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博 士 論 文

Optimal compressive force accelerates osteoclastogenesis in RAW264.7 cells

（至適圧縮力は **RAW264.7** 細胞において破骨細胞形成を促進する）

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Optimal compressive force accelerates osteoclastogenesis in RAW264.7 cells

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Abstract

Mechanical stress produced by orthodontic force is a factor in the remodeling of periodontal ligament (PDL) and alveolar bone, and consists of compressive and tensile forces. It has been reported that the expression of many cytokines associated with osteoclastogenesis, such as receptor activators of nuclear factor-κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF), are up-regulated when compressive forces act on osteoblasts and PDL cells. However, little is known about the direct effects of compressive forces on osteoclasts. Therefore, we investigated the effects of compressive force on osteoclasts in this study. The numbers of tartrate-resistant acid phosphatase (TRAP)-positive multinucleated osteoclasts derived from RAW264.7 cells were counted. The gene expression of osteoclasts associated with differentiation and fusion changed in response to compressive force, and was evaluated by real-time PCR. Differentiation and fusion of osteoclasts increased rapidly on the 4th to 5th days. We then examined the effects of compressive force on osteoclasts using 3, 5, 7, 9 and 14-layered cover glass slips (slips) on the 4th day for 24 h. The number of large osteoclasts with more than 8 nuclei peaked with 7 slips. The force generated by 7 slips was regarded as the optimal compressive force. mRNA expression of osteoclast-associated genes, including nuclear factor of activated T cells c1 (NFATc1), TRAP, matrix metalloproteinase-9 (MMP-9), dendritic cell specific trans membrane protein (DC-STAMP), osteoclast stimulatory trans membrane protein (OC-STAMP), cathepsin-K (cath-K), chloride channel 7 (ClC-7), ATPase H^+ transporting vacuolar proton pump member I (ATP6i), Integrin-αv and β3, increased significantly after 3 h of optimal compression. Thus, we were successful in directly applying

compressive force to osteoclasts. These findings suggest that osteoclastogenesis accelerates via up-regulated and advanced mRNA expressions when applying optimal compressive force.

1. Introduction

The purpose of orthodontic treatment is to establish the most suitable occlusion for an individual. Orthodontic force generates mechanical stress, which is a factor that brings about remodeling of the periodontal ligament (PDL) and alveolar bone [1, 2]. Previous studies revealed the response of various mechanical stimulations on osteoblasts and PDL cells. Mechanical stimuli, such as tensile force [3, 4, 5], compressive force [6, 7, 8, 9, 10], fluid flow [11, 12], hydrostatic pressure [13], rotative stress [14] and others [15, 16], are influential factors in bone remodeling. In particular, orthodontic force consists of compressive and tensile forces. Tensile force leads to bone formation, while compressive force causes bone resorption. Many studies have reported that applying such forces leads to secretion of cytokines associated with bone resorption and formation. Bone is a complex tissue whose remodeling involved both bone formation and resorption [17, 18, 19]. Many types of cells in periodontal ligament joined bone remodeling, such as osteoblasts, osteoclasts, fibroblasts and others.

Tensile force using a Flexercell tension system suppressed osteoclast differentiation and fusion via increased nitric oxide (NO) and promoted differentiation and fusion after release from this stress [4], while mechanical stress inhibited osteoclast differentiation and fusion though dendritic cell specific trans membrane protein (DC-STAMP) and other fusion-related molecules [5]. Furthermore, when compressive force is applied to osteoblasts and PDL cells, essential factors that promote osteoclast differentiation and activation, such as prostaglandin E_2 (PGE_2), interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, receptor activator of

nuclear factor-κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) [15, 16], are up-regulated. It has also been reported that the number of osteoclasts and osteoclast activity are increased by hypoxia, which up-regulates RANKL levels in osteoblasts [20]. However, it remains unclear whether directly compressive force regulates osteoclastogenesis.

Osteoclasts are derived from the monocyte/macrophage lineage and are formed by the fusion of mononuclear precursors. RANKL is an essential factor for osteoclast differentiation and activation [21]. RANKL is a member of the TNF family and is expressed as a transmembrane protein secreted by osteoblasts and stromal cells, and is bound to RANK, which is expressed on tartrate-resistant acid phosphatase (TRAP)-positive cells [19]. RANK is induced by M-CSF in osteoclastogenesis, and is an essential factor for the formation of mature osteoclasts [22]. RAW264.7 (RAW) cells express RANK and can differentiate into osteoclasts after RANKL stimulation without M-CSF. RANKL signaling leads to activation of TNF receptor-associated factor 6 (TRAF6), c-Fos and nuclear factor-κB (NF-κB) pathways, which induces the master transcription factor of nuclear factor of activated T cells c1 (NFATc1). NFATc1 is an essential regulator of numerous osteoclast-specific genes associated with differentiation, fusion and bone resorption [23].

Therefore, the aim of this study was to investigate the responses of osteoclast differentiation and fusion induced by RAW cells by applying various magnitudes of compressive force for various periods of time up to 24 h.

2. Materials and methods

2.1. Cell culture

We used the murine monocyte/macrophage cell line RAW cells (ATCC no. TIB-71™; Manassas, VA) as osteoclast precursors. RAW cells differentiated into osteoclast-like cells in the presence of RANKL. Cells were kept in Dulbecco's modified Eagle's medium (DMEM; Wako Pure Chemical, Osaka, Japan) added 10% heat-inactivated fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA) and 66.7 μg/ml kanamycine sulfate (Meiji Seika, Tokyo, Japan) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Cells were seeded onto 100-mm standard dishes (BD Falcon, Franklin Lakes, NJ) and cultured overnight. A 12-mm diameter glass cover slip (Fisher Microscope Cover Glass; Fisher Scientific, Pittsburgh, PA) was placed on the wells of a 24-well culture plate (BD Falcon, Franklin Lakes, NJ). To differentiate osteoclasts, RAW cells were transferred into 24-well culture plates at 1.0×10^4 cells/well and were cultured in α-minimum essential medium (α-MEM; Wako Pure Chemical) supplemented with 10% heat-inactivated FBS, 2mM L-alanyl-L-glutamine (Wako Pure Chemical), 284 μM L-ascorbic acid 2-phosphate (Sigma-Aldrich, St. Louis, MO), 66.7 μg/ml kanamycine sulfate and 50 ng/ml RANKL (Oriental Yeast Co., Ltd., Tokyo, Japan) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Medium containing these reagents was replaced every other day.

2.2. Reversing TRAP-positive cells on glass cover slips

Prior to culture, we prepared sterilized 15-mm diameter silicone tubes sliced to a thickness

of 2 mm (Fig. 1A). RAW cells were cultured on 12-mm diameter glass cover slips (slips) on sliced silicone tubes in 24-well culture plates until the 5th day, and the slips were then reversed in same wells filled with culture medium after 3 days for 48 h, or after 4 days for 24 h (Fig. 1B). The control group was cultured for the same time without reversing the slips.

2.3. Preparation of collagen gels

Acid-soluble collagen solution (Cellmatrix, Nitta Gelatin Inc., Osaka, Japan), ten-fold concentrated α-MEM, and reconstruction buffer (2.2 g $NaHCO_3$ + 4.77 g HEPES in 100 ml of 0.05 N NaOH) were mixed at a volume ratio of 8:1:1, and 10% heat-inactivated FBS, 284 μM L-ascorbic acid 2-phosphate and 2 mM L-alanyl-L-glutamine were added in an ice-cold bath kept at below 4°C. This prepared collagen mixture was dropped into 24-well culture plates at a volume of 500 μl/well, and subjected to gelation in a CO_2 incubator at 37°C for 30 min. After the collagen mixture had gelled, 50 ng/ml RANKL and 1 ml/well of culture medium were overlaid in each well.

2.4. Application of compressive forces

Prior to the application of compressive force, RAW cells were incubated on the slip until the 3rd day (Fig. 1C, left). Collagen gel layers were prepared in other 24-well culture plates. Subsequently, osteoclasts on the slips were reversed and placed on top of these collagen gel layers (Fig. 1C, right), and were continuously compressed with 3, 5, 7, 9 and 14 layered slips (about 43 mg/slip) for 24 h, respectively (Fig. 1D). The control group comprised osteoclasts

on the slips only reversed onto the collagen gel layer (Fig. 1C, right).

