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Impact of the Microgravity Environment in a Three–dimensional Clinostat on Osteoblast- and Osteoclast-like Cells

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Running title

Microgravity affects osteoblast and osteoclast differentiation.

Footnotes

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Abstract

Mechanical unloading conditions result in decreases in bone mineral density and quantity, which may be partly attributed to an imbalance in bone formation and resorption. To investigate the effect of mechanical unloading on osteoblast and osteoclast differentiation, and the expression of RANKL and OPG genes in osteoblasts, we used a three-dimensional (3D) clinostat system simulating microgravity to culture MC3T3-E1 and RAW264.7 cells. Long-term exposure (7 days) of MC3T3-E1 cells to microgravity in the 3D clinostat inhibited the expression of Runx2, Osterix, type I collagen αI chain, RANKL and OPG genes. Similarly, 3D clinostat exposure inhibited the enhancement of β3-integrin gene expression, which normally induced by sRANKL stimulation in RAW264.7 cells. These results, taken together, demonstrate that long-term 3D clinostat exposure inhibits the differentiation of MC3T3-E1 cells together with suppression of RANKL and OPG gene expression, as well as the RANKL-dependent cellular fusion of RAW264.7 cells, suggesting that long-term mechanical unloading suppresses bone formation and resorption.

Key words

Osteoblast cells, Osteoclast cells, 3D clinostat, Microgravity, RANKL, OPG

1. Introduction

The systematic balance between the synthesis of bone matrix by bone-forming cells/osteoblasts and bone resorption by bone-resorbing cells/osteoclasts controls the normal bone remodeling process that occurs throughout life (Lacey et al., 1998). By contrast, skeletal abnormalities such as osteopetrosis or osteoporosis can be caused by increased or decreased bone mass, respectively, resulting from an imbalance of osteoblast and osteoclast activities (Simonet et al., 1997). Physical forces are known to largely affect this balanced bone metabolism, because space flights have shown the effects of mechanical unloading on body tissues (Rambaut and Goode, 1985). Astronauts have been shown to develop osteopenia during their journeys around the earth, under the microgravity conditions (Vico et al., 2000). These findings suggest that transduction of physical forces, such as mechanical unloading, into a cellular response in bone, can vary the balance of both osteoblast or osteoclast cell differentiation causing decrease in bone mass.

Accumulated data on the molecular mechanisms of osteoclast differentiation have demonstrated that macrophage colony stimulating factor, the receptor activator of NF-κB ligand (RANKL)-RANK pathway, and OPG, which antagonizes RANKL function as a decoy receptor, are essential for osteoclast differentiation and development. It is known that one of the primary sources of RANKL and OPG is the matured osteoblasts in local bone tissues. The key regulators of the bone remodeling, acting through a coupling mechanism with bone resorption at local sites in the body, are not serum RANKL and OPG molecules but local RANKL and OPG produced by osteoblasts (Nakamura et al., 2003).

Studies to date have focused primarily on osteoblast differentiation in microgravity conditions (Rucci et al., 2002), (Ontiveros and McCabe, 2003). However, the mechanisms on the differentiation of osteoclasts, particularly on the communication between osteoblasts and osteoclasts, exposed to microgravity for the short and long

periods are still unclear. Therefore, i) the behavior of osteoclasts subject to microgravity, ii) cytokine production by osteoblasts in microgravity, and iii) their involvement in bone formation through a coupling mechanism with bone resorption, were investigated in the present study.

2. Materials and methods

2.1. Three-Dimensional Clinostat

The 3D clinostat (Mitsubishi Heavy Industries, Co., Ltd., Kobe, Japan) was used for cell culture with microgravity. By controlling the simultaneous rotation of two axes, the 3D clinostat cancels the cumulative gravity vector at the center around the device, producing an environment with an average of 10^{-3} G over time. This is accomplished by rotation of a chamber at the center of the device to disperse the gravity vector uniformly within a spherical volume at a constant angular velocity velocity (Japanese patent, publication number: P2000-79900A, date of filing: Sept. 22, 1998, publication number: 2001-197182A, date of filing: June. 28, 2001, and overseas patents, PCT [U.S.A., Canada, China, and Korea], P/E [Italy, U.K., Sweden, Germany, and France]).

