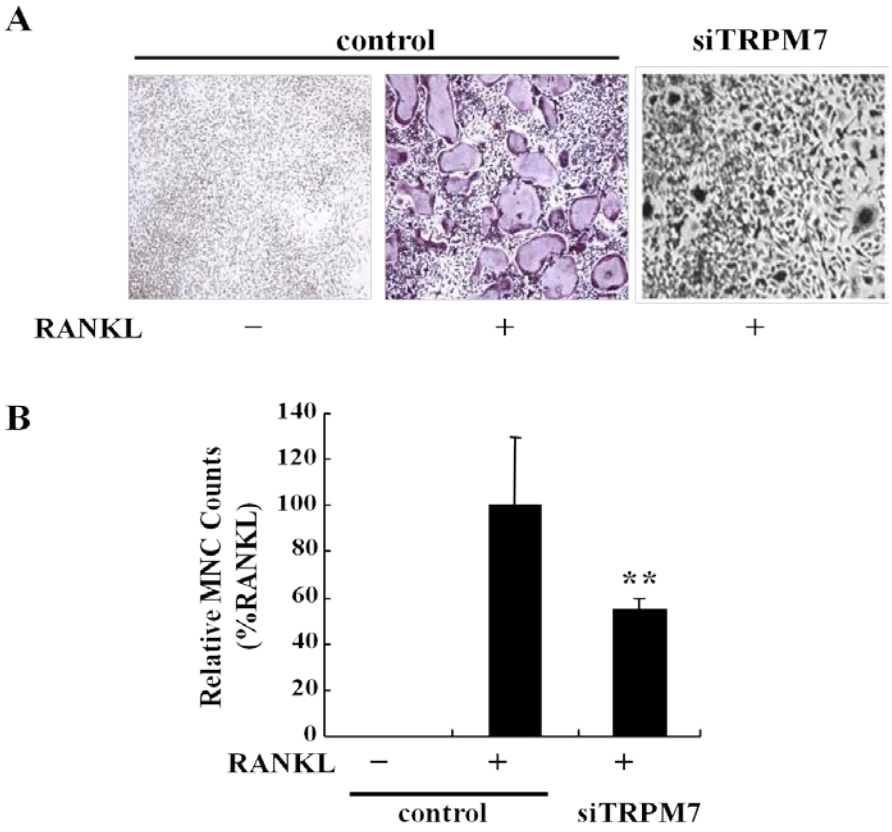


Fig. 9. Effects of deletion of TRPM7 on the formation of multinucleated cells. To verify the formation of multinucleated cells (MNCs, number of nuclear > 3), each sample was incubated for 6 days in the presence of RANKL. TRAP staining was performed as described in methods. And then MNCs in each well was counted.