2.5. TRAP staining

Cells were compressed for the indicated times. After compression, cells were fixed with 10% neutral formalin. They were then washed with distilled water and stained with Fast Red Violet LB Salt (Sigma Aldrich) in TRAP staining solution (pH 5.0). TRAP-positive cells with 2-7 nuclei were considered to be small osteoclasts, and those with >8 nuclei were considered to be large osteoclasts [3, 4]. The numbers of osteoclasts were counted with a light microscope (×100). Osteoclasts were counted in a 20-mm^2 rectangle within the circular field.

2.6. Pit assay

A disc (ϕ12-mm) made of Osteologic™ (BD Biosciences, Franklin Lakes, NJ) was coated with calcium phosphate. Osteoclasts were cultured for 3 days on these discs under the same conditions as described in cell culture paragraph, and the discs were reversed on the top of a collagen gel layer in 24-well culture plates on the 4th day for 24 h as a control group. Another group of reversed osteoclasts were subjected to compressive force by 7 layered slips. On the 5th day, the control and compressed osteoclasts on the discs were rinsed twice with distilled water. Then, 1 ml of bleach solution (6 % NaOCl, 5.2 % NaCl) was added to each well, followed by pipetting up and down to remove cells and incubation for five minutes at room temperature. Discs were washed with 2 ml of distilled water three times and examined

under a light microscope (×100) after drying [24, 25]. Resorption area was measured using image J software.

2.7. Reverse transcription-polymerase chain reaction and real-time PCR

Cells were compressed for 1, 3, 6, 12 and 24 h in 24-well culture plates. Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Reverse transcription was conducted with 1 μg of RNA to obtain cDNA. The cDNA was amplified by Rever Tra Ace-α FSK-101 (Toyobo, Osaka Japan). Primers (Applied Biosystems, Carlsbad, CA) for the following genes were used: NFATc1 (Mm00479445_m1); TRAP (Mm00475698_m1), matrix metalloproteinase-9 (MMP-9) (Mm00432271_m1); dendritic cell specific trans membrane protein (DC-STAMP) (Mm01168058_m1); osteoclast stimulatory trans membrane protein (OC-STAMP) (Mm00512445_m1); Integrin-αv (Mm00434506_m1); Integrin-β3 (Mm00443980_m1); cathepsin-K (cath-K) (Mm00484036_m1); chloride channel 7 (ClC-7) (Mm00442400_m1);, ATPase H^+ transporting vacuolar proton pump member I (ATP6i) (Mm00469395_g1); and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (Mm99999915_g1). Real-time PCR was performed using the ABI Prism 7300 sequence detection system (Applied Biosystems). Data obtained for each sample were standardized against the expression of GAPDH and were calculated using the $2^{-\Delta\Delta Ct}$ method [26, 27].

2.8. Statistical analysis.

All results are given as means ± SD. Comparisons between 2 groups were analyzed by 2-tailed unpaired Student's *t*-test. *P* values of <0.05 were considered to be statistically significant.

3. Results

3.1. Number of osteoclasts on various culture days

In order to investigate when osteoclast differentiation and fusion were activated, we assessed the number of osteoclasts for 5 days and the number of nuclei in osteoclasts. Osteoclasts induced from RAW cells were cultured with 50 ng/ml RANKL on 24-well culture plates for 5 days. The number of osteoclasts increased gradually on the 3rd and 4th days, and rapidly on the 5th day, but none were seen on the 2nd day (Fig. 2A). We counted the number of osteoclasts and the number of nuclei. Osteoclasts had 2-4 nuclei on the 3rd day, and began to appear with >5 nuclei on the 4th day. Subsequently, osteoclasts showed >6 nuclei (Fig. 2 B).

3.2. Effects of reversing glass cover slips on osteoclastogenesis

We investigated the effects of reversing the slips on osteoclastogenesis in sliced silicone tubes for 24 and 48 h (Fig. 1 B). Controls were cultured on slips without reversing and were fixed on the 5th day. We counted the number of TRAP-positive cells with 2-7 nuclei and those with >8 nuclei. The number of osteoclasts showed no significant differences when compared with the control group at either 24 h or 48 h (Fig. 2 C).

3.3. Effects of compressive force of various intensities for 24 h on osteoclastogenesis

Various intensities of compressive force were applied to osteoclasts for 24 h after 4-5 days of culture. RAW cells on slips were cultured as described above. Cells were reversed onto the

collagen gel layer in other plates on the 4th day. Furthermore, cells were loaded with layers of 3-14 slips after the cells were reversed. The number of osteoclasts was significantly increased when the cells were compressed with 7 slips (Fig. 3A). The number of osteoclasts with >8 nuclei were significantly elevated (Fig. 3B).

3.4. Bone resorption activity of osteoclasts subjected to compressive forces with 7 slips

We examined the bone resorption activity of osteoclasts when the cells were subjected to compressive force with 7 slips. RAW cells were cultured on Osteologic™ discs as described above. We then obtained light micrographs of Osteologic™ discs (×100) in the control and compressed (CF) groups (Fig. 3C). We measured the areas where calcium phosphates on discs were completely resorbed. The resorption areas of the discs by the control group and CF group are shown in the figure (Fig. 3C). The resorption areas in the compressed group were significantly larger when compared with the control group (Fig. 3 D).

3.5. Expression of osteoclast-associated mRNA

We examined the effects of compressive force on the mRNA levels of osteoclast-specific genes associated with differentiation, fusion and adherence using real-time PCR analysis (Fig. 4). The analysis showed that compressive force with 7 slips up-regulated mRNA levels in osteoclasts at 3 h when compared with control group.

mRNA expression in CF groups of TRAP, cath-K, ATP6i and DC-STAMP were significantly increased at 1, 3, 6 and 12 h. NFATc1, MMP-9, ClC7, integrin-αv and

OC-STAMP were significantly increased at 1, 3 and 6 h, whereas NFATc1, ClC7, DC-STAMP and Integrin β3 were significantly decreased at 24 h when compared with the control group. In addition, all mRNA levels peaked at 3 h in CF group, whereas, all mRNA levels peaked at 24 h in control group.

4. Discussion

In this study, we directly applied compressive force to osteoclasts for the first time. We also showed that the optimal compressive force up-regulated expression of various osteoclast associated genes and promoted osteoclast differentiation and fusion. The results shown in Fig. 2 B suggested that RAW cells cultured with RANKL started osteoclastogenesis on day 3 and advanced rapidly on day 5. A previous study demonstrated that both small osteoclasts with 2-7 nuclei appeared on the 3rd day [27]. Osteoclasts continued to increase up to the 6th day [27, 28], and osteoclasts began to undergo apoptosis from the 7th day [3]. Therefore, we determined the best time to apply compressive force was after 4-5 days.

The technical manual for collagen gel (Cellmatrix) noted that the gel was developed for tissue culture, allowing it to be used for the culture of various cells. We found that the collagen gel layer could act as a cushion material for PDL when compressive force was applied to osteoclasts. We were also able to avoid innutrition and cell death during application of the compression, ensuring that osteoclasts could be cultured on the bottom of a 24-well culture plate and subjected to compressive force by the collagen gel layer. However, we were unable to count the cell number and measure mRNA expression in these osteoclasts. We also found that RAW cells were able to differentiate and fuse only on a rigid surface. The cells did not promote osteoclastogenesis on the collagen gel [29], and only underwent cell division to form a mass of mononucleated cells. Therefore, we devised the methods used in this study.

Before applying compression, we examined the effects of reversing cover slips on

osteoclasts differentiayion when compared with osteoclasts on the same slips without reversal. Differences in the numbers of osteoclasts with or without reversing were not detected.

In order to culture and compress the osteoclasts, we cultured cells on slips and reversed them onto the collagen gel layer. The slips and collagen gel provided a similar micro circumstance of alveolar bone adjacent to PDL. In addition, osteoclasts on the slips reproduced osteoclasts on the bone surface. Application of compressive force for osteoclasts on slips via collagen gel *in vitro* was therefore similar to the application of orthodontic force to osteoclasts on the bone surface via PDL *in vivo*. In this method, we were able to culture and compress the cells, and measure the number of osteoclasts and their mRNA expression.

We examined effects of various weights (3-14 layering slips) on the number of osteoclasts. The number of osteoclasts was greatest when using 7 slips, and we considered this to be the optimal compressive force. Furthermore, the optimal compressive force may have particularly accelerated the fusion of osteoclasts. Compressive force also accelerated osteoclastogenesis in RAW cells, as compared with the control group.