In this study, OptiCell (127x85x5.8 mm, BioCrystal, Westerville, OH) was contained in the sample stage of the device and the rotation rate of two motors was changed at random from 5 to -5 (reverse rotation) rpm at every minute. The onset, the rate, and the duration of rotation of motors were controlled and observed with a personal computer connected to the device. These specific conditions produced a simulated environment of 10^{-3} G in eight minutes.

2.2. Culture of MC3T3-E1 and RAW264.7 cells

The MC3T3-E1 cell line, established from mouse C57BL/6 calvaria, was purchased from the ECACC. Cells were inoculated in OptiCell at a density of 1.0 x 10^5 cells/well, in α-MEM supplemented with an antibiotic mixture (Invitrogen), 10% Fetal Bovine Serum (FBS) (Biological Industries, Haemek, Israel) and 20 ug/ml L-ascorbic acid (Sigma) and maintained at 37°C under 5% CO_2/95% air. When the cells became just confluent, the medium was replaced with fresh culture medium. One group of

MC3T3-E1 cells (conventional condition; group C) was maintained in a 1G normal environment while another group (3D clinostat condition; group CL) was cultured in the 3D clinostat with microgravity.

The RAW 264.7 mouse macrophage/monocytes cell line (TIB-71; ATCC) was a generous gift from Dr. Atsushi Shimazu (Department of Mucosal Immunology, Hiroshima University Graduate School). Cells were inoculated in OptiCell at a density of 5.0×10^4 cells/well, in α-MEM supplemented with an antibiotic mixture (Invitrogen), 10% FBS and 1.5 g/L sodium bicarbonate (Invitrogen). Cells were cultured at 37°C under 5% CO_2/95% air for 1 day after inoculation (pre-culture). After replacing the medium with fresh medium, a group of RAW264.7 cells was maintained in a normal 1G environment (C) and another group (CL) was cultured in the 3D clinostat. In some experiments to go over the differentiation, cells were cultured in the presence of 50 ng/ml recombinant soluble murine RANKL (sRANKL, Peprotec, London, UK).

In the maintenance for both cultures of MC3T3-E1 and RAW 264.7cells until analyses, OptiCell was completely filled with 10 ml medium to eliminate air bubbles and to diminish turbulence and shear forces. No renewal of medium was done after the first medium replacement. Three numbers of OptiCell were used for each condition and triplicate independent experiments were carried out.

2. 3. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis and real-time quantitative RT-PCR

Total RNA was extracted with TRI reagent (Invitorgen). The cDNA was then amplified by BIOTAQ DNA polymerase (Bioline, Randolf, MA, USA). The sequences of the primers for Runx2, Osterix and β-actin used in the RT-PCR analysis were taken from previous reports (Kanno et al., 2007) (Makihira et al., 2007). For each gene, a cycle curve experiment was performed, and the optimal number of PCR cycles was selected according to the amplification. Amplified products were separated on 2.0%

agarose gels and visualized by ethidium bromide staining and subsequent ultraviolet light transillumination. Photographs of the gels were taken using a digital camera.

Real-time quantitative RT-PCR analyses for type I collagen αI chain (Col-IαI), RANKL, OPG, TRAP, the seven-transmembrane-region receptor dendritic cell-specific transmembrane protein (DC-STAMP), and β3-integrin were performed using an ABI PRISM 7700 Sequence Detection System instrument and software (PE Applied Biosystems, Inc., Foster City, CA). First-strand cDNA was synthesized using ReverTra Ace (Toyobo, Osaka, Japan) with total RNA (100 ng). β-actin was chosen as an internal control to standarize the variability in amplification due to differences in the starting total RNA concentrations. The sequences of all primers and probes purchased from Sigma and used in these analyses are listed in Table 1.

