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Comparison of the biological response of osteoblasts after tension and compression

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SUMMARY The aim of this study was to investigate the difference in the biological response of osteoblasts when stretched and compressed. A cellular cyclic tension and compression apparatus (CCTCA) was designed to stretch and compress cells under the same conditions. After stretching or compressing MC3T3-E1 with continuously increased strain for 5 hours, cellular cytoskeletal modulation was detected by immunohistochemical assay with actin antibody. Real-time polymerase chain reaction was performed at 1, 3, and 5 hours to detect local factors related to bone remodelling. Statistical analysis was undertaken with analysis of variance and the Kruskal–Wallis.

Following stretching or compression for 5 hours, MC3T3-E1 attached to the culture dishes grew well. Compared with the control, the microfilaments orientated parallel with each other and were clearly observed by laser scanning confocal microscope after 5 hours of stretching. The morphology of MC3T3-E1 cells was thinner and longer than the control. However, microfilaments presented a disordered arrangement after 5 hours of compression, and the MC3T3-E1 cells decreased in size. Gene expression of Wnt10b and Lrp5 increased during tension but more in the compression groups at 1, 3, and 5 hours. The ratio of osteoprotegerin to receptor activator for nuclear factor kappa B ligand increased in the tension group compared with the control but decreased in the compression group at 5 hours.

Introduction

The basic biological mechanism of orthodontic tooth movement is force leading to alveolar bone remodelling. Bone resorption occurs at the compression side and bone formation at the tension side (Persson, 2005).

Over recent years, several biomechanical force systems have been used to investigate the mechanotransduction process in bone or periodontal ligament cells, including fluid shear stress, hydrostatic compression, uniaxial strech, biaxial stretch, or a combination of two or more of these strain system (Basso and Heersche, 2002). However, few comparative studies of biological response of osteoblasts after compression and tension have been reported due to the lack of suitable equipment to apply different strains under the same condition.

During the mechanotransduction process, determining the mechanical signal of the cells is the first essential step. A cell cytoskeleton could serve as the mechanoceptor in response to mechanical stimulation either directly or indirectly (Formigli *et al.*, 2007). It links the next series of cellular responses to external stimulations, such as recognization, signal transduction, and gene expression.

Bone and bone cells are sensitive to even mild stress and many molecules are involved in the process (Rubin *et al.*, 2006). Recently, it was reported that Wnt/β-catenin signalling is a normal physiological response and activated under mechanical loading both *in vivo* and *in vitro* (Robinson *et al.*, 2006). Mice with null allele for the Wnt co-rereptor *Lrp5*, exhibit a low bone mass phenotype and decreased osteoblast activity (Kato *et al.*, 2002). An activation mutation in LRP5 is also associated with high bone mass and increased osteoblast proliferation (Boyden *et al.*, 2002; Little *et al.*, 2002). It is reported that receptor activator for nuclear factor kappa B ligand (RANKL) and osteoprotegerin (OPG), secreted by osteoblasts, play a critical role in regulating osteoclast differentiation and proliferation (Little *et al.*, 2002). RANKL activates its receptor, receptor activator of nuclear factor kappa B (RANK), which is expressed in osteoclasts and their precursors, resulting in osteoclast maturation and bone resorption. OPG, as a competitive inhibitor, binds RANKL and prevents RANKL from binding with RANK. Therefore, changes in the OPG/RANKL ratio determine osteoclast functions and bone remodelling (Boyce and Xing, 2008).

A cellular cyclic tension and compression apparatus (CCTCA) that can stretch and compress cells under the same conditions was developed to enable comparison of osteoblast cytoskeleton change and Wnt10b, Lrp5, OPG, and RANKL expression in osteoblasts primarily after tension and compression.