Bone resorption areas of osteoclasts with Osteologic™ were significantly greater when applying the optimal compressive force. Optimal orthodontic force [30, 31, 32] provides a maximum biologic response and maximum rate of tooth movement, and exceeding this force reduces the rate of tooth movement and damages PDL cells [33], while forces below the optimal level lead to non-response of PDL and alveolar bone [34]. This suggests that the optimal compressive force activates osteoclast function, such as bone resorption and osteoclastogenesis.

Previous studies have demonstrated tensile force using the Flexercell tension system, finding that suppressed osteoclast differentiation and fusion via increased nitric oxide (NO), and demonstrating differentiation and fusion after release of tensile stress [4]. In addition, the tensile force inhibited osteoclast differentiation and fusion though DC-STAMP and other fusion-related molecules [5]. Numerous studies have also reported that compressive force on osteoblasts and PDL cells up-regulates various factors that promote osteoclastogenesis, such as PGE_2, IL-1β, IL-6, TNF-α, RANKL and M-CSF [7, 9].

mRNA expression of osteoclast-associated genes, NFATc-1, TRAP, cath-K, ClC7, MMP-9, ATP6i, DC-STAMP, OC-STAMP, Integrin-αv and β3 were up-regulated and accelerated after 3 h of compression at optimal force when compared with controls in this study. Among them, NFATc1 is thought be an essential transcriptional factor for autoamplification. This indicates that the optimal compressive force may activate osteoclastogenesis and up-regulated osteoclast-associated mRNA expression.

There have been numerous reports suggesting that applying compressive force to osteoblasts and PDL cells promotes osteoclastogenesis. In conclusion, we succeeded in applying compressive force to osteoclasts and found that optimal compressive force accelerates osteoclastogenesis. There would be another way to think about optimal orthodontic force for effective tooth movement in orthodontic treatment.

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Fig. 1 A

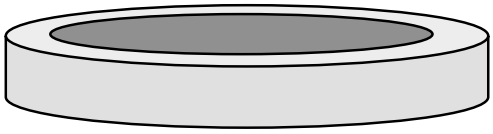


Fig 1 B

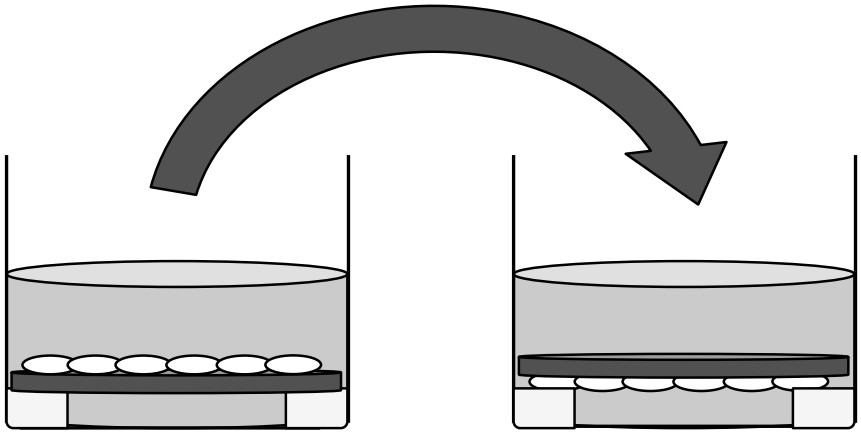
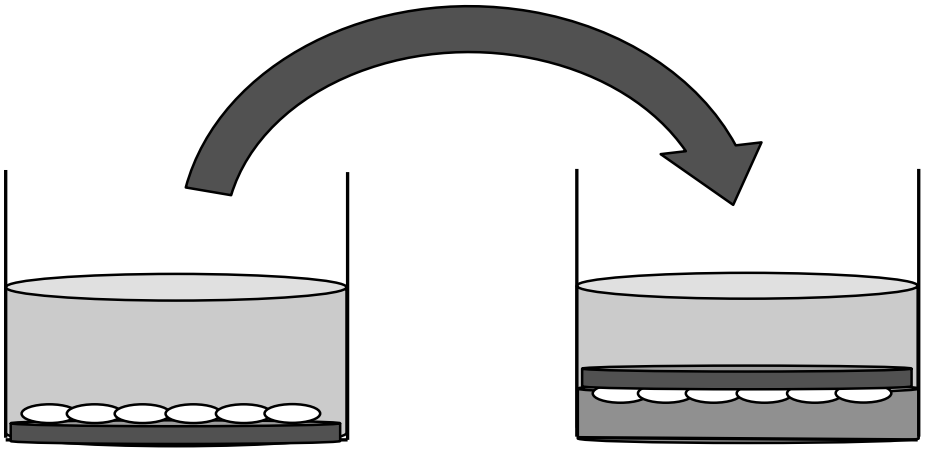


Fig. 1 C



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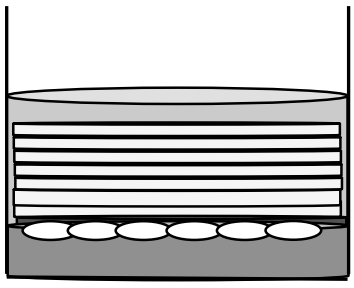


Fig. 2 A

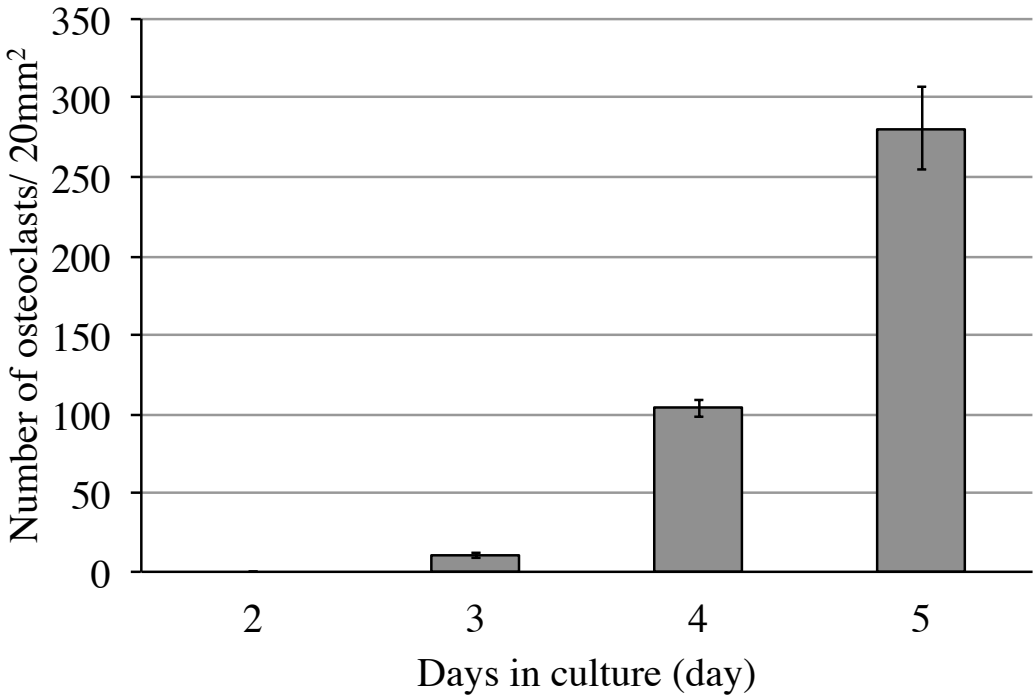

350
300
250
200
150
100
50
0
Number of osteoclasts/ 20mm2
2
3
4
5
Days in culture (day)