2.4. Assessment of tartrate-resistant and phosphatase (TRAP)–positive cells

RAW264.7 cells were cultured for 1 day in normal conditions, and then in a normal gravity environment (C) or the 3D clinostat condition (CL), for 7 days with sRANKL. Differentiated osteoclast-like cells were observed as red and multinucleated cells by cytochemical staining for tartrate-resistant acid phosphatase (TRAP Staining kit, Primarycell, Hokkaido, Japan).

2.5. Statistical Analysis

All the numerical data were subjected to a one-way analysis-of-variance (ANOVA) and Tukey’s multiple range test at 1 or 5% level.

3. Results

3.1. Effects of the 3D clinostat on the expression of Runx2, Osterix, Col-IαI, RANKL and OPG mRNAs in MC3T3-E1cells

The effect of a 3D clinostat on osteoblast differentiation was examined using the MC3T3-E1 osteoblastic cell line. First, the expression levels of osteoblast-specific transcriptional gene markers, namely Runx2 and Osterix, and Col-IαI, were analyzed to evaluate and standardize the differentiation stage of MC3T3-E1 cells in normal and 3D clinostat conditions during the periods of 1 to 7 days of culture after the cells had reached confluence. β-actin was chosen as an internal standard to control for variability in amplification due to differences in the starting total RNA concentrations. Analysis of RT-PCR products on an agarose gel revealed that Runx2 mRNA started to be expressed on 1 day after confluence (on day-1) in group C cells, but the Runx2 mRNA was observed on day 3 after confluence in group CL cells (Fig. 1A). The expression of Osterix mRNA was observed on day-1 and showed a maximum on day-3 in group C cells, decreasing again on day-7. On the other hand, in group CL cells, the expression of Osterix mRNA gradually increased through day-1 to day-7 (Fig. 1A). Exposure of cells to microgravity conditions had no effect on the level of β-actin expression, which was used as an internal control, compared with cells cultured in normal gravity conditions. Real-time quantitative RT-PCR results showed that the expression of Col-IαI mRNA in cells cultured in normal gravity conditions (group C cells) increased and reached a maximum on day-3, then decreased on day-7, while the expression of Col-IαI mRNA in cells cultured in 3D clinostat conditions (group CL cells) gradually increased in a time-dependent manner, reaching a maximum on day-7 (ANOVA, $p<0.01$). The significant difference was observed with Col-IαI mRNA in group C cells on day-3 and that in group CL cells (ANOVA, $p<0.01$) (Fig. 1B).

The expression levels of RANKL and OPG in MC3T3-E1 cells were

investigated by real-time quantitative RT-PCR. The expression of RANKL mRNA in group CL cells was noted on day-1, as compared with that in group C cells (ANOVA, $p<0.01$) (Fig. 1B). However, the expression of RANKL in group CL cells significantly decreased on day-3 or day-7. On the other hand, the significantly increased expression of RANKL mRNA was observed in group C cells on day-7 (ANOVA, $p<0.01$) (Fig. 1B). The expression of OPG mRNA increased time-dependently, in either group C or group CL cells. However, on day-7, the level of OPG mRNA expression in group CL cells was significantly lower than that in group C cells (ANOVA, $p<0.01$) (Fig. 1B).