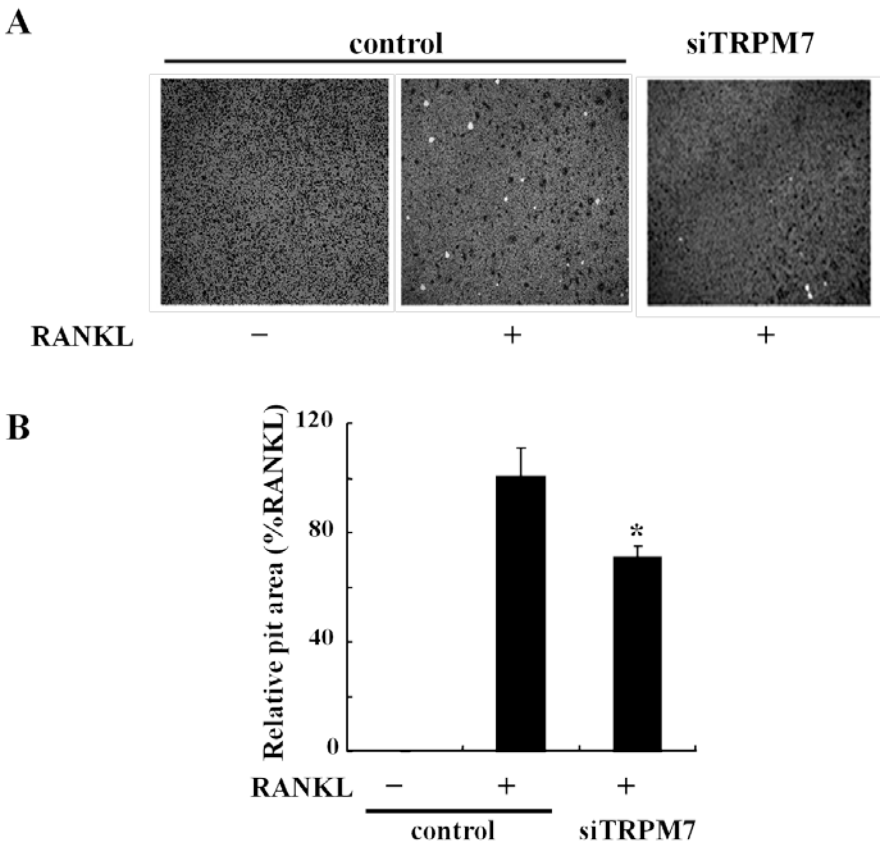


Fig. 10. Effects of deletion of TRPM7 on the bone resorptive activity. To determine the bone resorptive activity, cells were seeded on bone-slice covered plate in the presence of RANKL for 15 days. After incubation, whole image of each well was taken to calculate mineralized area as described in methods. Data were normalized to the expression level in RANKL treated control and expressed as the mean ± SEM. **p < 0.01, *p < 0.05 compared with RANKL treated control.

Ⅳ. DISCUSSION

As tools for communication, intracellular Ca^{2+} concentration determine various cellular responses such as proliferation, movement, differentiation, etc (Berridge et al., 2003). Two separated Ca^{2+} sources, internal and external Ca^{2+} sources, are virtually being used for triggering and maintaining all the Ca^{2+} responses including osteoclastogenesis. The property as a Ca^{2+} conducting channels raised the possibility that TRPM7 may be involved in osteoclastogenesis, based on the requirement for Ca^{2+} responses. In present study, the potential use of TRPM7 in modulating the bone resorptive activities of osteoclast was examined. The first finding of this study is that TRPM7 is being expressed in osteoclast precursor cells and the constitutive activities are being negatively regulated by intracellular Mg^{2+} concentration. In osteoclastogenesis, precursor cells present long-lasting Ca^{2+} oscillations in response to RANKL stimulation. Signals leading differentiation clearly require sustained extracellular Ca^{2+} influx through certain Ca^{2+} permeable channels. Hence, this result suggests that TRPM7 must be considered as a candidate for mediating the long-

lasting Ca^{2+} oscillations. Notably, RANKL-induced Ca^{2+} oscillations and extracellular Ca^{2+} influx mediated by reduction of intracellular Mg^{2+} concentration were completely diminished by deletion of TRPM7. In previous work, reactive oxygen species (ROS), which is generated by RANKL stimulations, induce Ca^{2+} oscillations through activating the enzymatic reaction such as PLC (Kim et al., 2010; Runnels et al., 2002). Based on its characteristics as a kinase and channel, this results strongly suggest that function of TRPM7 as a kinase and cation channel is tightly linked with RANKL-induced ROS generation. Taken together, constitutive expression of TRPM7 would help precursor cells immediately sense the ROS generation and then ROS mediated activation of TRPM7 contributes the induction of Ca^{2+} oscillations by allowing extracellular Ca^{2+} influx. In terms of Mg^{2+} homeostasis in blood plasma, it is well established that bone is also considered as Mg^{2+} store in a body. Negative regulation of TRPM7 by Mg^{2+} concentration may explain the regulation of osteoclast activation. Present study strongly proposes that Mg^{2+} concentration in blood plasma is one of determinants for osteoclast activity by modulating the TRPM7 conductance.

It has been reported that NFATc1 expression is enhanced in response to RANKL

stimulation at transcriptional level. Considering main focus of this study, which is on identifying the possibility as a candidate for modulating the bone metabolism, I investigated the effects of deletion of TRPM7 on late-stage of osteoclastogenesis like forming MNCs and bone resorption. Only if TRPM7 has roles in a process of being differentiated into fully-activated osteoclasts, I may use it as a target molecule to treat bone diseases. This results indicate that extracellular Ca^{2+} influx through TRPM7 is regarded as a nodal point which sequentially regulates in the activity of NFATc1, forming MNCs, and physiological activity of osteoclasts. These findings address missing links between generation of ROS and induction of Ca^{2+} oscillations, which are required for triggering late-stage of osteoclastogenesis. TRPM7, which is activated by sensing both intracellular ROS generation and extracellular Mg^{2+} concentration, is acting as a mediator of extracellular Ca^{2+} entry in RANKL-induced osteoclastogenesis. Based on these results, adjusting the activities of TRPM7 by genetic modification and Mg^{2+} ingestion has to be considered as a novel way to treat bone diseases.