Materials and methods

Theory of CCTCA

For culturing cells on the elastic membrane, the CCTCA was designed. Given the elastic deformation of the membrane that occurred, cells could be exposed to stress proportional to the strain of the membrane since cells anchor to it. The strain of membrane due to applied force would cause membrane stress. Tensile strain due to expanding the membrane would cause tensile stress. Also, compressive strain due to relaxing the expanded membrane would cause compressive stress. In this strain model, cells were anchored on the membrane so that the membrane's stress would be transmitted to the cells and cause cell deformation (Figure 1A).

Construction of the CCTCA

The force-loading dish was refitted from a 60 mm cell culture dish (Greiner Bio-one GmbH, Frickenhausen, Germany) in which the bottom part was replaced with a medical elastic silicone membrane (Institute of Rubber Products, Shanghai, China; Figure 1B). The relationship between the load on the membrane and the strain that occurred was measured by an 3367 universal testing machine (Instron, Norwood, Massachusetts, USA) to determine the stress–strain curve during elastic deformation. The stress–strain curves were linear with almost the same slope in three randomly selected regions of the membrane (Figure 2).

The dish was fixed above the protruding base of the carrier rod. The hybrid linear actuator (Haydon Kerk Motion Solutions, Waterbury, Connecticut, USA; Part No. 43H4J-05-101), controlled by the SC100 Central Processing Unit (Haydon Switch and Instrument, Waterbury, Connecticut USA), drove the carrier rod to precisely shift up and down (Figure 1C). With the carrier rod shifting up or down, the elastic silicone membrane was expanded or relaxed (Figure 1D and 1E). The relationship between displacement of the carrier rod and membrane deformation was calculated using the Solidworks three-dimensional software (Dassault Systémes, Velizy-Villacoublay, France; Table 1). Thus, the carrier rod displacement could be converted into stress on cells so the stress applied on the osteoblasts could be reproducibly controlled.

Cell culture

The MC3T3-E1 subclone 4(ATCC® CRL-2593™) was cultured in Alpha Modified Minimum Essential Medium (Invitrogen Corporation, Carlsbad, California, USA; Catalog No.11900024) supplemented with 10 per cent foetal bovine serum (Biochrom AG, Berlin, Germany) and 1 per cent penicillin/streptomycin. On reaching 50–70 per cent confluence, the cells were detached and seeded in the force-loading dish at a density of 1×10^5 cells/cm^2. After fixing the dish above the protruding base of the mechanical strain device, the CCTCA was maintained at 37°C in a humidified atmosphere of 5 per cent CO_2 and 95 per cent air.

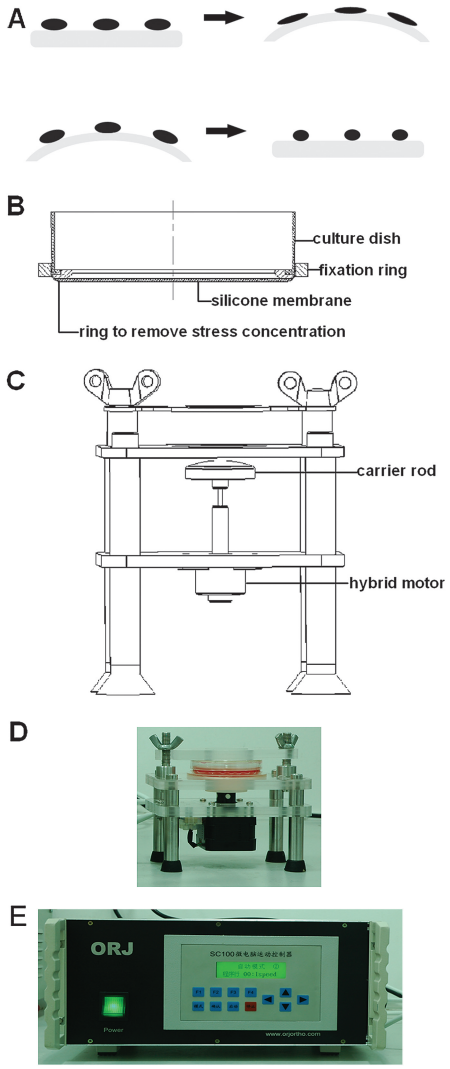


Figure 1 (a) Strain cell theory. Cells were stretched when the membrane was expanded since the cells were seeded on the relaxed membrane and compressed when the membrane was relaxed. (b) The force-loading dishes with the bottom part of the cell culture dishes replaced with a medical silicone membrane. (c) The carrier rod was driven by a hybrid motor.(d) The cell culture dish was fixed above the carrier rod to accomplish cell compression or stretching. (e) A central processing unit was used to control the hybrid motor precisely with the prescription programming.