3.2. Effects of the 3D clinostat on the expression levels of TRAP, DC-STAMP and β3-integrin mRNAs in RAW264.7 cells

To examine the effect of sRANKL stimulation on osteoclast precursor cells under the normal and microgravity environments, RAW264.7 cells, obtained from murine ascitic fluids, were used in this study. RAW264.7 is well known as a useful cell line to differentiate into functional and mature osteoclasts easily in the presence of certain concentrations of sRANKL. In the present study, RAW264.7 cells were inoculated on OptiCell and pre-cultured for 1 day in the normal condition to obtain the cell attachment to the OptiCell surface, and subsequently incubated in either normal gravity or microgravity conditions for 5 days, with or without the supplementation of 50 ng/ml sRANKL. To evaluate and standardize osteoclast differentiation, a real-time quantitative RT-PCR strategy was employed to measure the levels of TRAP mRNA, which is known as a marker for osteoclast differentiation. sRANKL significantly enhanced the expression of TRAP mRNA in RAW264.7 cells grown on OptiCell surfaces in conventional conditions for 5 days (ANOVA: $p<0.01$). The 3D clinostat had no effect on the level of TRAP mRNA in cells cultured with or without RANKL stimulation (Fig. 2A). sRANKL enhanced the expression of DC-STAMP and β3-integrin mRNAs in both groups of cells (ANOVA: $p<0.05$). Exposure to the 3D clinostat only significantly

inhibited the enhancement of β3-integrin expression induced by sRANKL stimulation (ANOVA: $p<0.05$) (Fig. 2A).

Further incubation of the cells with sRANKL for up to 7 days after pre-culture in normal and 3D clinostat conditions provoked the emergence of gigantic multinucleated cells, as detected by TRAP staining, though the size of the gigantic multinucleated cells in group CL cultures was smaller than those observed in group C cultures (Fig. 2B).

4. Discussion

In this study, to elucidate whether alteration of RANKL and OPG expression occurs in osteoblasts subjected to microgravity during short and long periods and whether a change in RANKL–dependent osteoclast differentiation occurs in the same conditions, as compared with cells subjected to conventional gravity, we cultured both MC3T3-E1 and RAW264.7 cells in a 1G normal device or subjected them to clinorotation for 1 to 7 days after pre-culture. These cell lines were chosen as differentiated MC3T3-E1 cells are known to express RANKL and OPG (Lamghari et al., 2006) and the RAW264.7 cell line is a very useful *in vitro* model of osteoclast differentiation dependent on optimal RANKL stimulation (Makihira et al., 2007).

The clinostat has been considered to be a device for simulating a microgravity environment on the earth; 1D clinostats have been developed to mimic the microgravity environment by “nulling the gravitational vector” through continuous averaging (Kobayashi et al., 2000). However, it was difficult to null the gravitational vector using these classical clinostats. By controlling the simultaneous rotation of two axes, the three-dimensional clinostat (3D clinostat) used in this study cancels the cumulative gravity vector at the center of the device, producing an environment with an average of 10^{-3}G over time, as previously described (Yuge et al., 2003). Therefore, the 3D clinostat is considered to be the instrument of choice for generating a microgravity environment (Ichigi and Asashima, 2001).

Long or short impaction of microgravity simulated by a NASA-approved rotating wall vessel bioreactor (RWV) inhibited the differentiation of rat osteoblast-like cells (ROS.SMER#14) (Rucci et al., 2002), MC3T3-E1 cells (Ontiveros and McCabe, 2003), and human mesenchymal stem cells (Zayzafoon et al., 2004), suggesting that the suppression of osteoblastic-lineage development and osteoblast differentiation was related to the decrease in ERK and p38 phosphorylation through decreased Runx2 and AP-1 transactivation, or by disruption of integrin signaling under microgravity. Recently,

we also revealed that the inhibition of human osteoblast differentiation induced by exposure to the microgravity environment simulated by our 3D clinostat over the long term was due to the suppression of p38 phosphorylation (Yuge et al., 2003). Thus, our results that the exposure of MC3T3-E1 cells to microgravity caused delay in the expression peaks of Runx2, Osterix and Col-IαI would be attributed to the latter phenomenon (Fig. 1).