Fig. 2 B

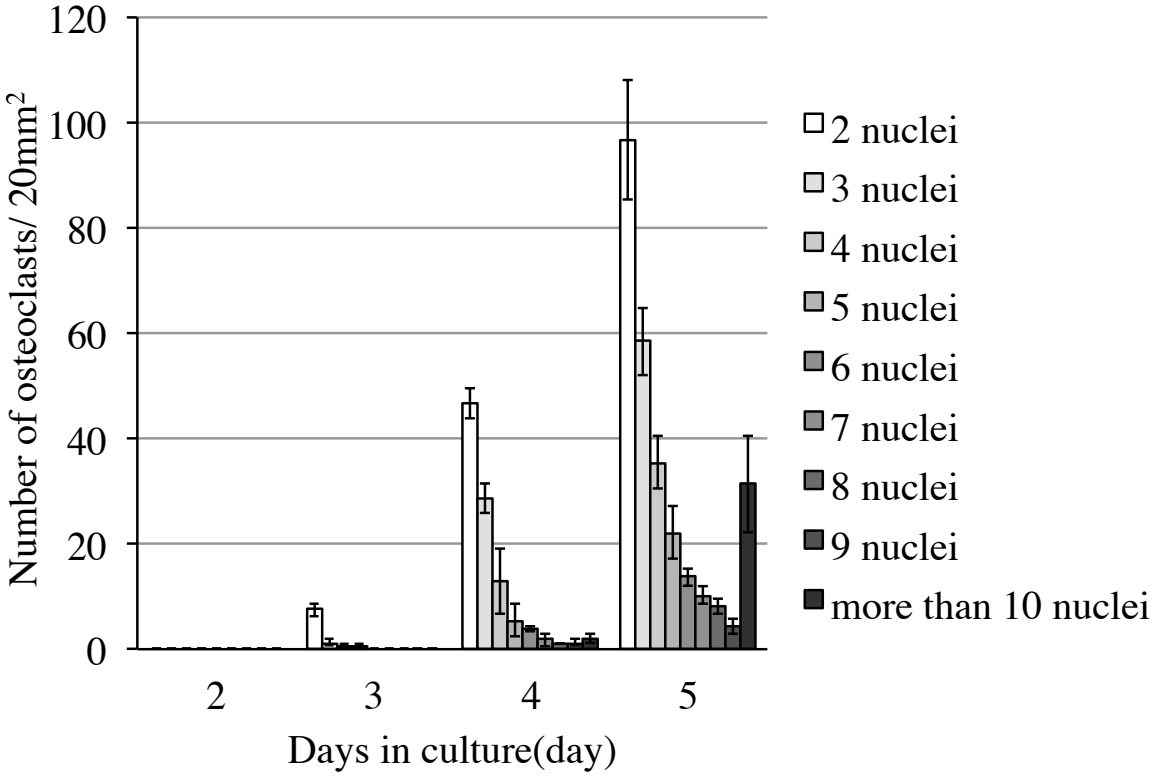

120
100
80
60
40
20
0
Number of osteoclasts/ 20mm2
2
3
4
5
Days in culture(day)
2 nuclei
3 nuclei
4 nuclei
5 nuclei
6 nuclei
7 nuclei
8 nuclei
9 nuclei
more than 10 nuclei

Fig. 2 C

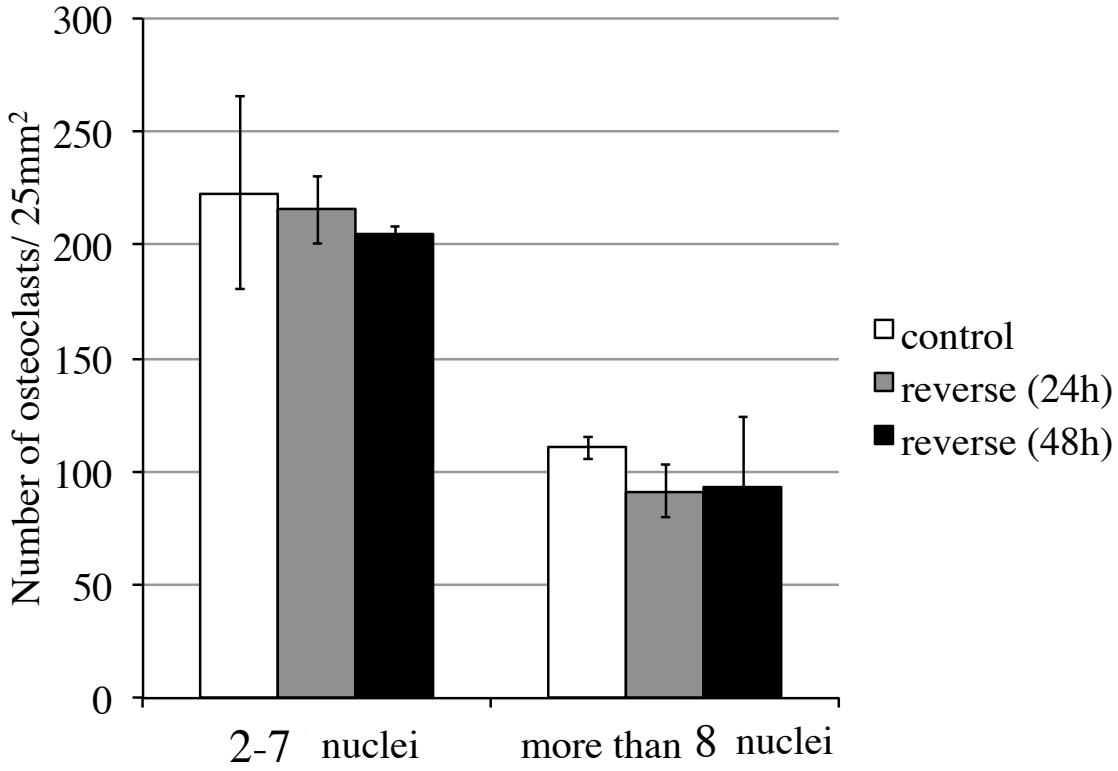

300
250
200
150
100
50
0
Number of osteoclasts/ 25mm2
2-7 nuclei
more than 8 nuclei
control
reverse (24h)
reverse (48h)

Fig. 3 A

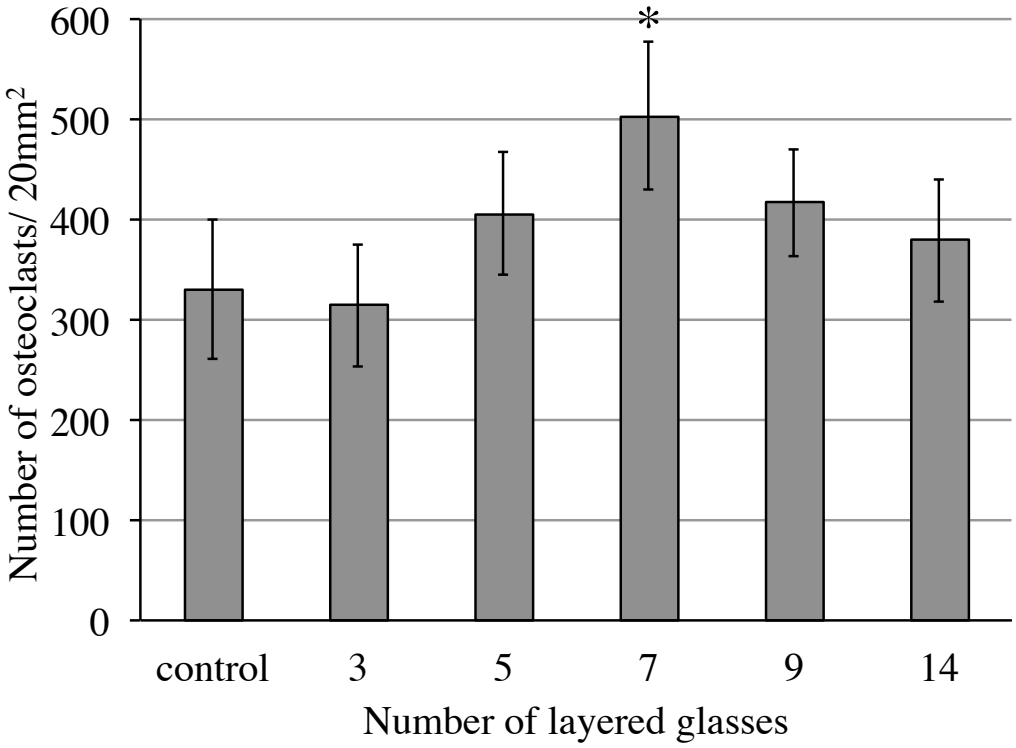

600
500
400
300
200
100
0
Number of osteoclasts/ 20mm2
*
control
3
5
7
9
14
Number of layered glasses