Strain mode

The cells were exposed to biaxial compressive or tensile strain with the CCTCA (Figure 1D and 1E). The following mechanical parameters were applied: either continuous tensile or compressive strain at a magnitude of 200, 1600, 3400, 5200, and 7000 μs for 1 hour each sequentially. Unstrained cells were cultured in a flexible membrane dish as the control. The cells were harvested immediately after loading at 1, 3, and 5 hours.

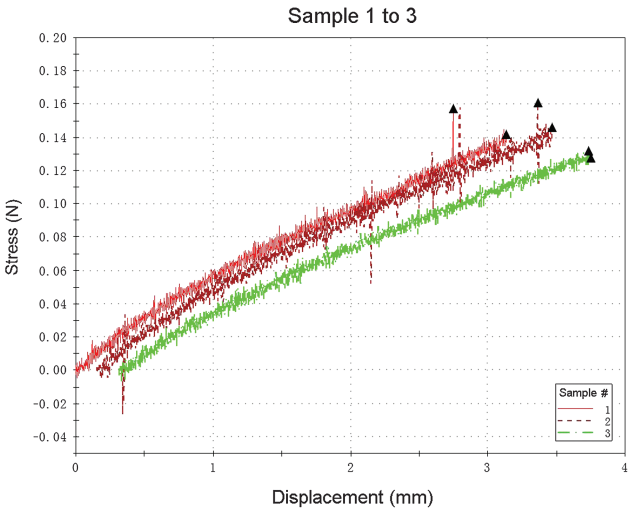


Figure 2 Stress–strain curves were linear with almost the same slope in three randomly selected regions of the membrane.

Table 1 Relationship between the displacement of the carrier rod and membrane deformation (0.1 per cent length changes = 1000 microstrain).

Displacement (mm)	Length (mm)	Variation ratio	Membrane deformation (μs)	Stress on cells (N)
0	1963.5			
0.5	1963.9	1.0002	200	0.0195
1.5	1967.3	1.0018	1600	0.1657
2.5	1974.58	1.0052	3400	0.3549
3.5	1986.31	1.0104	5200	0.5718
4.5	2003.11	1.0174	7000	0.8190

Actin staining and laser scanning confocal microscopy observation

After stimulation, the cells on the flexible membrane dishes were washed twice with phosphate-buffered saline (PBS), fixed in 4 per cent paraformaldehyde solution at 37°C for 30 minutes and in 70 per cent ethanol solution at −20°C for 2 hours, washed in PBS for 2 hours, and then permeabilized with 0.5 per cent Triton X-100 in PBS for 20 minutes at room temperature. The cells were washed in a blocking solution consisting of 5 per cent albumin from bovine serum and 0.2 per cent Triton X-100 for 1 hour. For labelling, the fixed cells were incubated with specific antibodies against actin (1:100; Santa Cruz Biotechnology, Inc., Santa Cruz, California, USA; Lot# L1908) in the blocking solution at 4°C over night. After washing three times in the blocking solution, the cells were incubated with tetramethylrhodamine isothiocyanate-conjugated goat anti-rabbit IgG (1:100; Santa Cruz Biotechnology Inc.) then in the blocking solution at 4°C over night followed by three washes in PBS. The cells were then incubated for 10 minutes at room temperature with 5 mg/ml Hoechst 33342 (Molecular Probes, Eugene, Oregon, USA). After three washes in PBS, the cells were mounted in a 90 per cent glycerol–PBS mixture. Laser scanning confocal microscopy (LSCM) was performed at room temperature using a Leica TCS SP2 (Leica Mikrosysteme Vertrieb GmbH, Wetzler, Germany).

