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# Mechanisms involved in regulation of osteoclastic differentiation by mechanical stress-loaded osteoblasts

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ABSTRACT

Mechanical stress is known to be important for regulation of bone turnover, though the detailed mechanisms are not fully understood. In the present study, we examined the effect of mechanical stress on osteoblasts using a novel compression model. Mouse osteoblastic MC3T3-E1 cells were embedded in three-dimensional (3D) gels and cultured with continuous compressive force (0–10.0 g/cm$^2$) for 48 h, and the conditioned medium were collected. RAW264.7 cells were then incubated with the conditioned medium for various times in the presence of receptor activator of nuclear factor-κB ligand (RANKL). Conditioned medium was found to inhibit the differentiation of RAW264.7 cells into osteoclasts induced by RANKL via down-regulation of the expression of tumor necrosis factor receptor-associated factor 6 (TRAF6), phosphorylation of IκBα, and nuclear translocation of p50 and p65. Interestingly, the conditioned medium also had a high level of binding activity to RANKL and blocked the binding of RANK to RANKL. Furthermore, the binding activity of conditioned medium to RANKL was reduced when the 3D gel was supplemented with KN-93, an inhibitor of non-canonical Wnt/Ca$^{2+}$ pathway. In addition, expression level of osteoprotegerin (OPG) mRNA was increased in time- and force-dependent manners, and remarkably suppressed by KN-93. These results indicate that osteoblastic cells subjected to mechanical stress produce OPG, which binds to RANKL. Furthermore, this binding activity strongly inhibited osteoclastogenesis through suppression of TRAF6 and the nuclear factor-kappa B (NF-κB) signaling pathway, suggesting that enhancement of OPG expression induced by mechanical stress is dependent on non-canonical Wnt/Ca$^{2+}$ pathway.



## 1. Introduction

Bone mass homeostasis is regulated by an interaction of various factors, including growth factors, hormones and mechanical loading [1,2], and some researchers have reported that mechanical stress applied to several types of cells maintained bone metabolism including bone formation and bone resorption [3–5].

Receptor activator of nuclear factor-κB ligand (RANKL), identified as a membrane-bound protein, is an essential factor for osteoclastogenesis produced by osteoblasts and stimulates osteoclast precursors to differentiate via binding to the receptor, RANK. OPG is a member of tumor necrosis factor (TNF) receptor family that acts as a decoy receptor of the RANKL [6].

RANKL interacts with RANK, resulting in recruitment of intracellular tumor necrosis factor receptor-associated factor 6 (TRAF6) and activation of signaling pathways including nuclear factor of kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) [7–9]. Furthermore, RANKL activates the nuclear translocation and DNA binding of the NF-κB proteins (p50 and p65) via phosphorylation and degradation of IκBα [10,11].

Wnt pathway, confirmed to play critical roles in bone development and homeostasis, is classified into 3 pathway groups; the β-catenin-dependent canonical Wnt pathway, non-canonical planar cell polarity pathway, and non-canonical Wnt/Ca$^{2+}$ pathway [12]. It has been demonstrated that canonical Wnt pathway modulates several aspects of osteoblast physiology including proliferation, differentiation, bone matrix formation and apoptosis [13–16]. Findings in a recent genetic study indicated that Wnt/β-catenin pathway is involved in the expression of both RANKL and OPG [17]. On the other hand, mechanical loading was shown to induce differentiation of mesenchymal progenitor cells through the non-

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canonical Wnt/$Ca^{2+}$ pathway [18]. However, the role of non-canonical Wnt/$Ca^{2+}$ pathway in regard to mechanical stress-induce osteoclastogenesis has not been fully elucidated. In the present study, we investigated the mechanisms of the non-canonical Wnt/$Ca^{2+}$ pathway involved in osteoclastogenesis induced by compressive force.

## 2. Materials and methods

### 2.1. Reagents

Human recombinant RANKL was purchased from Oriental Yeast Co., Ltd. (Shiga, Japan). Anti-p38 MAPK polyclonal and anti-phosphorylated p38 MAPK polyclonal antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, CA, USA). Anti-p50 monoclonal, anti-p60 monoclonal, anti-IκBα monoclonal, anti-phospho-IκBα monoclonal, anti-TRAF6 monoclonal, and anti-RANK monoclonal antibodies were obtained from Santa Cruz Biotechnology. (Santa Cruz, CA, USA).

