European Journal of Orthodontics 32 (2010) 698–705
doi:10.1093/ejo/cjq010
Advance Access Publication 4 June 2010

Functional analysis of core binding factor a1 and its relationship with related genes expressed by human periodontal ligament cells exposed to mechanical stress

Ying Yang*, Yanqi Yang**, Xiaotong Li*, Liang Cui*, Minkui Fu*, A. B. Rabie** and Ding Zhang***

Orthodontics, *Peking University School and Hospital of Stomatology, Beijing, **Faculty of Dentistry, University of Hong Kong and ***Department of Stomatology, Peking Union Medical College Hospital, Beijing, China

Correspondence to: Ding Zhang, Department of Stomatology, Peking Union Medical College Hospital, 1 Shuai Fu Yuan, Wang Fu Jing, Beijing 100730, China. E-mail: dingz77@sina.com

SUMMARY Mechanical stress induces human periodontal ligament (PDL) cells to express an osteoblastic phenotype *in vitro*. Core binding factor a1 (CBFA1) is a key regulator of osteoblast differentiation. This study was designed to investigate the role of CBFA1 in alveolar bone remodelling, specifically the expression of CBFA1 messenger RNA (mRNA) in human PDL cells under mechanical stress and its up- and downstream relationships with other bone remodelling markers. Cultured human PDL cells were exposed to mechanical stress. The expressions of CBFA1 and alkaline phosphatase (ALP), osteopontin (OPN), osteoprotegrin (OPG), and receptor activator nuclear factor kappa B ligand (RANKL) were detected before and after RNA interference (RNAi) of CBFA1. The data were analysed using a *t*-test and one-way analysis of variance.

After mechanical stress loading, CBFA1 mRNA expression was raised initially, followed by an increased expression of ALP and RANKL, decreased expression of OPG, and a change in OPN expression. After CBFA1 knock-down in human PDL cells by small hairpin (sh) RNA, the expression of ALP, OPN, OPG, and RANKL also changed. These findings suggest that in the present model system CBFA1 may play an important role in PDL-mediated bone remodelling in response to mechanical stimulation. Mechanical stress: CBFA1–ALP and OPG–PDL homeostasis may be one of the signal transduction pathways of human PDL cell differentiation under mechanical stress without exclusion of the involvement of other pathways.

Introduction

Mechanical stress has long been recognized to be an important regulatory factor in bone homeostasis and a determinant of skeletal morphology both during development and post-natally. Orthodontic tooth movement necessitates bone resorption at the pressure side with concomitant bone formation at the tension side (Yokoya *et al.*, 1997). Ankylosed teeth, in which the cementum of the tooth root is fused with alveolar bone, cannot be moved by therapeutic mechanical stress because of absence of a periodontal ligament (PDL), suggesting that PDL cells are the direct target cells for orthodontic force (Mitchell and West, 1975). However, it has been unclear how PDL cells induce alveolar bone remodelling after being exposed to mechanical force. In bone metabolism, osteoclasts are the only cells that are responsible for bone resorption, while the formation and activity of osteoclasts are regulated by osteoblasts/stromal cells (Suda *et al.*, 1992, 1995). Therefore, osteoblasts play an important role in bone remodelling.

A 'key' regulator of osteoblast differentiation is core binding factor a1 (CBFA1) also known as RUNX2, a member of the runt homology family of transcription factors (Bae *et al.*, 1995; Ducy *et al.*, 1997). CBFA1 binds to the osteoblast-specific *cis*-acting element 2, which is found in the promoter regions of all the major osteoblast-specific genes: osteocalcin (OCN), type I collagen, bone sialoprotein, osteopontin (OPN), alkaline phosphatase (ALP), and collagenase-3, and controls their expression (Ducy *et al.*, 1997; Thirunavukkarasu *et al.*, 2000). Conceivably, CBFA1 plays a pivotal role during osteoblast differentiation and skeletogenesis (Ducy *et al.*, 1997; Rodan and Harada, 1997).