Table 2 Primers of real-time polymerase chain reaction.

Gene	Forward primer	Reverse primer
Wnt10B	5′-GCGGGTCTCCT-GTTCTTGG-3′	5′-GCTGTCGCTTACTC-AGTCCG-3′
Lrp5	5′-GCAGGGATGGAT-GGCAGTA-3′	5′-TGGCACGGTGGAT-GAAGC-3′
OPG	5′-TCCCTTGCCCTG-ACCACT-3′	5′-TCCTGCTTCACG-GACTGC-3′
RANKL	5′-TGAAAGGAGGG-AGCACGAA-3′	5′-TCCAGCAGGGAAG-GGTTG-3′

Real-time polymerase chain reaction

Total RNA was isolated with TriZol (Invitrogen, Cat. No. 15596-026) and reverse transcribed with Moloney Murine Leukemia Virus reverse transcriptase (Promega Corporation, Madison, Wisconsin, USA). The primers are shown in Table 2. Triplicate aliquots of serially diluted amplicon were used in a reaction mixture that contained 10 μM of each primer in a reaction volume of 15 μl using the SYBR (R) Green RT PCR Master Mix (Toyobo Bio-Technology, Osaka, Japan). ABI Prism 7300 SDS software (Applied Biosystems, Carlsbad, California, USA) was used for these experiments. The thermal cycling was started with an initial 5 minutes at 95°C for 30 seconds and 60°C for 2 minutes. Glyceraldehyde 3-phosphate dehydrogenase expression was used as an endogenous control. The expression of messenger RNA is represented as a fold increase ($2^{-\Delta\Delta Ct}$).

Statistical analysis

The real-time polymerase chain reaction (RT-PCR) data at 5 hours time were presented in terms of the mean and standard deviation. Analysis of variance and Kruskal–Wallis test were performed using the Statistical Package for Social Sciences version 14.0 (SPSS Inc., Chicago, illinois, USA). $P < 0.05$ was considered statistically significant.

Results

LSCM observation

When exposed to tensile or compressive strain for 5 hours, the change in the cell skeleton suggests that different mechanical signalling was sensed by the osteoblasts then distinguished from each other. In the tension group, microfilaments were orientated parallel with each other and seemed to be straightened after 5 hours of stretching. The arrangement of microfilaments was clearer than in the control. The morphology of MC3T3-E1 became thinner and

longer compared with the control (Figure 3A and 3B). The morphology of MC3T3-E1 after 5 hours compression showed shrinkage, and the microfilaments presented a vague outline with a disordered arrangement (Figure 3C and 3D). Tensile and compressive strain caused different cellular skeletal modulation. Since the cellular skeleton links a series of cellular responses, for example gene expression, it was hypothesized that osteoblast-related gene expression might respond differently to tensile and compressive strain.

Real-time polymerase chain reaction

It has been reported that Wnt/β-catenin signalling is involved in response to mechanical loading. To determine whether Wnt signalling molecules were differently expressed in MC3T3-E1 after being stretched and compressed, RT-PCR was carried out. Gene expression of Wnt10b and Lrp5 in the present study increased gradually in the tension and compression group at 1, 3, and 5 hours and their expression was upregulated more in the compression than in the tension group at all time points (Figure 4A and 4C). At 5 hours, the expression of Wnt10b and Lrp5 was significantly upregulated ($P < 0.01$) in the compression and tension groups compared with the control (Figure 4B and 4D). Their expression in the compression group was also more significantly upregulated ($P < 0.05$)