### 2.2. Cell cultures

The murine monocyte/macrophage cell line RAW264.7 was maintained in α-minimal essential medium (α-MEM; Gibco, Gland Island, NY, USA) containing 10% fetal bovine serum (FBS), with penicillin G (100 U/ml) and streptomycin (100 μg/ml). The murine osteoblastic cell line MC3T3-E1 was cultured in α-MEM supplemented with 10% FBS and antibiotics. The cells were maintained at 37 °C in an atmosphere containing 5% $CO_2$.

### 2.3. Application of compressive force

To examine the effect of static compressive force, MC3T3-E1 cells were cultured in a three-dimensional (3D) cell culture system [19]. Briefly, collagen gel cultures were assembled by mixing 7 volumes of 0.3% type I-A collagen solution (Nitta-gelatin, Osaka, Japan), 1 volume of 20 mM HEPES buffer containing 2.2% sodium bicarbonate and 0.05% sodium hydroxide, and 1 volume of cell suspension to provide a final cell density of $1 \times 10^6$ cells/ml. The gel mixtures were allowed to polymerize for 1 h, following transfer to 6-well plates to promote nutrient diffusion from their surroundings. The gel mixtures in each well were cultured with 2 ml of α-MEM containing 1% FBS, and allowed to set for 24 h prior to force loading. Compressive force was applied using a sterile titanium plate (32 mm in diameter) and plastic cylinder placed over the gels, which was adjusted by adding lead granules to the cylinder. In some experiments, KN-93, a selective $Ca^{2+}$/calmodulin-dependent protein kinase II inhibitor (Sigma–Aldrich, St. Louis, MO, USA), was added to the collagen gels.

### 2.4. Kinetic analysis using quartz-crystal microbalance (QCM)

A 27-MHz QCM (Affinix Q; Initium Inc., Tokyo, Japan) was employed to analyze the affinity of RANKL and conditioned medium harvested from 3D cultures of MC3T3-E1 cells. RANKL (2 μl; $10^{-11}$ M) was immobilized directly on the gold electrode surface of the QCM ceramic sensor chip, after which the sensor chip was soaked in a chamber containing 8 ml of distilled water at 25 °C until frequency equilibrium was attained. Conditioned medium (volume 800 μl) was added to the equilibrated solution containing the RANKL-immobilized sensor chip. The binding of conditioned medium to RANKL was determined by monitoring the alterations in frequency resulting from changes in mass on the electrode surface [20].

### 2.5. Western blot analysis

MC3T3-E1 cells were mixed into the collagen gels and subjected to 7.5 g/$cm^2$ of compressive force for indicated times, and conditioned medium were collected. Next, RAW 264.7 cells ($2.5 \times 10^5$ cells/well) were cultured in 6-well plates in α-MEM containing 10% FBS in the presence or absence of RANKL (40 ng/ml) along with the conditioned medium. The cells were then washed twice with phosphate buffer saline (PBS; pH 7.2) and lysed in lysis buffer (75 mM Tris–HCl containing 2% SDS and 10% glycerol, pH 6.8). In some experiments, nuclear factors were isolated using a NucBuster™ Protein Extraction Kit (EMD Biosciences Inc., Darmstadt, Germany) according to the manufacturer's instructions. Protein contents were measured using a DC protein assay kit (Bio-Rad, Hercules, CA, USA). Equivalent sample volumes were subjected to 10% SDS–PAGE and transferred to polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA, USA). Non-specific binding sites were blocked by immersing the membrane in 10% skimmilk in PBS for 1 h at room temperature, after which the membrane was washed 4 times with PBS, followed by incubation with the diluted primary antibody at 4 °C overnight. After washing the membrane, chemiluminescence was produced using enhanced chemiluminescent (ECL) reagent (Amersham Pharmacia Biotech, Uppsala, Sweden) and detected with Hyperfilm-ECL (Amersham Pharmacia Biotech).