It has previously been demonstrated, using an established system for applying calibrated stretch, that a low level intermittent mechanical strain to human PDL cells rapidly induces expression of osteoblastic-like phenotypes and functional proteins, such as the upregulation of ALP and OCN, and influences the expression of osteoprotegrin (OPG) and receptor activator nuclear factor kappa B ligand (RANKL; Yang *et al.*, 2006). The latter two proteins are believed to be closely related to osteoclastogenesis (Akatsu *et al.*, 1998; Yasuda *et al.*, 1999). However, the function of CBFA1 in mechanical signal transduction in human PDL cells is not fully understood. The objective of this study was

to determine the expression of CBFA1 at the messenger RNA (mRNA) level in cultured human PDL cells subjected to mechanical stress at different time points and to investigate the up- and downstream relationships between CBFA1 and other bone remodelling markers.

Materials and methods

Cell culture

Human PDL fibroblasts were cultured *in vitro* (Somerman *et al.*, 1988; Zhang *et al.*, 2004). The cells were obtained from healthy permanent premolars extracted for orthodontic reasons (20 teeth from five donors, aged: 10–13 years). Informed consent was obtained prior to extraction. Cells that proliferated from the extracts were passaged four to six times.

Exposure of cultured human PDL cells to mechanical stress

Human PDL cells were loaded with intermittent mechanical strain *in vitro* (Yang *et al.*, 2006). Briefly, the cells were subcultured onto a silicone membrane (39.5 × 26 × 0.2 mm) coated with type I collagen. The silicone membrane, attached by approximately an 80 per cent confluent PDL cell layer, was suspended with racks, which provided a uniform uniaxial strain, and immersed in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, California, USA) containing 10 per cent (v/v) foetal bovine serum (HyClone, Logan, Utah, USA) supplemented with antibiotics (100 μg/ml penicillin and 100 μg/ml streptomycin) at 37°C in a 5 per cent CO_2 incubator. The cells were strained with an intermittent in-plane substrate deformation of 12 per cent by a cycle of 3 minutes for 0, 0.5, 1, 2, 4, 6, 12, and 24 hours. The cells and culture medium were harvested at the tested time points. Since the expression of the examined genes in human PDL cells remained unchanged within 24 hours without external stimuli and β-actin, the housekeeping gene was used as the internal control to balance the effects of cell amount. Cells at 0 hour (no loading) were used as the control for the mRNA test.

RNA extraction and semi-quantitative RT-PCR reaction analysis

Total RNA was extracted from the cultured human PDL cells using the Trizol reagent (Invitrogen). The RNA concentrations were calculated spectrophotometrically. All samples had an optical density ratio (OD_{260}:OD_{280}) of at least 1.8. Total RNA from the cells was used to synthesise first-strand complementary DNA (cDNA) by M-MLV (Promega Corporation, Madison, Wisconsin, USA). Polymerase chain reaction (PCR) circulates were determined according to the linear range to ensure the accuracy of semi-quantitative analysis. Primers and PCR conditions are shown in Table 1. Blank control (cDNA was replaced by dH_2O in the PCR mixtures) and negative control [the samples in the PCR mixtures were obtained by reverse transcription (RT) without reverse transcriptase in the RT reaction system] were run for each sample to exclude contamination of the nucleic acid. Cells with non-mechanical stress loading (0 hour) were used as the control to cells with mechanical stress loading for different time periods. PCR products were semi-quantified by ImageMaster 2D Platinum software version 7.0 (General Electric Company Bio-Sciences Corporation, Piscataway, New Jersey, USA) to measure relative integral OD. Each product was normalized with that of β-actin from the same template and shown as a ratio. The experiments were repeated three times.

RNA interference

Plasmid expressing anti-CBFA1 small hairpin RNAs (shRNA, RNAs with a self-complimentary stem loop) were constructed with the sequences of 5′-GTAGCAAGGTTCAACGATC-3′, 5′-GCTTGATGACTCTAAACCT-3′, and 5′-CCAGAA TGATGGTGTTGAC-3′. A mismatch shRNA (non-sense shRNA) of 21 base pairs was used as a blank control with the sequence of 5′-AAAGCTTCATAAGGCGCATGC-3'. A plasmid was constructed with pGenesil-1 as the vector. After 24 hours of transfection, mechanical force was applied to the transfected human PDL cells and the mRNA expressions of CBFA1, ALP, OPN, OPG, and RANKL were detected by semi-quantitative RT-PCR. Cells with non-mechanical stress loading (0 hour) were used as the control.