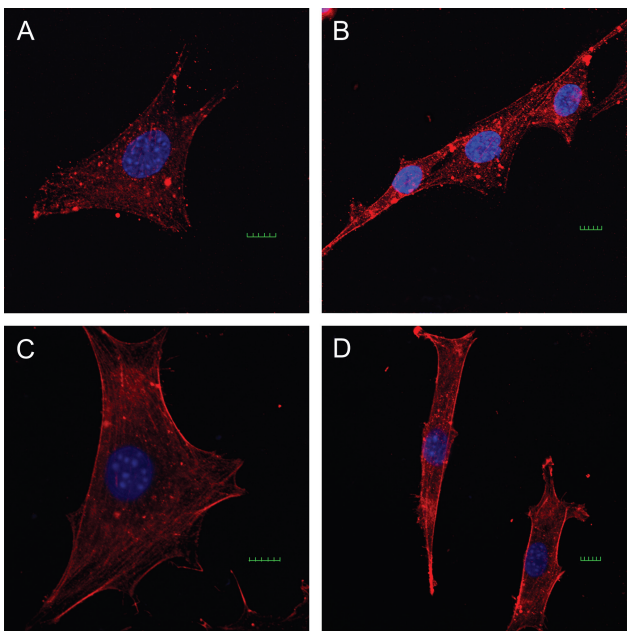


Figure 3 Changes in microfilament and osteoblast morphology observed with laser scanning confocal microscopy suggesting a different response of osteoblasts to stretching and compression. Unstimulated control cell (A). Cells stretched for 5 hours (B). The morphology of MC3T3-E1 was thinner and longer than the control. The microfilaments were orientated parallel to each other and seemed to be straightened after 5 hours of stretching. The microfilaments arrangement was more clear than in the control (A) and (B). The control cell was unstimulated (C). Cells were compressed for 5 hours (D). Compared with control, the morphology of MC3T3-E1 after 5 hours compression was size reduction; the microfilaments presented a vague outline with a disordered arrangement (C and D). Bar = 10 μm.

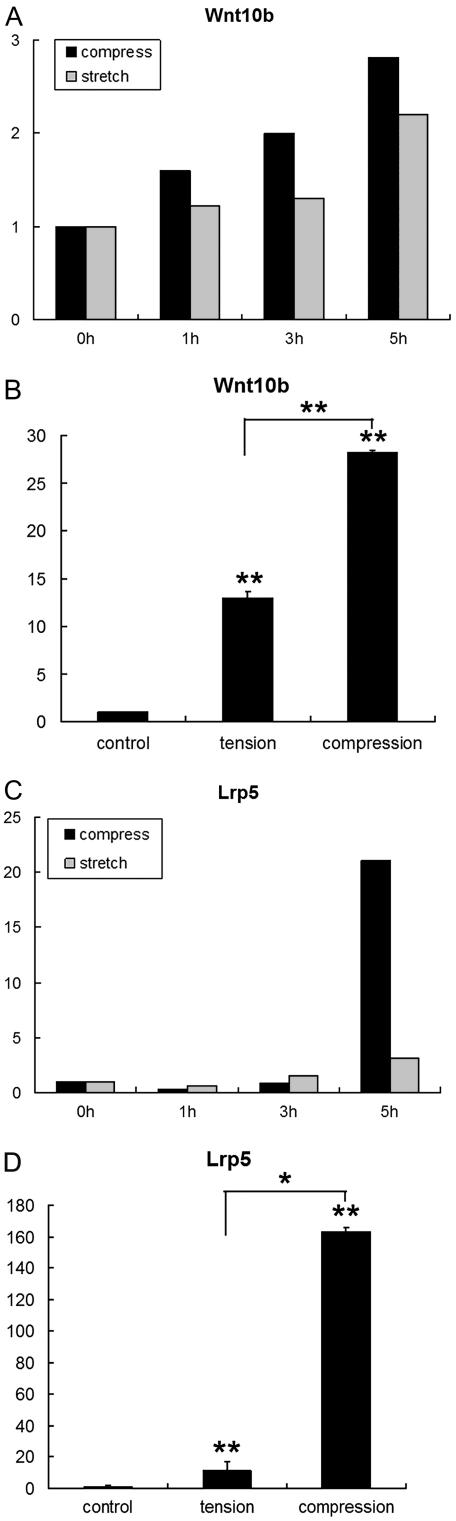


Figure 4 Gene expression of Wnt10b (A and B) and Lrp5 (C and D) demonstrated a different biological response of MC3T3-E1 after being stretched and compressed. Gene expression of Wnt10b and Lrp5 increased gradually in the tension and compression groups at 1, 3, and 5 hours. After 5 hours stimulation, Wnt10b and Lrp5 were activated in both experimented groups but more in compression group (B and D). *$P < 0.05$; **$P < 0.01$.