V. CONCLUSION

The present study was aimed to investigate the involvement of TRPM7 in RANKL-induced Ca^{2+} Oscillations as a Ca^{2+} permeable channel and the role in physiological activities of osteoclasts.

1. The removal of Mg^{2+} in extracellular solutions led to activation of TRPM7-mediated currents in eGFP-transfected cells but not showed in siTRPM7-transfected cells.
2. Reduced mRNA expression of TRPM7 by siTRPM7 treatment resulted in diappearance of Ca^{2+} oscillations in response to RANKL stimulation.
3. The $[Ca^{2+}]_i$ increases by Mg^{2+} free solution were not observed in TRPM7 knock-down cells.
4. Lack of TRPM7 diminished RANKL-induced NFATc1 translocation into nucleus and reduced NFATc1 expression in RAW264.7 cells and BMM cells. .

5. Deletion of TRPM7 significantly reduced RANKL-induced responses, formation of multi-nucleated cells (MNCs) in BMMs and bone resorptive activity in RAW264.7 cells.

Therefore, TRPM7 plays a key role in the RANKL-induced $[Ca^{2+}]_i$ oscillations which triggers the late stages of osteoclastogenesis.

VI. REFERENCE

Aarts M, Iihara K, Wei WL, Xiong ZG, Arundine M, Cerwinski W, et al. (2003). A key role for TRPM7 channels in anoxic neuronal death. *Cell* 115(7): 863-877.

Abed E, Moreau R (2007). Importance of melastatin-like transient receptor potential 7 and cations (magnesium, calcium) in human osteoblast-like cell proliferation. *Cell Prolif* 40(6): 849-865.

Bae CY, Sun HS (2011). TRPM7 in cerebral ischemia and potential target for drug development in stroke. *Acta Pharmacol Sin* 32(6): 725-733.

Berridge MJ, Bootman MD, Roderick HL (2003). Calcium signalling: dynamics, homeostasis and remodelling. *Nat Rev Mol Cell Biol* 4(7): 517-529.

Boesmans W, Owsianik G, Tack J, Voets T, Vanden Berghe P (2011). TRP channels in neurogastroenterology: opportunities for therapeutic intervention. *Br J Pharmacol* 162(1): 18-37.

Boyle WJ, Simonet WS, Lacey DL (2003). Osteoclast differentiation and activation. *Nature* 423(6937): 337-342.

Clark K, Middelbeek J, Lasonder E, Dulyaninova NG, Morrice NA, Ryazanov AG, et al. (2008). TRPM7 regulates myosin IIA filament stability and protein localization by heavy chain phosphorylation. *J Mol Biol* 378(4): 790-803.

Dorovkov MV, Ryazanov AG (2004). Phosphorylation of annexin I by TRPM7 channel-kinase. *J Biol Chem* 279(49): 50643-50646.

Jans D, De Weer P, Srinivas SP, Lariviere E, Simaels J, Van Driessche W (2002). Mg(2+)-sensitive non-capacitative basolateral Ca(2+) entry secondary to cell swelling in the polarized renal A6 epithelium. *J Physiol* 541(Pt 1): 91-101.

Kim BJ, Lim HH, Yang DK, Jun JY, Chang IY, Park CS, et al. (2005). Melastatin-type transient receptor potential channel 7 is required for intestinal pacemaking activity. *Gastroenterology* 129(5): 1504-1517.

Kim MS, Yang YM, Son A, Tian YS, Lee SI, Kang SW, et al. (2010). RANKL-mediated reactive oxygen species pathway that induces long lasting Ca2+ oscillations essential for osteoclastogenesis. *J Biol Chem* 285(10): 6913-6921.

Kraft R, Harteneck C (2005). The mammalian melastatin-related transient receptor potential cation channels: an overview. *Pflugers Arch* 451(1): 204-211.