In contrast, Rucci et al. have shown that the short exposure (1 day) to RWV failed to affect the differentiation of primary osteoblast cells derived from calvaria, though the short-term exposure of osteoblast cells to RWV induced an increase in the ratio of RANKL to OPG (Rucci et al., 2007). Similarly, Kanematsu et al. have shown that the exposure of bone marrow stromal cells to RWV induced an increase in RANKL gene expression and a decrease in OPG gene expression, and suggested that the phenomenon may be responsible for the increase in osteoclast activity in bone tissue (Kanematsu et al., 2002). Thus we employed osteoblastic cells to examine the effects of the microgravity on their RANKL and OPG expression. Our results showed that 1-day exposure of MC3T3-E1 cells to the 3D clinostat enhanced the expression of RANKL mRNA. Conversely, further exposure (7 days) to the 3D clinostat significantly suppressed expression of both RANKL and OPG mRNAs, compared to the expression levels in conventional conditions (Fig.1B), implying that the effects on signal transduction in osteoblast cells induced by mechanical unloading are complicated, and that there might be some differences in cellular adaptation to mechanical unloading as an early or late responses.

The enhanced expression of DC-STAMP and αVβ3 integrin mRNAs induced by RANKL is known to be involved in the emergence of multinucleated gigantic cells. Induction of DC-STAMP by RANKL is required for cell-cell fusion of osteoclasts (Yagi et al., 2006). αVβ3 integrin forms ruffled borders, provoked by the activation of the RANK-RANKL signal pathway (Boissy et al., 1998). Based on the data shown in Fig. 2, it is possible that the reduction in the enhancement of β3 integrin through the activation

of the RANKL-RANK signal pathway under microgravity might result in a lack of cellular fusion of pre-osteoclast cells. Recently, it was reported that the impaction of microgravity using RWV enhanced osteoclastogenesis *in vitro* (Saxena et al., 2007). TRAP-positive cells was reported to be increased in cells pre-cultured in RWV, as compared with those pre-cultured in normal conditions (Saxena et al., 2007).

As stated, the effect of exposure to RANKL and/or microgravity varies depending upon the timing or duration of exposure. We can offer the some reasons for the variety of responses of RAW264.7 cells, exposed to sRANKL and/or microgravity, that microgravity might enhance a RANKL-independent signal pathway and suppress a RANKL-dependent one. Further analysis is required to clarify the mechanisms involved in the phenomenon.

In conclusion, the data presented here, taken together, suggest that long-term exposure to microgravity causes the decrease in osteoblast differentiation coupled with a decrease in osteoclast differentiation, possibly by suppression of the RANKL-dependent signal pathway and a reduction in RANKL expression in osteob lasts.

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Figure legends

Fig. 1. Runx2, Osterix and β-actin gene expressions in MC3T3-E1 cells cultured on OptiCell surfaces in conventional (C) or 3D clinostat (CL) environments, for 1, 3 or 7 days after reaching confluence (on day-1, -3 and -7), analyzed by RT-PCR. Data are representative of three experiments. The expression levels of Col-IαI, RANKL and OPG mRNAs in conventional conditions (open circles) or in the 3D clinostat (closed circles) were determined by real-time quantitative RT-PCR analysis. The findings of real-time quantitative RT-PCR analysis of Col-IαI, RANKL and OPG gene expression were normalized to the expression level of β-actin mRNA. Data represent the means +/- S.D of triplicate experiments. Three independent experiments were carried out. Different letters indicate that mean values were significantly different and identical ones indicate no significant difference (ANOVA: $P<0.01$).

Fig. 2. A: Gene expression profiles including TRAP, DC-STAMP and β3-integrin mRNAs in RAW264.7 cells cultured on OptiCell surfaces in normal (C) or 3D clinostat (CL) conditions, for 5 days, after 1-day culture in the normal condition, determined by real-time quantitative RT-PCR. Cells were cultured in the absence or presence of sRANKL. Data represent the means +/- S.D of triplicate experiments. Three independent experiments were performed. *ANOVA: $p<0.05$ and ** ANOVA: $p<0.01$ versus control samples in the conventional condition. B: Phase-contrast morphological analysis of RAW264.7 cells cultured on OptiCell surfaces with sRANKL stimulation in conventional (C) or 3D Clinostat (CL) environments. TRAP-positive cells were stained according to the protocol described in the materials and methods section.