### 2.6. Evaluation of osteoclastic differentiation

RAW264.7 cells were cultured in 24-well plates ($7 \times 10^4$ cells/well) with RANKL (40 ng/ml) in the presence of conditioned medium from the 3D cultures of MC3T3-E1 cells, for 3 days. Adherent cells were fixed and stained with tartrate-resistant acid phosphatase (TRAP) (Sigma Chemical Co., St. Louis, MO, USA). TRAP-positive multinucleated cells containing three or more nuclei were considered to be osteoclasts and counted under a microscope.

### 2.7. Real-time RT-PCR analysis

Total RNA was isolated from compressed 3D-gels using ISOGEN-LS (Nippon Gene, Tokyo, Japan). Briefly, collagen gels containing cells were washed extensively with PBS and minced in ISOGEN-LS, then RNA was isolated according to the manufacturer's instructions. Extracted total RNA was reverse transcribed and subjected to real-time RT-PCR, in which the PCR products were detected using FAST SYBR® Green Master Mix (Applied Biosystems, Foster City, CA). The primer sequences used were as follow; β-actin forward, 5′-CTGAACCCTAAGGCCAACCGTG-3′ and reverse 5′-GGCATACAGGGACAGCACAGCC-3′, and OPG forward, 5′-GCCTGGGACCAAAGTGAATG-3′ and reverse 5′-CTTGTGAGCTGTGTCTCCGTTT-3′. Thermal cycling and fluorescence detection were done using a StepOne™ Real-Time PCR System (Applied Biosystems). Real-time RT-PCR efficiency ($E$) was calculated according to the equation provided by Rasmussen [21], as follows: $E = 10[-1/\text{slope}]$, for β-actin and various target genes. The slope was determined from the graph of ng of the cDNA substrate ($x$-axis) versus the cycle number at the crossing point (CP) ($y$-axis). The CP value was the PCR cycle number that represented the CP in SYBR® Green fluorescence intensity above the automatic noise-based threshold. The fold increase in copy numbers of mRNA was calculated as the relative ratio of target gene to β-actin, following the mathematical model presented by Pfaffl [22].

$$\text{Fold increase} = \frac{(E_{\text{TARGET}})^{\text{CP TARGET(MEAN control-MEAN subject)}}}{(E_{\beta}-\text{actin})^{CP\beta-\text{actin(MEAN control-MEAN subject)}}}$$

### 2.8. OPG measurement

The amounts of OPG in the conditioned medium were determined using an OPG ELISA kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

### 2.9. Statistical analysis

Statistical differences were determined using an unpaired Student's *t*-test with Bonferroni correction for multiple comparisons. All data are expressed as the mean ± standard deviation of three examinations, with similar results obtained in each experiment.

## 3. Results

### 3.1. Effect of conditioned medium derived from MC3T3-E1 cells on osteoclastogenesis induced by RANKL

To determine whether mechanical stress in our compression model had effect on osteoclast differentiation, we first evaluated the number of osteoclasts by counting TRAP-positive multinucleated cells (Fig. 1A). Conditioned medium derived from MC3T3-E1 cells inhibited the differentiation of RAW264.7 cells into osteoclast-like cells in a loading force-dependent manner (Fig. 1B). The inhibitory effect of the conditioned medium began to be seen at 7.5 g/cm$^2$ of loading force (Fig. 1C). In addition, the effect of conditioned medium on proliferation of RAW264.7 cells was examined using a WST-1 assay. However, no effect on cell growth was seen for up to 48 h (data not shown).

### 3.2. Interaction between RANKL and conditioned medium derived from MC3T3-E1 cells

To examine the interaction of conditioned medium and RANKL, we investigated the affinity between them using a QCM technique. Medium conditioned by 0 g/cm$^2$ of compressive force decreased the frequency by 745 Hz, while it was decreased by 2200 Hz when we used medium conditioned by 7.5 g/cm$^2$ of compressive force (Fig. 2A). To investigate whether MC3T3-E1 cells under mechanical stress produce OPG, we examined OPG mRNA expression in MC3T3-E1 cells in the collagen gels. Mechanical stress caused an up-regulation of OPG mRNA expression at 6 h (Fig. 2B). These enhancement was in time- and dose-dependent manners (Fig. 2C).