Assay of secreted ALP

After 24 hours of transfection, the transfected (test group) and non-transfected (control group) cells were seeded onto the silicone membranes at 3×10^5 cells per membrane and exposed to a stretching force. The culture medium was harvested at 0 (non-mechanical force loading), 0.5, 1, 2, 4, 12, and 24 hours after mechanical force loading for both the test and control groups. Secreted ALP was measured by biochemistry testing (Yang *et al.*, 2006). Briefly, 100 μl medium was taken out and mixed with 1 ml of *p*-nitrophenyl phosphate solution (16 mmol/l, Diagnostic Kit 245; Sigma-Aldrich Corporation, Saint Louis, Missouri, USA) at 30°C for up to 5 minutes. The light absorbance at 405 nm of *p*-nitrophenol product formed as a result of ALP-*p*-nitrophenyl phosphate substrate complex was measured on a microplate reader and compared with serially diluted standards. The experiments were repeated three times.

Statistical analysis

Values were expressed as the mean ± standard deviation (SD). The data were analysed using a *t*-test and one-way analysis of variance with the Statistical Program for Social Sciences, version 10.0 (SPSS Inc., Chicago, Illinois, USA). Data with a P value < 0.05 were considered significant.

Table 1 Primers and polymerase chain reaction (PCR) conditions specific for human core binding factor a1 (CBFA1), alkaline phosphatase (ALP), osteopontin (OPN), osteoprotegrin (OPG), receptor activator nuclear factor kappa B ligand (RANKL), and β-actin.

	Primer sequence	PCR condition	Product size (bp)
CBFA1 (GI:2245523)	Sense: 5′-CCACCTCTGACTTCTGCCTC-3′ antisense: 5′-GACTGGCGGGGTGTAAGTAA-3′	95°C, 30 s, 54°C, 30 s, 72°C, 1 min. 35 cycles	172
ALP (GI:40787773)	Sense: 5′-GCCCTCTCCAAGACATATA-3′ antisense: 5′-CCATGATCACGTCGATATCC-3′	94°C, 30 s, 50°C, 30 s, 72°C, 30 s. 30 cycles	372
OPN (GI:16924232)	Sense: 5′-CCAAGTAAGTCCAACGAAAG-3′ antisense: 5′-GGTGATGTCCTCGTCTGTA-3′	94°C, 30 s, 50°C, 30 s, 72°C, 30 s. 30 cycles	348
OPG (GI:15029632)	Sense: 5′-TCAAGCAGGAGTGCAATCG-3′ antisense: 5′-AGAATGCCTCCTCACACAGG-3′	94°C, 30 s, 58°C, 30 s, 72°C, 30 s. 20 cycles	342
RANKL (GI: 21536432)	Sense: 5′-GGCTCATGGTTAGATCTGGC-3′ antisense: 5′-TGACCAATACTTGGTGCTTCC-3′	94°C, 30 s, 54°C, 30 s, 72°C, 30 s. 30 cycles	351
β-actin (GI:15928802)	Sense: 5′-GTGGGGCGCCCCAGGCACCA-3′ antisense: 5′-CTTCCTTAATGTCACGCACGATTTC-3′	95°C, 45 s, 55°C, 45 s, 72°C, 45 s. 30 cycles	540

Results

CBFA1 expression induced by mechanical stretch in human PDL cells

As depicted in Figure 1, unstretched cells (lane 0 hour) exhibited marginally detectable levels of CBFA1 mRNA. These levels were increased after 30 minutes of intermittent applied mechanical load and reached their peak at 1 hour. After 2 hours, the level of CBFA1 began to decline and returned to the unstreched level after 6 hours (Figures 1A, B, and C). Hence, mechanical stimulation evoked an early and rapid induction of CBFA1 mRNA in human PDL cells.