McNulty S, Fonfria E (2005). The role of TRPM channels in cell death. *Pflugers Arch* 451(1): 235-242.

Negishi-Koga T, Takayanagi H (2009). Ca^{2+}-NFATc1 signaling is an essential axis of osteoclast differentiation. *Immunol Rev* 231(1): 241-256.

Nilius B, Owsianik G, Voets T, Peters JA (2007). Transient receptor potential cation channels in disease. *Physiol Rev* 87(1): 165-217.

Paravicini TM, Chubanov V, Gudermann T (2012). TRPM7: a unique channel involved in magnesium homeostasis. *Int J Biochem Cell Biol* 44(8): 1381-1384.

Park S, Lee SI, Shin DM (2011). Role of regulators of g-protein signaling 4 in Ca^{2+} signaling in mouse pancreatic acinar cells. *Korean J Physiol Pharmacol* 15(6): 383-388.

Runnels LW, Yue L, Clapham DE (2002). The TRPM7 channel is inactivated by PIP(2) hydrolysis. *Nat Cell Biol* 4(5): 329-336.

Son A, Kim MS, Jo H, Byun HM, Shin DM (2012). Effects of Inositol 1,4,5-triphosphate on Osteoclast Differentiation in RANKL-induced

Osteoclastogenesis. *Korean J Physiol Pharmacol* 16(1): 31-36.

Su LT, Chen HC, Gonzalez-Pagan O, Overton JD, Xie J, Yue L, et al. (2010). TRPM7 activates m-calpain by stress-dependent stimulation of p38 MAPK and c-Jun N-terminal kinase. *J Mol Biol* 396(4): 858-869.

Takayanagi H, Kim S, Matsuo K, Suzuki H, Suzuki T, Sato K, et al. (2002). RANKL maintains bone homeostasis through c-Fos-dependent induction of interferon-beta. *Nature* 416(6882): 744-749.

Yang YM, Kim MS, Son A, Hong JH, Kim KH, Seo JT, et al. (2009). Alteration of RANKL-induced osteoclastogenesis in primary cultured osteoclasts from SERCA2$^{+/-}$ mice. *J Bone Miner Res* 24(10): 1763-1769.

Yu WG, Sokabe M (1997). Hypotonically induced whole-cell currents in A6 cells: relationship with cell volume and cytoplasmic Ca^{2+}. *Jpn J Physiol* 47(6): 553-565.

ABSTRACT (IN KOREAN)

RANKL 유도성 파골세포분화에서 TRPM7의 역할

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정 회 훈

Transient receptor potential melastatin type 7 (TRPM7)은 C-터미널의 alpha kinase 도메인의 융합을 동반하는 비특이성 양이온통로이며 포유류에서 Mg^{2+} 항상성에 관여한다. 그럼에도 불구하고 RAW264.7 세포주와 골수세포 기원 단핵세포/대식세포 전구물질 (BMM)의 파골세포분화 과정에서 TRPM7 의 역할은 아직 명확하지 않다. 이에 본 연구에서는 siRNA, RT-PCR, 전기생리, 칼슘 이미징 기법을 이용해 TRPM7 의 역할에 대해 알아보고자

하였다. HEK293 세포주와 RAW264.7, BMM 세포에서 RANK 에 의한 파골세포 분화 중에 TRPM7 의 발현과 TRPM7 매개 전류는 변화가 없었다. HEK293 세포에서 si RNA 를 통한 TRPM7 의 발현 저하는 0 mM $[Mg^{2+}]_e$ 유출을 이용한 세포 내 Ca^{2+} 농도 증가를 낮추었고, RAW264.7 세포에서 RANKL-유도성 칼슘 진동 (Ca^{2+} oscillations)을 억제하였다. 마지막으로 TRPM7 의 발현 저하는 NFATc1 의 활성과 전좌, 다핵세포의 형성, 그리고 골질의 분해 활동 같은 RANKL-매개 파골세포 분화 과정 등을 연속적으로 억제하였다. 이와 같은 결과를 통해 TRPM7 이 파골세포 분화의 후기 단계에서 RANKL-유도성 칼슘 진동에 필수적인 역할을 하는 것으로 생각된다.

핵심되는 말 : TRPM7, 파골세포분화, RANKL, 칼슘신호전달