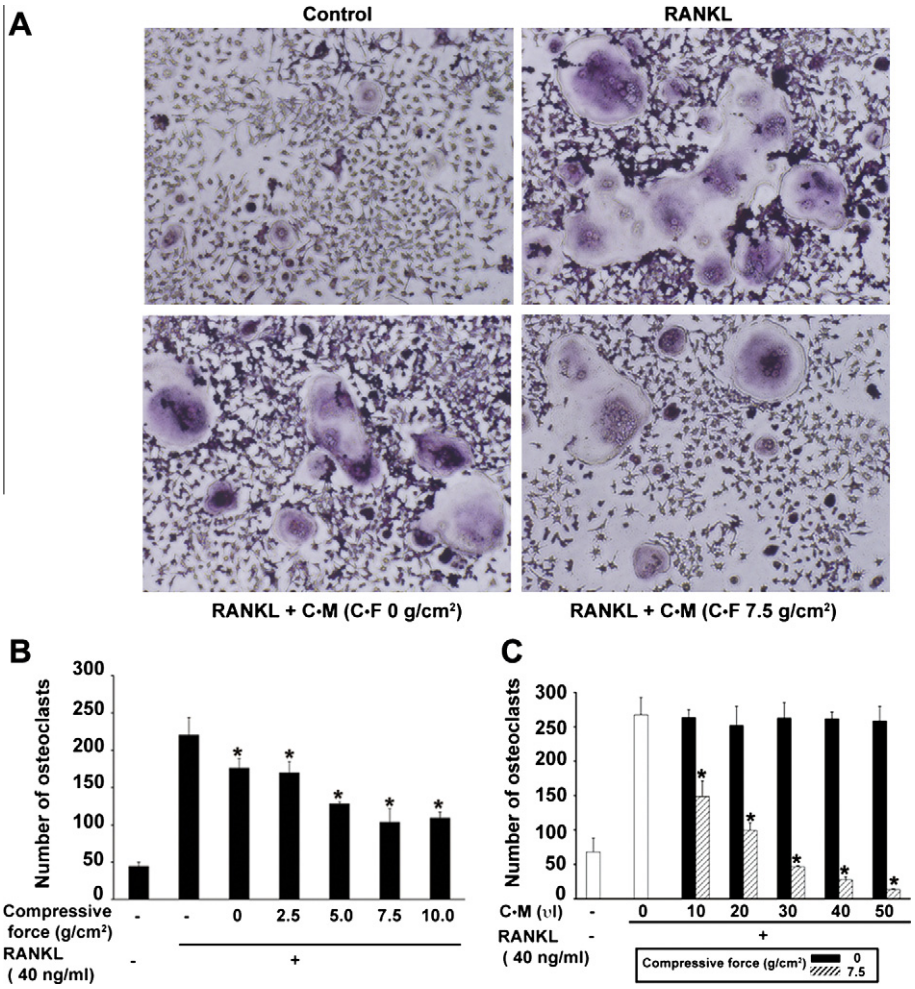


**Fig. 1.** Effect of conditioned medium derived from MC3T3-E1 cells on osteoclastogenesis induced by RANKL RAW264.7 cells ($7.0 \times 10^4$ cells/ml) were cultured with conditioned medium derived from MC3T3-E1 cells subjected to 7.5 g/cm$^2$ of compressive force and RANKL (40 ng/ml). The number of TRAP-positive multinucleated cells was counted. (A) Images showing osteoclast formation. (B) MC3T3-E1 cells were subjected to 0–10 g/cm$^2$ of compressive force, then the conditioned medium was collected and used as stimulants. (C) Medium conditioned by 7.5 g/cm$^2$ of compressive force (0–50 μl) were added to RAW264.7 cells. Data are expressed as the mean ± SD of triplicate cultures. Student's *t*-test, $^*P < 0.05$.

### 3.3. Effect of conditioned medium derived from MC3T3-E1 cells on RANKL-induced activation of NF-κB signaling pathway in RAW264.7 cells

We investigated the effect of mechanical stress on signal transduction in the process of osteoclast differentiation in RAW264.7 cells cultured with conditioned medium derived from MC3T3-E1 cells. Addition of medium conditioned by 7.5 g/cm$^2$ of force suppressed the expression of TRAF6, whereas the expression of RANK was not changed (Fig. 3A). Furthermore, we evaluated the effect of conditioned medium on phosphorylation of p38 MAPK and IκBα and expression of p50/p65, the most common NF-κB dimer, during osteoclast differentiation of RAW264.7 cells. Conditioned medium did not affect the phosphorylation of p38 MAPK (Fig. 3B). In contrast, it inhibited the phosphorylated levels of IκBα and expression of p50/p65 in the nuclear fraction of RAW264.7 cells induced by RANKL (Fig. 3C).