Fig. 3 B

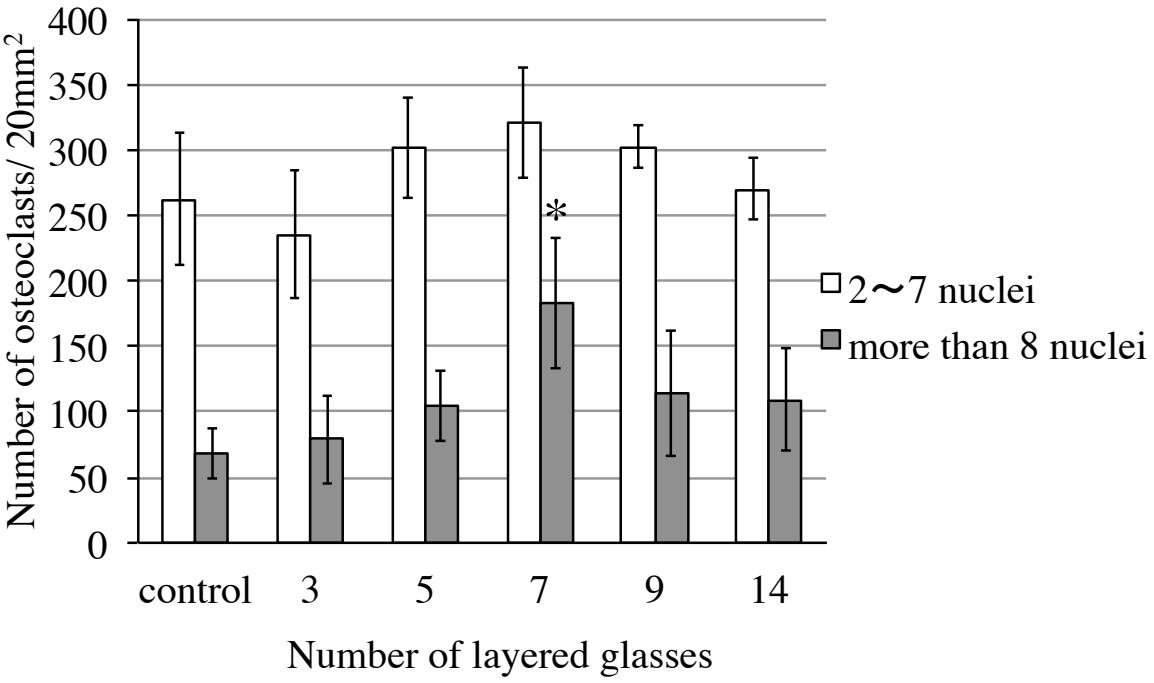
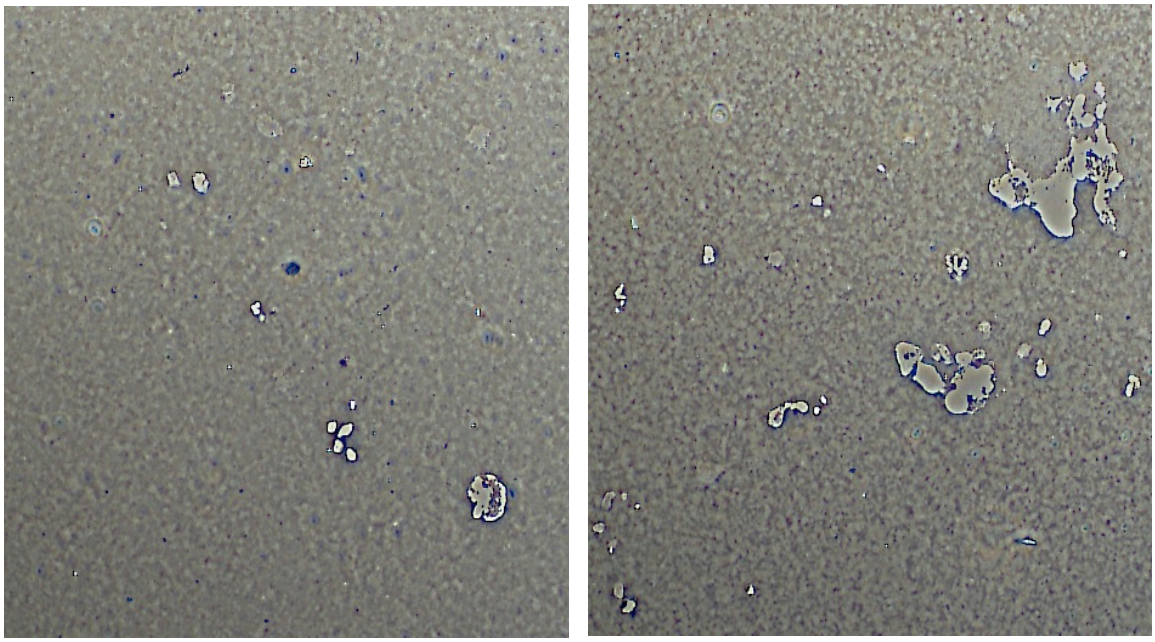

400
350
300
250
200
150
100
50
0
Number of osteoclasts/ 20mm2
*
control
3
5
7
9
14
Number of layered glasses
2～7 nuclei
more than 8 nuclei