At the same time, ALP mRNA expression was obvious after being stretched for 2 hours and reached its peak at 4 hours (Figure 1B). The expression of OPN was more complicated with three peaks at 0.5, 4, and 12 hours (Figure 1B). The level of OPG declined after 4 hours of mechanical stress loading, while that of RANKL was upregulated (Figure 1C). The changes in both ALP and OPG were retarded compared with that in CBFA1: the expression trendline of ALP was similar to that of CBFA1 but retarded (Figure 1B), while OPG dropped dramatically after CBFA1's fastigium. RANKL showed the same changing tendency as CBFA1 (Figure 1C). Thus, the ratio of OPG:RANKL was significantly decreased during the first hour after mechanical stress loading, which was opposite to the changing tendency of CBFA1 (Figure 1D).

mRNA expressions of CBFA1, ALP, OPN, OPG, and RANKL in anti-CBFA1 shRNA-transfected human PDL cells under intermittent mechanical stress

The expression of CBFA1 in human PDL cells transfected with anti-CBFA1 shRNA was significantly downregulated to 71.9 per cent of the control (data not shown). At the expression peak of CBFA1, after 1 hour of mechanical stress loading, the expression of CBFA1 was reduced to 87.0 per cent of the control (Figure 2A and 2B). Along with the downregulation of CBFA1, the expression of ALP became undetectable by RT-PCR and OPG fell to a low level (Figure 2C and E). Compared with the control, the first expression peak of OPN disappeared. At 6–12 hours, OPN increased more than in the control (Figure 2D). The expression of RANKL declined early under mechanical stress and then increased to the control level, higher than the control (Figure 2F). From the trendline of these factors, ALP and OPG declined with the downregulation of CBFA1 (Figure 2C and 2E). The alteration of OPG:RANKL ratio in anti-CBFA1 shRNA-transfected human PDL cells was opposite to that in the control in the early stage. After 6 hours, the ratio tended to flatten and was slightly less than the control (Figure 2G).

Secretion of ALP protein in anti-CBFA1 shRNA-transfected human PDL cells under intermittent mechanical stress

ALP protein in the culture medium secreted by non-transfected human PDL cells under intermittent mechanical stress was detected after 2 hours of stress loading and then accumulated until 24 hours (Figure 3). However, the secreted ALP became undetectable in the culture medium for anti-CBFA1 shRNA-transfected human PDL cells under intermittent mechanical stress, even after 24 hours accumulation.

Discussion

PDL cells are known to be heterogeneous (Lekic *et al.*, 2001) and have the potential to differentiate into various phenotypes, including osteoblasts, chondrocytes, and adipocytes (Gay *et al.*, 2007). They are indispensable for the regeneration of periodontal tissues, including unmineralized PDL, and two mineralized tissues, i.e. cementum and bone. It has been suggested that human PDL cells exposed to mechanical strain *in vitro* can express an