### 3.4. Involvement of non-canonical Wnt/$Ca^{2+}$ pathway in RAW264.7 cells under the mechanical stress

To investigate the relationship between the non-canonical Wnt/$Ca^{2+}$ pathway and osteoclast differentiation, we examined the effect of KN-93, a selective $Ca^{2+}$/calmodulin-dependent protein kinase II inhibitor, on the expression of OPG mRNA in RAW264.7 cells. MC3T3-E1 cells in collagen gel were cultured with KN-93 for 24 h and subjected to 7.5 g/cm$^2$ of compressive force for 12 h. Quantitative real-time RT-PCR analysis revealed that the expression of OPG mRNA induced by mechanical stress was remarkably suppressed by KN-93 (Fig. 4A). Finally, we determined the amount of OPG protein in conditioned medium by ELISA. Mechanical stress increased OPG secretion from MC3T3-E1 cells in a force-dependent manner, which was significantly suppressed by KN-93 (Fig. 4B). To examine the interaction between conditioned medium and RANKL, the affinity between them were determined using a QCM technique. In the presence of KN-93, the reduction of frequency by conditioned medium (7.5 g/cm$^2$) was recovered by 1618–958 Hz (Fig. 4C).

## 4. Discussion

It has been reported that mechanical stress functions as a critical regulatory factor in bone metabolism, and is also a postnatal determinant of bone homeostasis and skeletal morphology [23]. Although mechanical stress generates response from mechanosensitive cells, including bone cells, fibroblasts and epithelial cells have also been found to have responsiveness to mechanical stress [23,24]. Furthermore, recent studies have shown that osteoclast differentiation of RAW264.7 cells induced by RANKL was significantly decreased with oscillatory fluid flow [25]. Mechanical stress was also found to inhibit the expression of RANKL by murine stromal cells [26]. We previously reported that compressive mechanical force promoted osteoclast formation through RANKL expression in synovial cells derived from rat knee joints [4], while another study demonstrated that compressive force stimulation increased the levels of soluble RANKL and decreased those of OPG [27]. However, accurate details of the mechanisms by which mechanical