compared with tension group. It is suggested that the Wnt signalling pathway in both experimental groups was activated compared with the control and that compressive strain could activate Wnt pathway more.

OPG and RANKL, as two of the key regulators of osteoclastogenesis secreted by osteoblasts, were also selected in the experiment. It was found that the OPG expression decreased gradually in the compression group at 1, 3, and 5 hours. Generally, its expression decreased in the tension group over time (Figure 5A). In the compression group, the expression of RANKL decreased at 1 hour and then increased gradually at 3 and 5 hours. In the tension group, its expression decreased with time (Figure 5C). At 5 hours, the expression of OPG decreased significantly in the compression group compared with control and tension group (Figure 5B). The expression of RANKL decreased significantly in the tension group compared with the control (Figure 5D). Therefore, the results suggest that tension and compression affect the expression of RANKL and OPG differently. Since the ratio of OPG/RANKL has been reported to be critical in bone remodelling, the ratio of OPG/RANKL was compared at 5 hours in the present study. The ratio increased in the tension group but decreased in the compression group compared with the control (Figure 5E). A significant difference existed between the compression and tension groups, suggesting that tension and compression might direct bone remodelling to formation or resorption by changing the ratio of OPG/RANKL.

Discussion

Mechanical apparatus and strain mode

Various loading systems *in vitro* have been used to generate mechanical strain on cells, including tensile strain or fluid shear stress (Bottlang *et al.*, 1997; Garvin *et al.*, 2003; Appelman *et al.*, 2009). However, few researchers have compared the different effects between tensile and compressive strain on cells. In the present study, a CCTCA was constructed to stretch and compress cells under the same conditions. With the conversion of the carrier rod displacement into membrane strain and membrane strain into stress, stress on the cells could be controlled.

Since tooth movement in orthodontics combines both pathologic and physiologic processes in alveolar remodelling (Wise and King, 2008), strains of 200–7000 μs, which included the pathologic and physiologic response scale according to the Frost's (1987) theory, were used in this study. In previous investigations, various frequencies of strain were used from 0.05 to 2 Hz on bone cells (Wozniak *et al.*, 2000; Waters *et al.*, 2007) and a continuous static strain was applied on the alveolus during orthodontic treatment. In order to mimic the clinic situation, the force applying mode in this study was designed as continuous stretching or compression. The subsequent cytoskeletal observation proved the reliability of strains on cells with the CCTCA.