Fig. 3 C



control　　compressive force: CF

Fig. 3 D

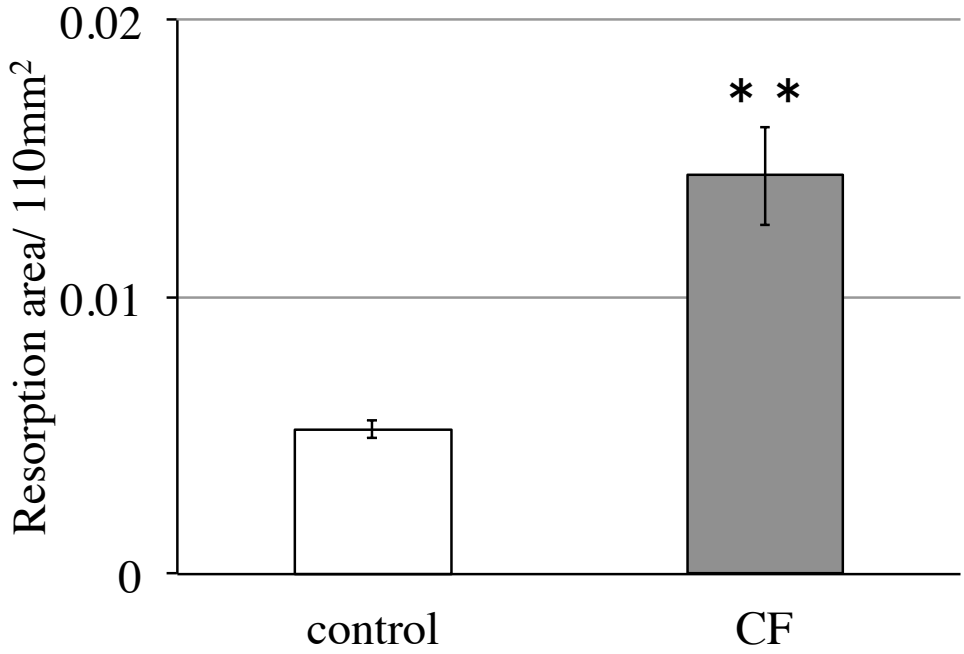
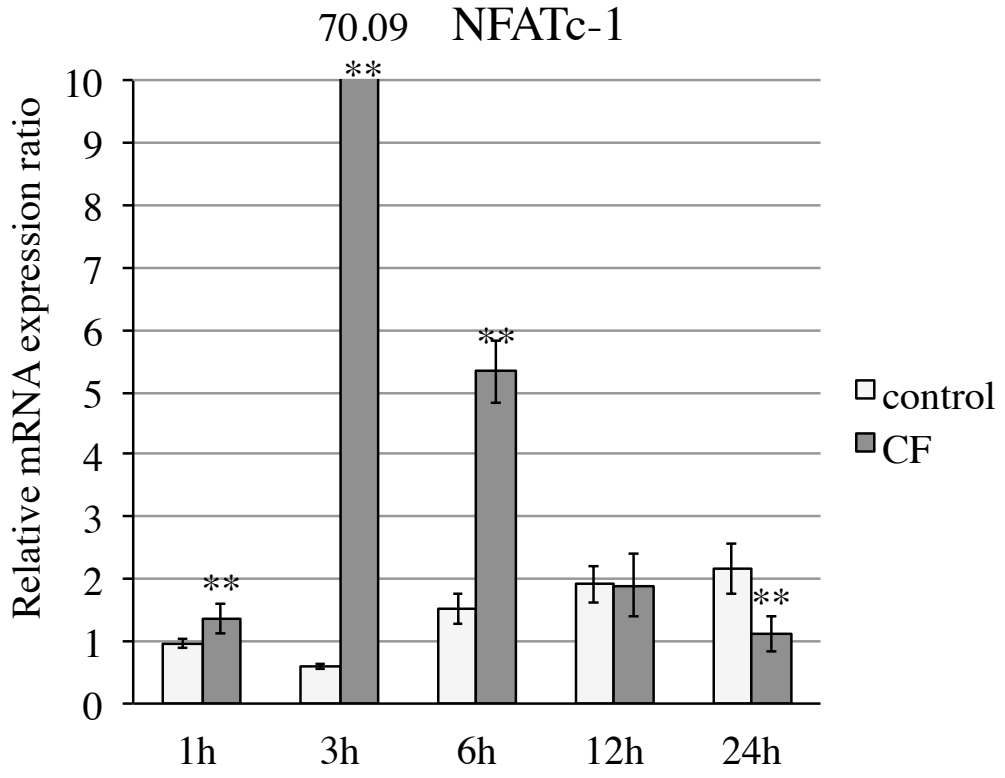
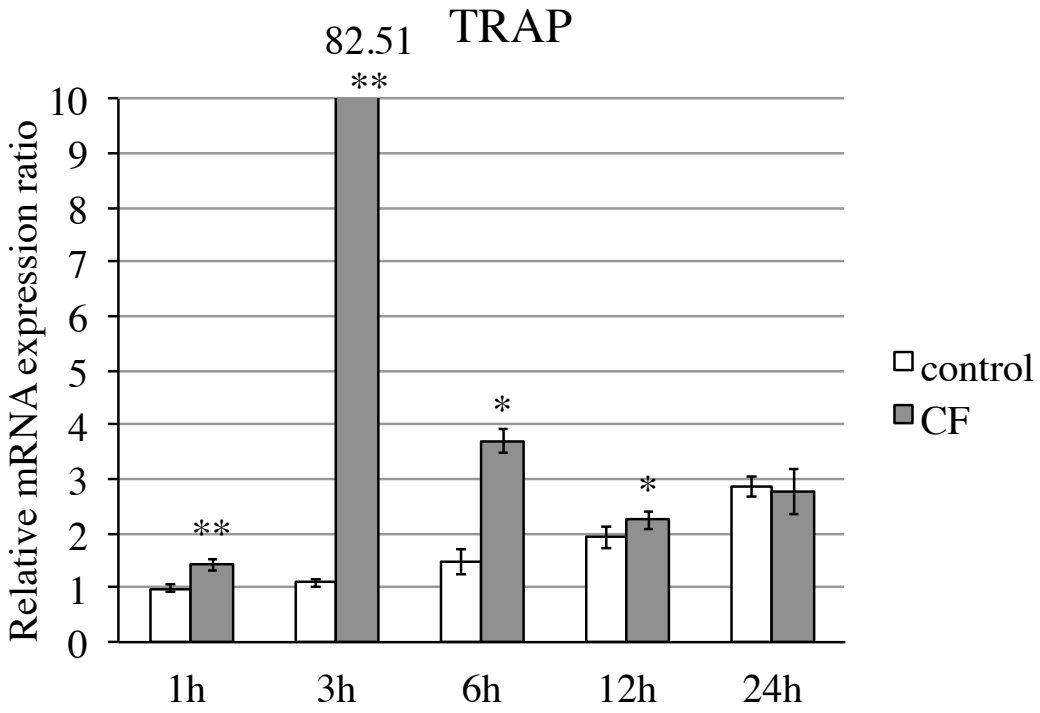
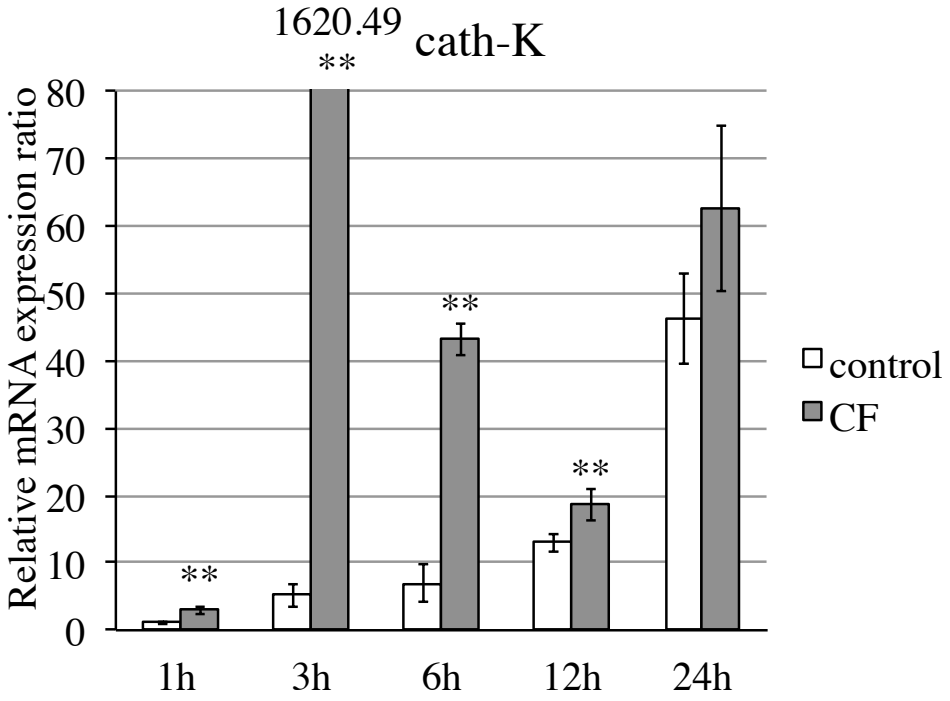
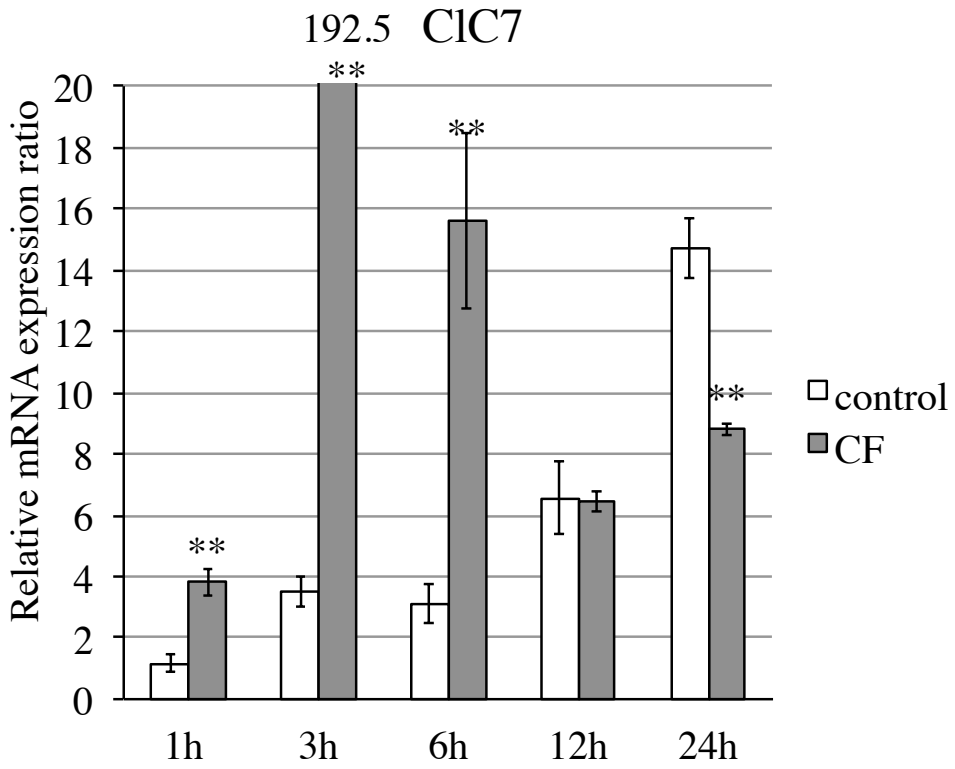