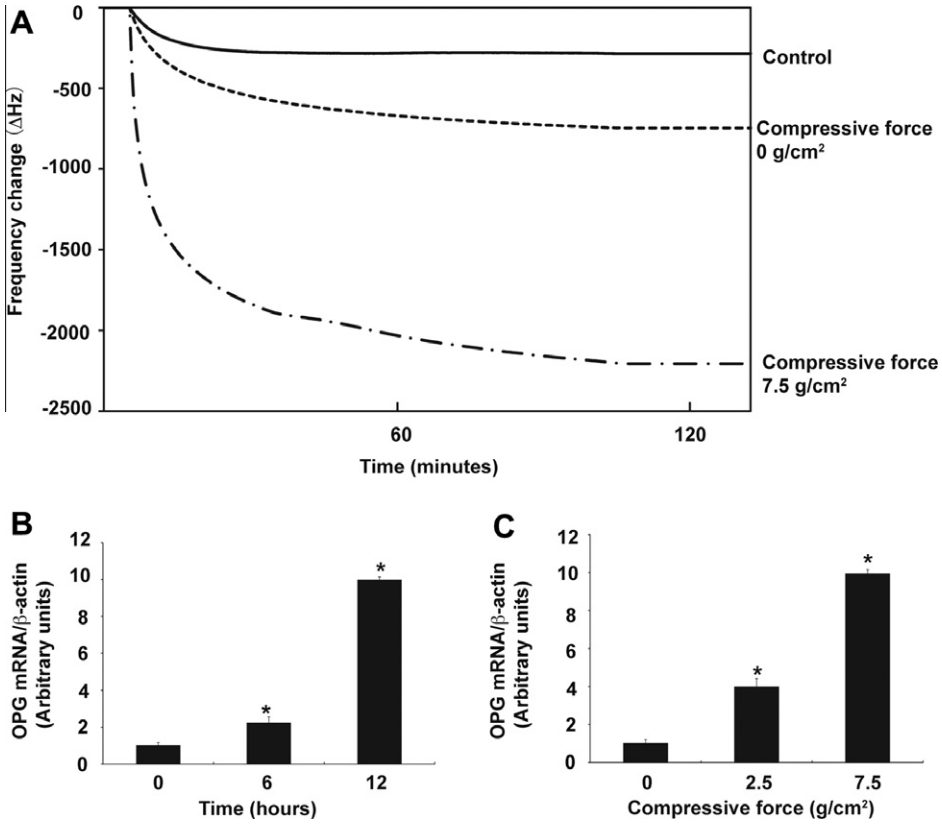


**Fig. 2.** Conditioned medium derived from MC3T3-E1 cells blocks binding of RANK to RANKL via enhancement of OPG expression MC3T3-E1 cells were cultured in collagen gels and subjected to 7.5 g/cm$^2$ of compressive force for 48 h, then conditioned medium were collected. (A) The binding ability of RANKL to conditioned medium was assessed using a QCM, as described in Section 2. MC3T3-E1 cells were cultured in collagen gels and subjected to compressive force. The fold change in OPG copy number between control and treated culture was determined by real-time RT-PCR, as described in Section 2. (B) Representative results from a time-dependent experiment with a compressive force of 7.5 g/cm$^2$. (C) Representative results from a force-dependent experiment at the time point of 12 h. Data are expressed as the mean ± SD of triplicate culture. Student's *t*-test, $^*P < 0.05$.

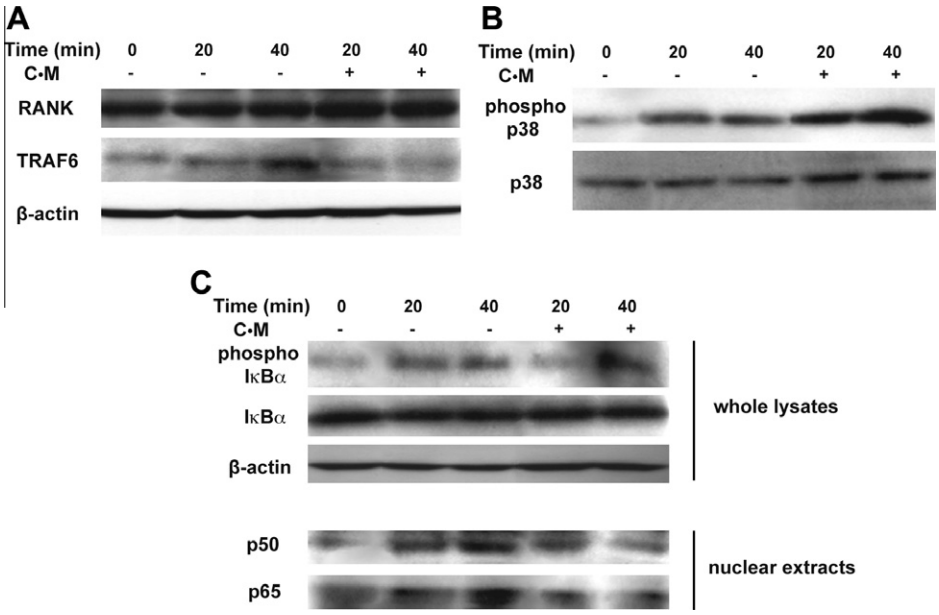


**Fig. 3.** Conditioned medium derived from MC3T3-E1 cells subjected to compressive force interferes with NF-κB signaling pathway in RAW264.7 cells RAW264.7 cells were stimulated with RANKL (40 ng/ml) in the presence or absence of conditioned medium derived from MC3T3-E1 cells subjected to compressive force for the indicated times. Whole cell lysates or nuclear fractions of RAW264.7 cells were subjected to immunoblotting analysis. (A) Expressions of RANK and TRAF6. (B) Expressions of p38 MAPK and phosphorylated p38 MAPK. (C) Expression of IκBα, phosphorylated IκBα, and expression of p50 and p65.