A

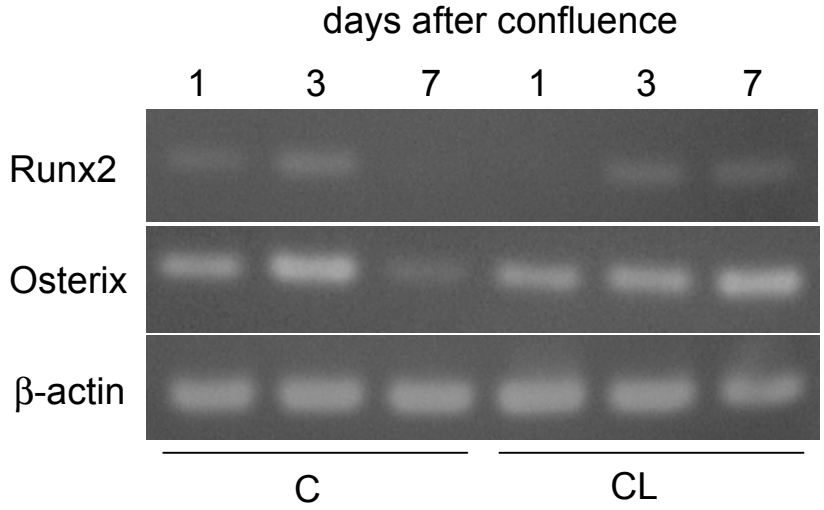
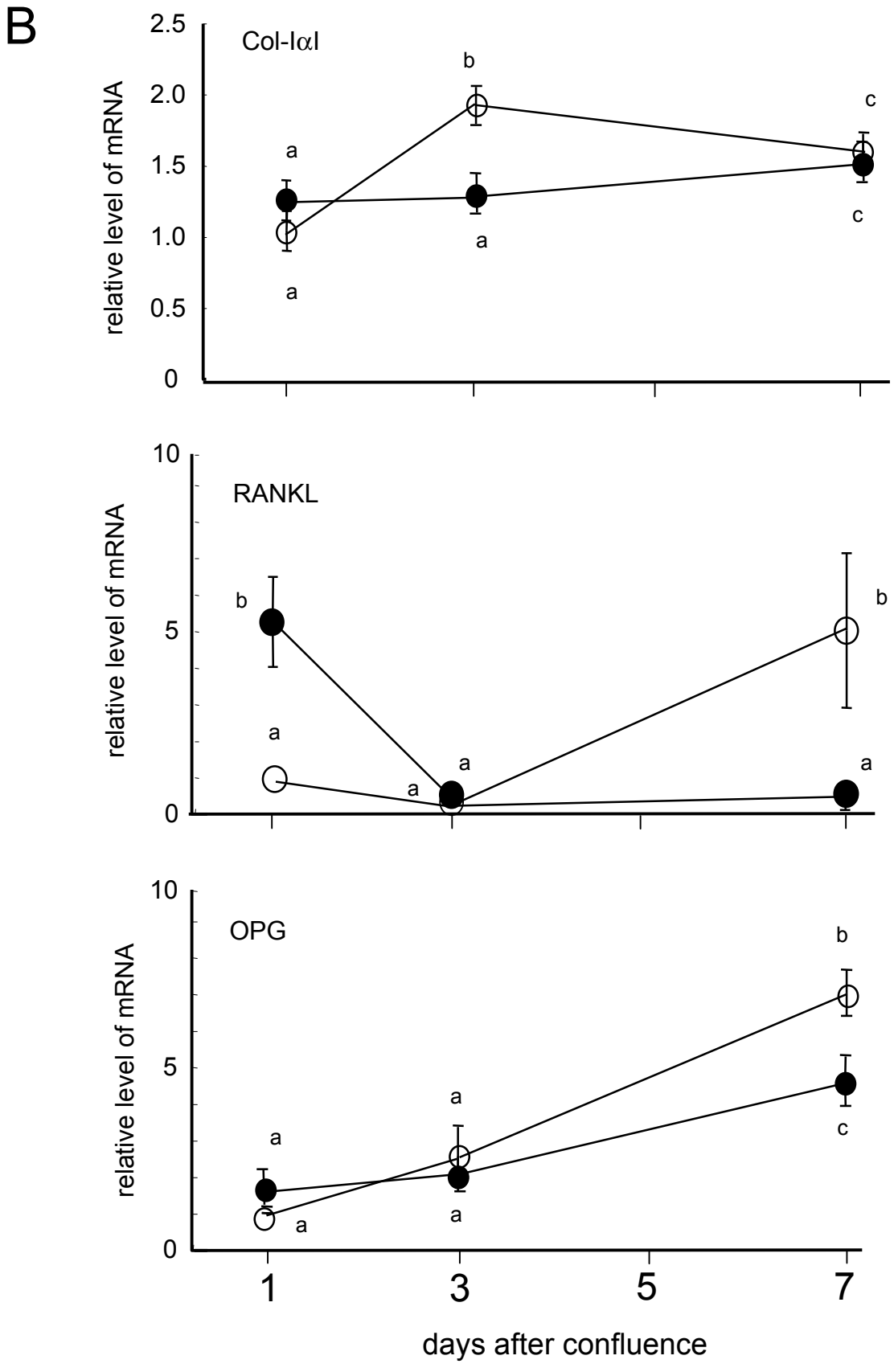