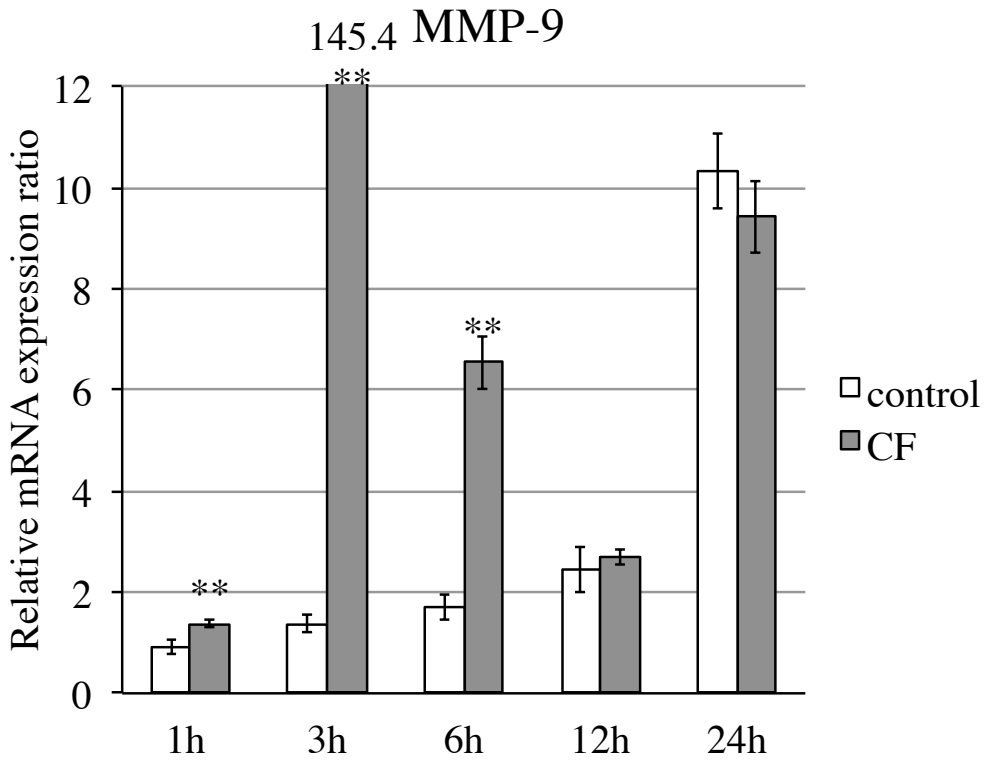
Fig. 4

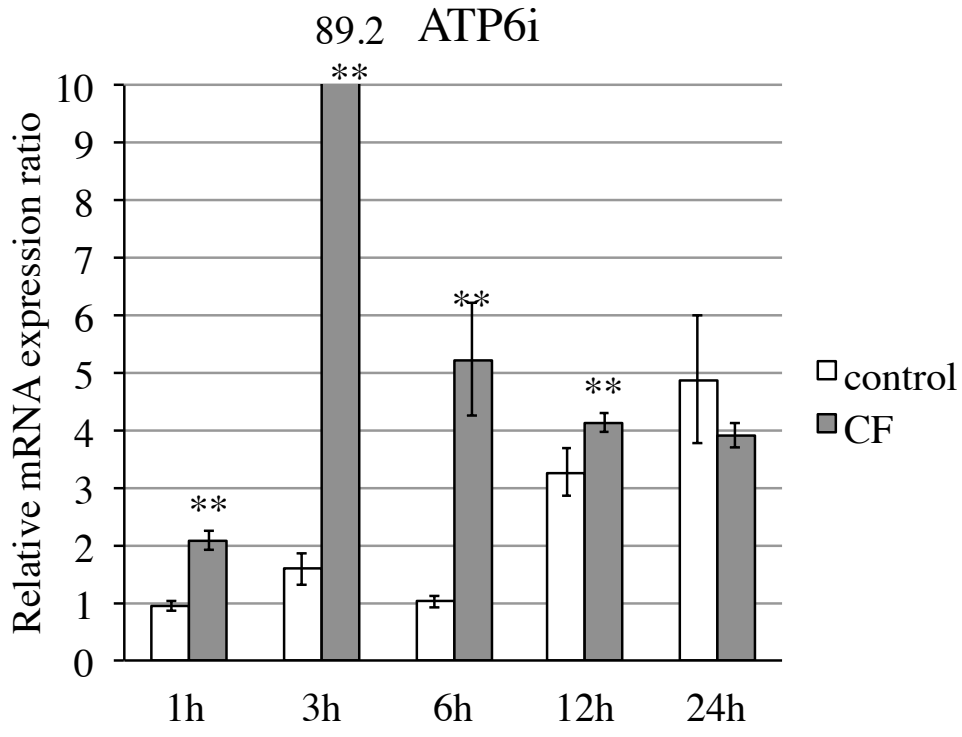

70.09
NFATc-1
Relative mRNA expression ratio
10
9
8
7
6
5
4
3
2
1
0
**
**
**
**
1h
3h
6h
12h
24h
control
CF

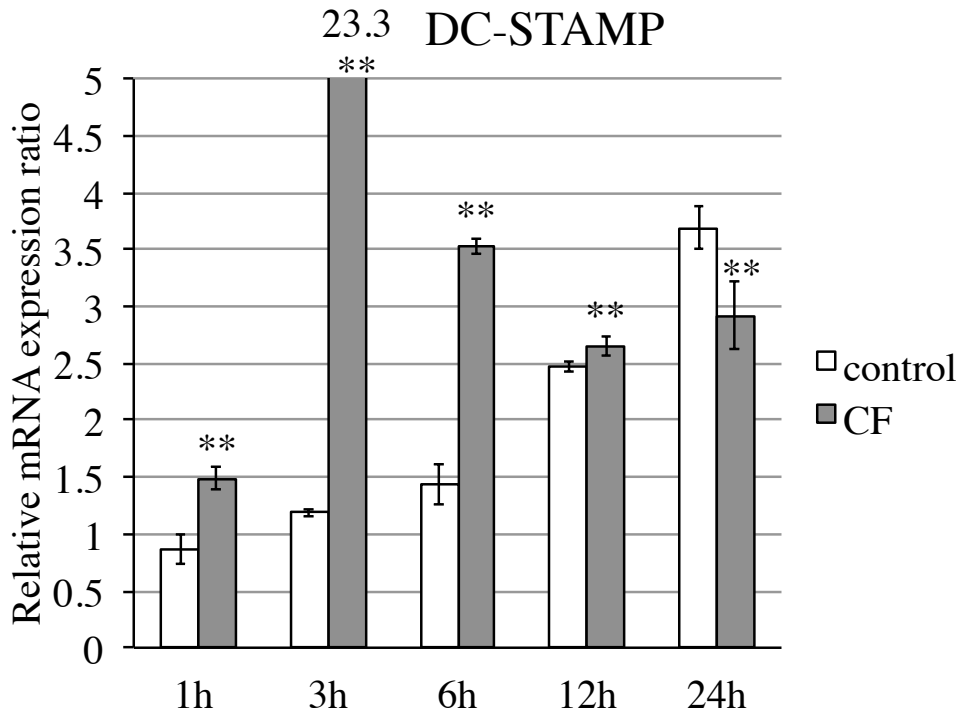

82.51
TRAP
Relative mRNA expression ratio
10
9
8
7
6
5
4
3
2
1
0
**
**
*
*
1h
3h
6h
12h
24h
control
CF

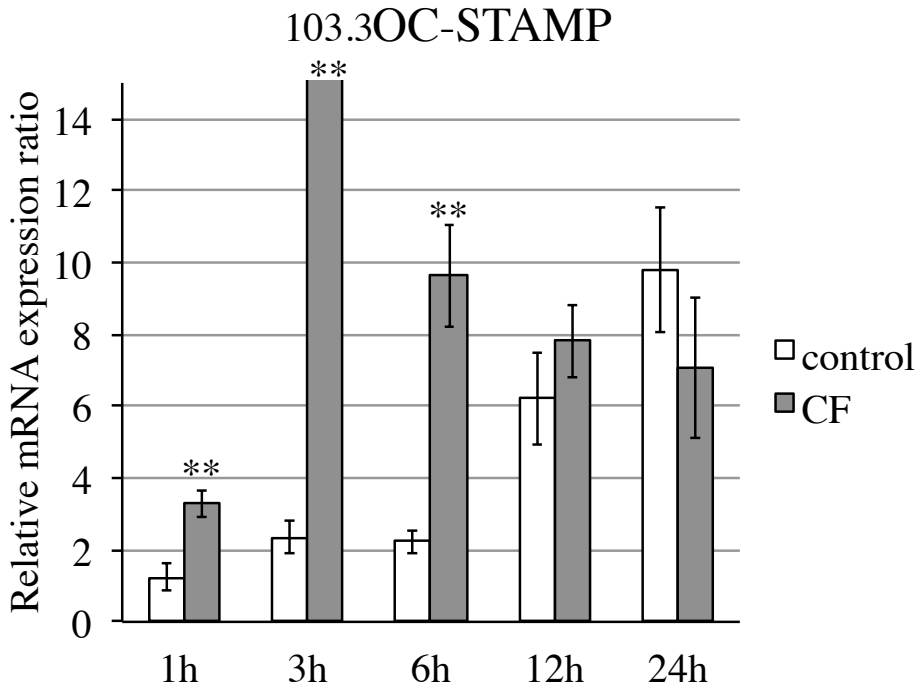

1620.49
cath-K
**
Relative mRNA expression ratio
80
70
60
50
40
30
20
10
0
**
**
**
1h
3h
6h
12h
24h
control
CF