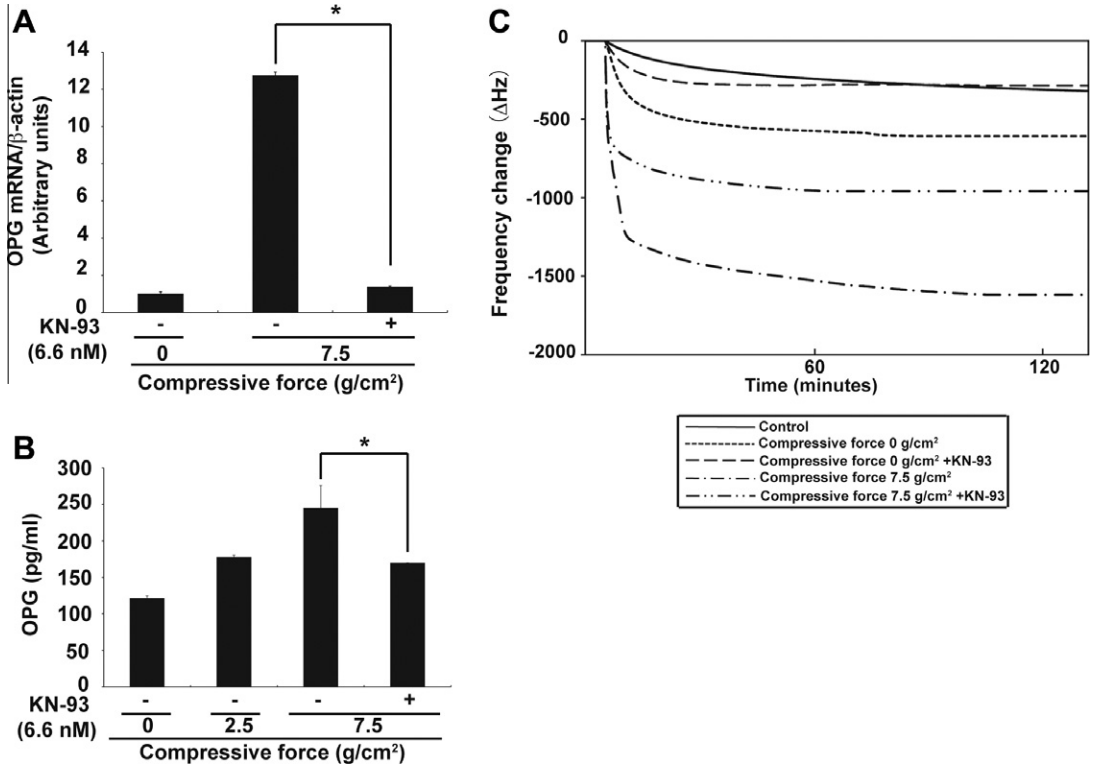


**Fig. 4.** Involvement of non-canonical Wnt/$Ca^{2+}$ pathway in the induction of OPG in MC3T3-E1 cells by compressive force MC3T3-E1 cells were cultured in collagen gels and subjected to compressive force in the presence or absence of KN-93. (A) The fold change in OPG copy number between control and treated culture was determined by real-time RT-PCR, as described in Section 2. (B) MC3T3-E1 cells were cultured in collagen gels and subjected to 0–7.5 g/$cm^2$ of compressive force for 24 h in the presence or absence of KN-93, then culture medium were collected. The amount of OPG in the culture medium was determined as described in Section 2. (C) MC3T3-E1 cells were cultured in collagen gels and subjected to 7.5 g/$cm^2$ for 48 h in the presence or absence of KN-93, then culture medium samples were collected. The binding ability of RANKL to conditioned medium was assessed using a QCM, as described in Section 2. Data are expressed as the mean ± SD of triplicate cultures. The experiment was performed 3 times, with similar results obtained in each. Student's *t*-test, $^*P < 0.05$.

stress affects the productions of RANKL and OPG have yet to be reported.