Fig. 1 Makihira et al.



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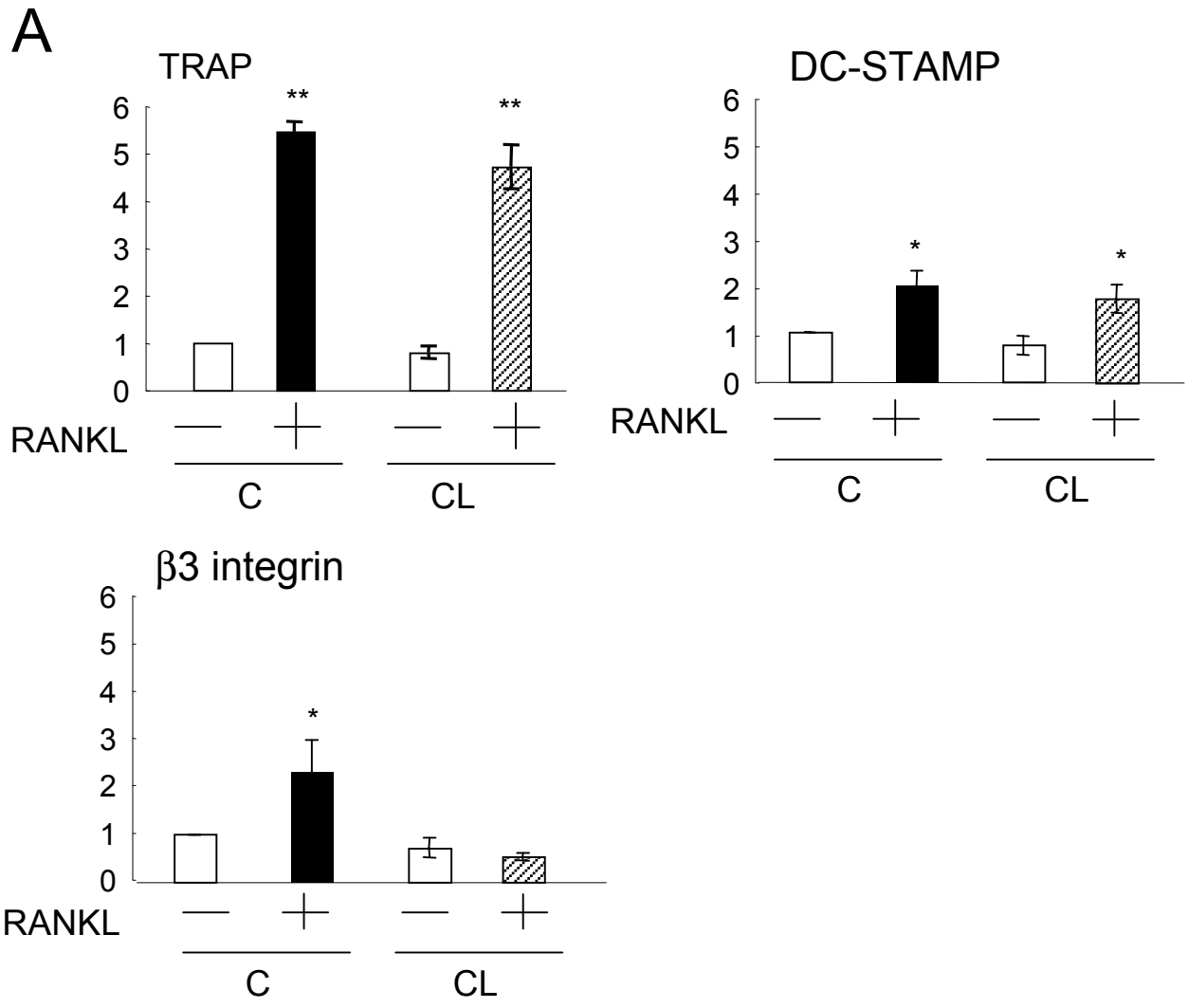

Fig. 2 Makihira et al.

Table 1 Primers and probes used for real time PCR

Target name	Primer and probe	Sequence (5'-3')
Col-Iαl	Primer F	AACCCGAGGTATGCTTGATCT
	Primer R	CCAGTTCTTCATTGCATTGC
	Probe	6FAM- CACGGCTGTGTGCGATGACG -TAMRA
RANKL	Primer F	AGCCATTTGCACACCTCACC
	Primer R	GGTACCAAGAGGACAGAGTGAC
	Probe	6FAM- TGCCAGCATCCCATCGGGTTCCCA -TAMRA
OPG	Primer F	GCGTTACCTGGAGATCGAATTC
	Primer R	AAGTCTCACCTGAGAAGAACCC
	Probe	6FAM- CTTGAAGCACCGGAGCTGTCCCC -TAMRA
TRAP	Primer F	GGAGCTTAACTGCCTCTTGC
	Primer R	CCGTGGGTCAGGAGTGG
	Probe	6FAM- TTTGTAGGCCCAGCAGCACCACCC-TAMRA
DC-STAMP	Primer F	GCTGTGGACTATCTGCTGTATCG
	Primer R	GACACGAGAATTTTAAGCTCTCCAC
	Probe	6FAM- AGTGAACTTCCAGCCCTGGCAAGCT-TAMRA
β3-integrin	Primer F	TGTGAGTGCGATGACTTCTCC
	Primer R	TGTAGTACAGTTGCAGTAGTAGCC
	Probe	6FAM- AGTCCGAGTCACACACGCAGTCCC-TAMRA
β-actin	Primer F	CCACACTGTGCCCATCTACG
	Primer R	GTGGTGGTGAAGCTGTAGCC
	Probe	6HEX- CCTGCGTCTGGACCTGGCTGGC-TAMRA

Note. Forward primers (Primer F) and reverse primers (Primer R) are listed.