LSCM observation

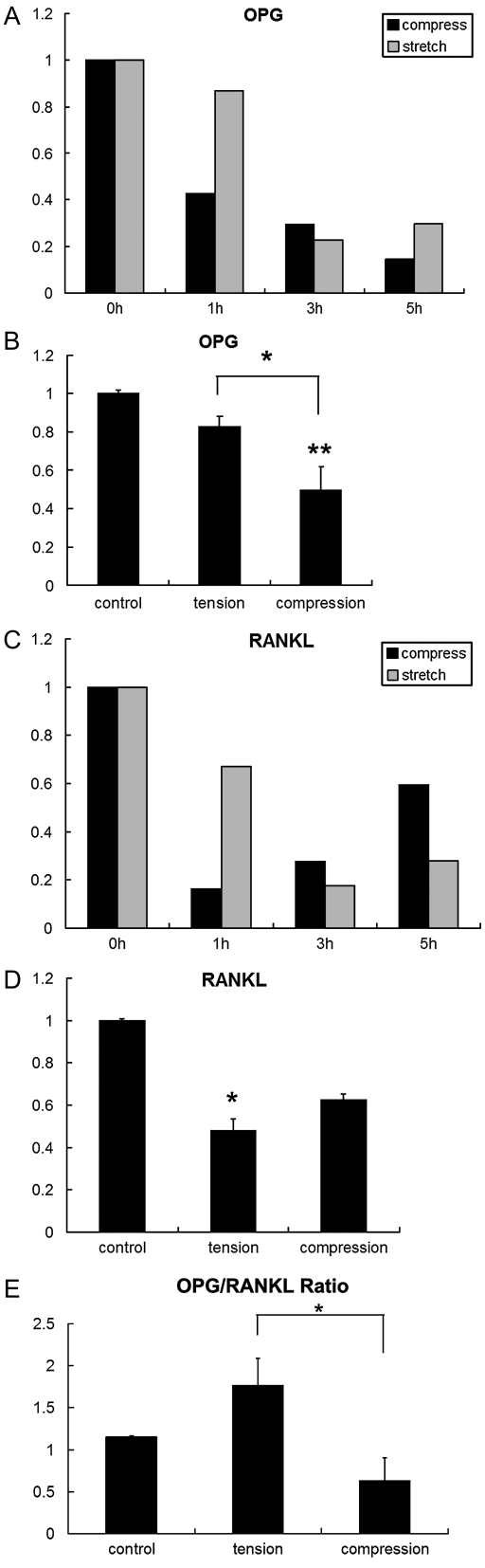
Using immunohistochemical assay, an attempt was made to observe whether there was a difference between osteoblasts when stretched and compressed in terms of the cytoskeleton. The cytoskeleton is composed of microfilament, microtubule, and intermediate filament. Actin, in the form of filamentous (F-) and unpolymerized (G-) actin, are the basic ingredients of microfilament (Akisaka *et al.*, 2001). It is reported that organized networks of actin filaments determine cell stiffness and transmit force during mechanotransduction, cell motility, and other cellular shape changes (Chaudhuri *et al.*, 2007). After 5 hours of stretching, microfilaments orientated parallel to each other seemed to be straightened and opaque compared with the control. This is consistent with the findings of Pender and McCulloch (1991), who reported that stretching of cells leads to rapid and large increases in F-actin. The morphology of MC3T3-E1 was thinner and longer than the control, which is also consistent with the findings of Neidlinger-Wilke *et al.* (2001) who reported that the substrate strain tended to lengthen the cell.

After 5 hours compression, the microfilaments showed a vague outline with a disordered arrangement. Similarly, Li *et al.* (2007) found that the fluorescence intensity of F-actin decreased when osteoblasts were compressed which could be explained as a reversible stress-softening behaviour in the actin network to resist compression. It was also found that the effect of compression was distinguished from stretching as the morphology of MC3T3-E1 cells after 5 hours compression reduced in size. In this study, the different response of microfilaments after stretching and compression suggest that cells could recognize different mechanical signals under the same conditions.

RT PCR

In order to determine if tension and compression could affect the expression of some related genes, RT-PCR was performed. The expression of Wnt10b and its receptor Lrp5 was both upregulated by tension and compression. In a study of mandibular osteodistraction and osteocompression, it was reported that a large number of osteoblasts could be observed in both the distraction area (Ge *et al.*, 2006) and the osteocompression region of an animal's mandible (Huang and Zeng, 2005). The G171V mutant mice, in which Lrp5 is activated, showed increased numbers of active osteoblasts with an increased functional lifespan (Bennett *et al.*, 2005). Therefore, increasing Wnt10b and Lrp5 secreted by osteoblasts could stimulate more osteoblastogenesis of mesenchymal precursors in alveolar bone after stretching and compression.