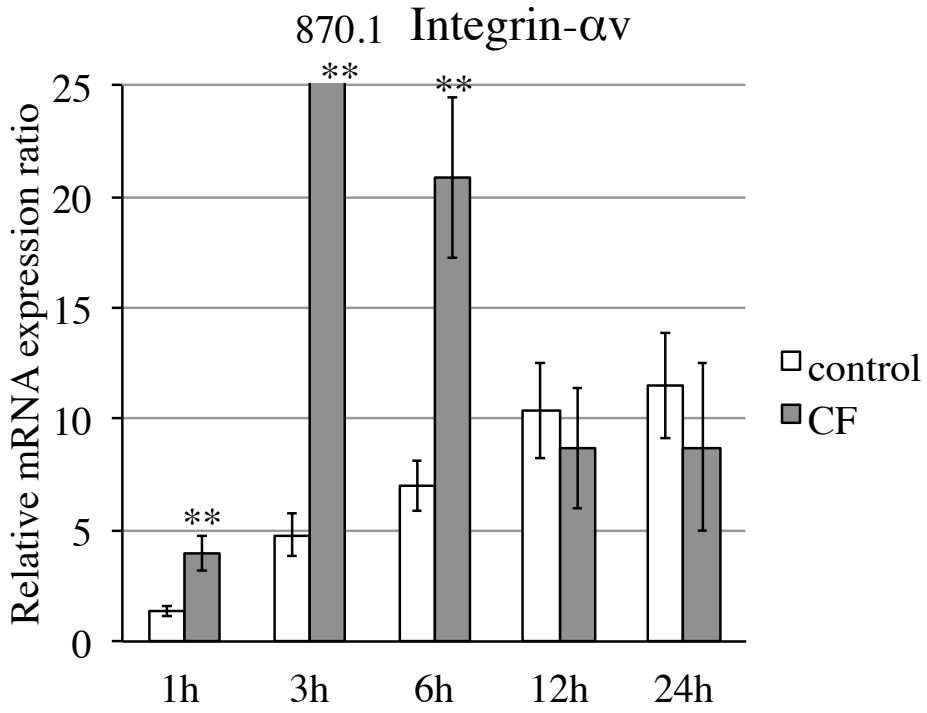

192.5
ClC7
**
Relative mRNA expression ratio
20
18
16
14
12
10
8
6
4
2
0
**
**
**
1h
3h
6h
12h
24h
control
CF


145.4
MMP-9
Relative mRNA expression ratio
12
10
8
6
4
2
0
**
**
**
1h
3h
6h
12h
24h
control
CF


89.2
ATP6i
Relative mRNA expression ratio
10
9
8
7
6
5
4
3
2
1
0
**
**
**
1h
3h
6h
12h
24h
control
CF


23.3 DC-STAMP
Relative mRNA expression ratio
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
**
**
**
**
**
1h
3h
6h
12h
24h
control
CF


103.3OC-STAMP
Relative mRNA expression ratio
14
12
10
8
6
4
2
0
**
**
**
1h
3h
6h
12h
24h
control
CF


870.1
Integrin-αv
Relative mRNA expression ratio
25
20
15
10
5
0
**
**
**
1h
3h
6h
12h
24h
control
CF

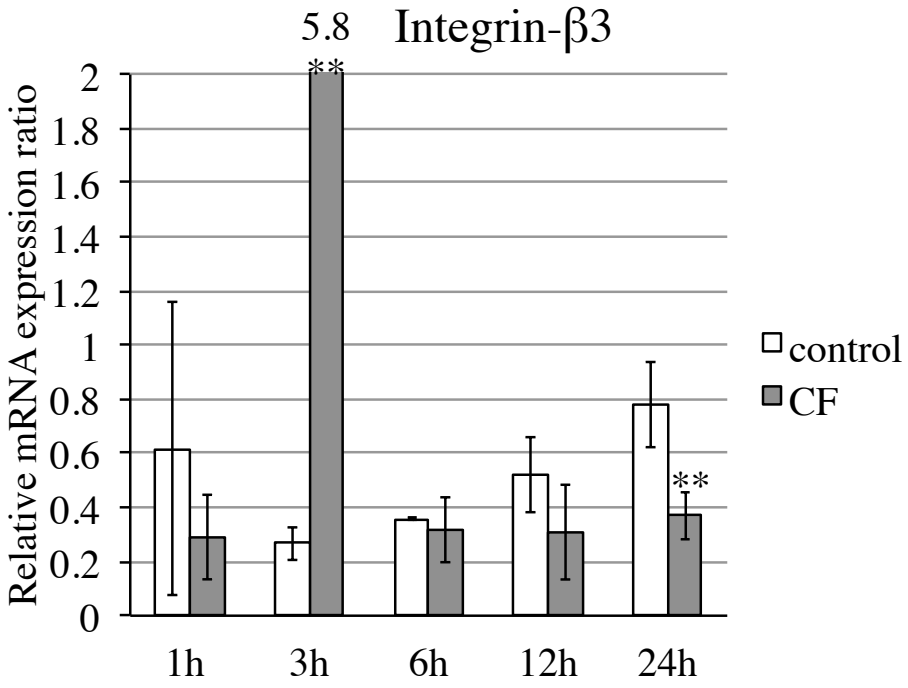

5.8
Integrin-β3
Relative mRNA expression ratio
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
**
1h
3h
6h
12h
24h
control
CF

Figure Legends

Fig. 1. Cell culture methods.

A. Silicone tube had 15-mm diameter and was sliced at 2-mm thicknesses. The tubes sterilized by autoclave.

B. RAW cells were cultured on 12-mm diameter glass cover slips (slips). Slips were placed on sliced silicon tubes in 24-well culture plate. RAW cells on slips were then reversed on sliced silicone tube using sterilized forceps.

C. Schema of cell culture methods for applying compressive force. RAW cells on glass cover slips were cultured in the bottom of 24-well culture plate. Cells on glass cover slips were reversed on the collagen gel layer using sterilized forceps.

D. Reversed cells were compressed continuously by layered slips.

Fig. 2. Number of osteoclasts on each culture day up to day 5.

A. Number of osteoclasts was assessed for 5 days on indicated points.

B. Number of osteoclasts with various numbers of nuclei up to day 5.

C. Number of small and large osteoclasts in response to reversal of slips at either 24 or 48 h when compared with control group on day 5. Control group was cultured on slips without reversing and were fixed on day 5. We counted the number of TRAP-positive cells with 2-7 nuclei and those with >8 nuclei. The numbers of small and large osteoclasts are shown.

Results are given as means ± SD (n=4).

Fig. 3. Effects of compressive force of various weights for 24 h on osteoclastogenesis.

RAW cells (1.0×10^4cells/well) on slips were pre-cultured with culture medium (50 ng/ml of RANKL) in 24-well culture plates for 3 days.

Cells on slips were reversed onto the collagen gel layer after 4 days.

Compressive forces were applied with 3, 5, 7, 9 and 14 layered slips for 24 h from days 4 to 5, while the control group was not loaded.

A. Total numbers of osteoclasts.

B. Number of small (2-7 nuclei) and large (>8 nuclei) osteoclasts.

Results are given as means ± SD (n=4). $^{*}P<0.05$.

C. Pit assay.

RAW cells on Osteologic™ discs were pre-cultured in 24-well culture plates for 3 days. Cells on the discs were reversed onto collagen gel layers after 4 days. Compressive forces were applied via 3-14 layered slips for 24 h from days 4 to 5, while the control group was not loaded.

Light micrograph Osteologic™ discs (×100).

Resorption areas of the discs in the control and compressed groups (CF) are shown. The left image shows the control group and the right image shows CF.

D. Measurement of resorption area was performed using Image J software.

Results were given as means ± SD (n=3). $^{**}P<0.01$. CF, compressive force.

Fig. 4. Effects of compressive force on mRNA levels of osteoclast-associated genes.

Force applied to cells for 1-24 h.

mRNA levels of osteoclast-associated genes were evaluated by quantitative RT-PCR.

The control group, which was cultured for the same duration without compressive force, was defined as the standard.

Results are given as means ± SD (n=4). $^{**}P<0.01$; $^{*}P<0.05$. CF, compressive force.