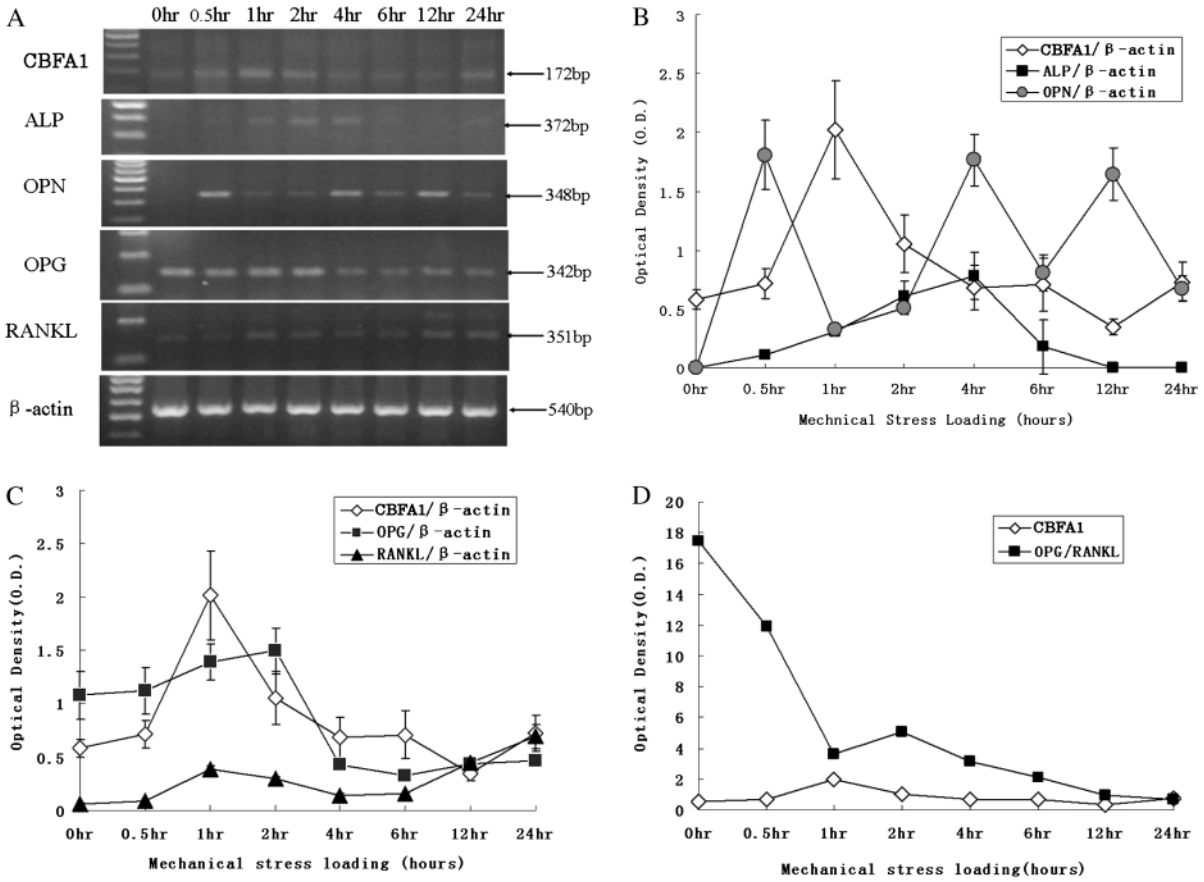
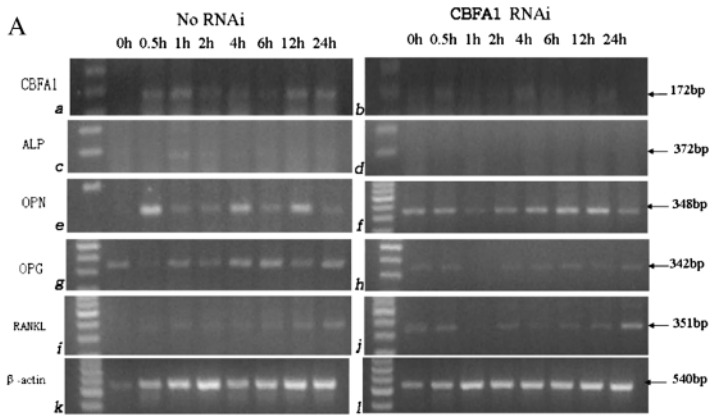
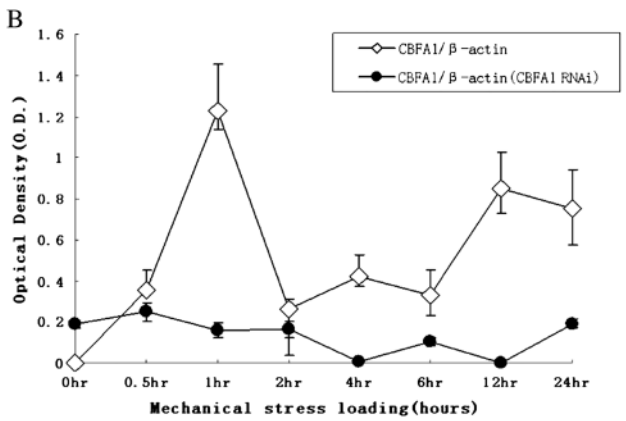