Robinson *et al.* (2006) reported that activation of Wnt/β-catenin is a normal physiological response of osteoblasts to mechanical stretching from 4 to 24 hours. In the present research, it was found that Wnt10b and Lrp5 were fast responsive genes following stretching for 1 hour. To date,



there has been no report concerning the effect of compression on Wnt/β-catenin signalling in osteoblasts. In this study, the activation of Wnt signalling after compression was more significant than tension on osteoblasts. Yet, why compression upregulated the Wnt pathway more significantly requires further investigation.

Tsuda *et al.* (1997) found that OPG secreted by osteoblasts could inhibit osteoclastogenesis. It is known that RANKL is a specific and essential differential factor for osteoclastogenesis and bone remodelling (Kong *et al.*, 1999; Hofbauer *et al.*, 2000). OPG and RANKL were determined by RT-PCR in the present study. In the tension group, OPG and RANKL expression decreased with time. At 5 hours, RANKL decreased significantly in the tension group compared with the control. This is similar to a previous report (Yang *et al.*, 2006) that observed that the expression of OPG was downregulated in osteoblasts after stretching. It has also been reported that strain magnitude dose-dependently downregulated RANKL gene expression under stretching (Rubin *et al.*, 2003). The present results are in agreement with these findings. However, little is known about the effect of compression on OPG and RANKL expression in osteoblasts. Only Sanchez *et al.* (2009) reported that after 28 days culture, OPG was downregulated by compression after 4 hours loading. In the current study, OPG expression decreased gradually in the compression group at 1 hour loading and was significant at 5 hours. This difference in timing of OPG expression may due to the different culture duration. Sanchez *et al.* (2009) found that RANKL expression was not affected by compression. In the present study, expression of RANKL decreased at 1 hour and then increased gradually and finally, there was no difference at 5 hours compared with the control.

Since OPG acts as a decoy receptor for RANKL to inhibit osteoclastogenesis (Roodman, 1999), the expression level of both RANKL and OPG produced by the marrow microenvironment is essential to regulate osteoclast formation and osteoclast activity. The present results demonstrated a significant difference in the OPG/RANKL ratio between the tension and compression groups at 5 hours. In the tension group, as the ratio in the OPG/RANKL increased (Figure 5E), it will inhibit osteoclastogenesis and osteoclast maturation.

Figure 5 Gene expression of osteoprotegerin (OPG) (A and B), receptor activator of nuclear factor kappa B (RANK)L (C and D), and the ratio of OPG to RANKL (e) demonstrated different biological response of MC3T3-E1 after being stretched and compressed. OPG expression demonstrated a decreasing tendency in both the experimental groups at 1, 3, and 5 hours (A). At 5 hours, expression of OPG decreased significantly in the compression group compared with the control and tension groups (B). Expression of RANKL decreased at 1 hour and then increased gradually at 3 and 5 hours in the compression group. In the tension group, there was a decreasing tendency with time (C). The expression of RANKL decreased significantly in the tension group compared with the control (D). The OPG/RANKL ratio increased in the tension group but decreased in the compression group compared with the control. A significant difference existed between the compression and tension group (Figure 3E). $*P < 0.05$; $**P < 0.01$.

Together with the effect of Wnt signalling activation to promote osteoblast number and functional lifespan, it is presumed that tensile strain would direct alveolar bone remodelling to formation. In the compression group, the ratio in the OPG/RANKL decreased (Figure 5E) so it will promote bone resorption through osteoclastgenesis and mature osteoclast activation. With upregulation of Wnt signalling to activate osteoblasts, it could be presumed that compressive strain would direct alveolar bone remodelling to resorption.

Conclusions

Under mechanical loading, MC3T3-E1 cells recognize tensile and compressive strain signals. Compression has a more significant effect on activation of Wnt signalling than tension. In MC3T3-E1 cells, tensile strain and compressive strain resulted in a difference in the OPG/RANKL ratio. Tensile strain would lead to alveolar bone formation while compressive strain would result in alveolar bone resorption. This indicates the possibility of a difference in the molecular mechanism of different alveolar adaptation to tension and compression in orthodontics.

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