We previously showed that our 3D culture system made it possible to study the role of loaded osteoblasts in initiation of the bone

remodeling process, as it partially mimicked the *in vivo* environment [19]. 3D gel-embedded cultures of various cells were reported to support cell proliferation as well as differentiation into several different types of cells [19,28,29]. In the present study, mouse osteoblast lineage, MC3T3-E1 cells were embedded in 3D gels and cultured with mechanical stimuli, after which the conditioned medium were collected and added to RAW264.7 cells. We found that conditioned medium significantly decreased osteoclast differentiation (Fig. 1). Furthermore, compressive force enhanced the gene expression of OPG in MC3T3-E1 cells in both time- and force-dependent manners (Fig. 2B and C).

OPG is produced by several types of cells including osteoblastic cells, and has been shown to be a soluble decoy receptor for RANKL that blocks osteoclast formation by inhibiting RANK–RANKL interactions. In the present study, we clarified that mechanical stress increases the expression of OPG in MC3T3-E1 cells and then inhibits osteoclastogenesis. Other inhibitory factors for osteoclastogenesis such as interferon-γ (IFN-γ and Toll-like receptor (TLR) ligands have been shown to function by suppression of RANK signaling in osteoclast precursors [30]. We also evaluated the release of IFN-γ protein derived from MC3T3-E1 cells into the conditioned medium, however, no significant stimulation by mechanical stress was observed (data not shown). Together, these results suggest that the inhibitory effect of medium conditioned by mechanical stress on osteoclastogenesis is mainly dependent on the up-regulation of OPG expression.

A number of studies have investigated signaling pathways induced by RANK–RANKL binding. The cytoplasmic domain of RANK was shown to contain a binding site for TRAF6 [31]. In another study, NF-κB, MAPK, c-Jun N-terminal protein kinase (JNK), p38, and extracellular signal-regulated kinase (ERK) were found to be activated downstream of TRAF6 and induced osteoclast differentiation [32]. NF-κB is present in the cytoplasm as an active heterotrimer consisting of p50, p65, and IκBα subunits. Upon activation of the complex, phosphorylation and degradation of IκBα exposes nuclear localization signals on the p50/p65 complex, leading to nuclear translocation and binding to specific regulated sequences in DNA [33]. We found that mechanical stress suppressed the expression of TRAF6 protein, phosphorylation of IκBα, and nuclear translocation of p50 and p65 (Fig. 3). These results suggest that down-regulation of TRAF6 and NF-κB-mediated signaling pathway is correlated with inhibition of osteoclastogenesis by conditioned medium of mechanical-loaded osteoblast.

Mechanical stress is also known to stimulate multiple transduction cascades in several types of cells. It has been demonstrated that application of mechanical stress activates MAPKs, JNK, and ERK [34–36]. Furthermore, the canonical Wnt/β-catenin pathway was shown to have an important role in regulating osteoblast and osteoclast functions, as well as involvement in mechanotransduction [12]. Among the three Wnt pathways, non-canonical Wnt/$Ca^{2+}$ pathway is well known to regulate two different downstream signaling pathways, the $Ca^{2+}$/calmodulin-dependent protein kinase (CaMK) and calcineurin, $Ca^{2+}$/calmodulin-dependent phosphatase (CaMP) pathway [37]. Although calcineurin regulates osteoclast differentiation via activity of the nuclear factor of activated T cells [38], the effect of CaMK in osteoclastogenesis is not clear. Yu et al. [18] reported that mechanical stress-mediated OPG induction was regulated by the non-canonical Wnt/$Ca^{2+}$ pathway and especially the CaMK II-NLK cascade in myoblast lineage cells. Interestingly, we clearly demonstrated that the expression level of OPG mRNA and protein induced by mechanical stress was remarkably suppressed by KN-93, a selective $Ca^{2+}$/calmodulin-dependent kinase II inhibitor (Fig. 4A and B). Furthermore, the enhanced binding ability of medium conditioned by mechanical stress was diminished by the addition of KN-93 (Fig. 4C). Together, these findings suggest that stimulation of OPG mRNA and protein expression by mechanical stress is dependent on CaMK.

In conclusion, we found that compressive force enhanced the expression of OPG in osteoblasts by activation of the non-canonical Wnt/$Ca^{2+}$ pathway. These results suggest that osteoblasts have the capacity to sense changes in mechanical stress, resulting in regulation of osteoclastogenesis.

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