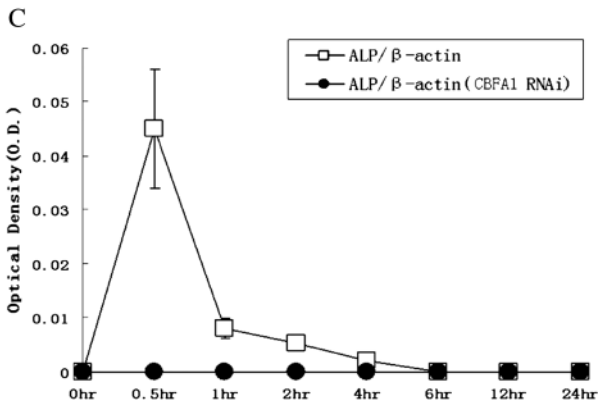
Figure 1 Effect of mechanical stress on expressions of core binding factor a1 (CBFA1), alkaline phosphatase (ALP), osteopontin (OPN), osteoprotegerin (OPG), and receptor activator nuclear factor kappa B ligand (RANKL) in human periodontal ligament (PDL) cells estimated by reverse transcription–polymerase chain reaction (PCR). The sizes of the PCR products were compared with a 100 bp DNA ladder (left lane; A). Human PDL cells were harvested at 0, 0.5, 1, 2, 4, 6, 12, and 24 hours, respectively, after mechanical stress loading. Each product was normalized with that of β-actin from the same template and shown as a ratio (B, C, and D). Unstretched cells (lane 0 hour) exhibited marginally detectable levels of CBFA1 messenger mRNA (mRNA). These levels were increased after 0.5 hours of intermittent applied mechanical load and reached their peak at 1 hour. After 2 hours, the level of CBFA1 began to decline and returned to an unstreched level after 6 hours (A, B, and C). At the same time, ALP mRNA expression was obvious after being stretched for 2 hours and reached its peak at 4 hours (A and B). The expression of OPN was more complicated with three peaks at 0.5, 4, and 12 hours, respectively (A and B). The level of OPG declined after 4 hours of mechanical stress loading, while that of RANKL was upregulated with the application of mechanical force (A and C). The changes of both ALP and OPG were retarded compared with that of CBFA1: the expression trendline of ALP was similar to that of CBFA1 but retarded, while OPG decreased significantly after the CBFA1 fastigium. RANKL had the same changing tendency as CBFA1 (A, B, and C). The ratio of OPG:RANKL was downregulated during the first hour after mechanical stress loading, which was opposite to the changing tendency of CBFA1 (D). Experiments were repeated three times with similar results (n = 3).

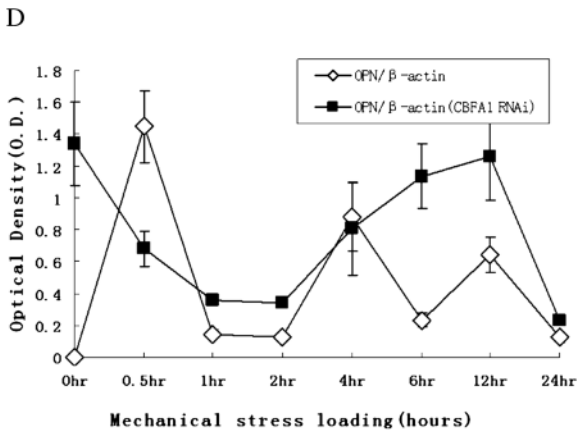
osteogenic and chondrogenic transcriptional profile (Yang *et al.*, 2006; Wescott *et al.*, 2007; Zhao *et al.*, 2008). The aim of the present study was to explore the up- and downstream relationships among the mechanoresponsive factors.

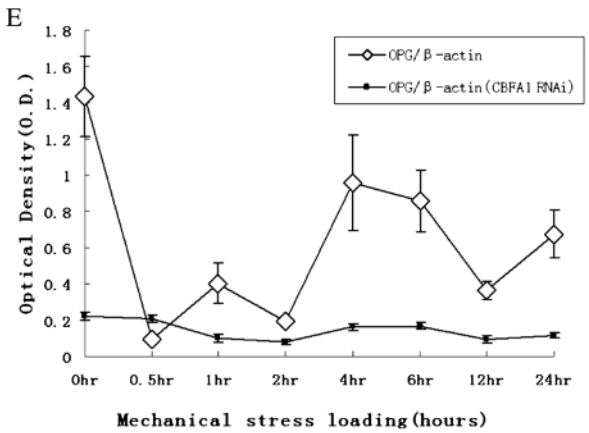
Several studies have established the importance of CBFA1 in osteoblast differentiation and function, and cememtoblast differentiation (Rodan and Harada, 1997; Thirunavukkarasu *et al.*, 2000; Yang *et al.*, 2006). Previous research has provided evidence that CBFA1, a pivotal transcriptional regulator of osteoblast differentiation and bone formation, is a key target of mechanical stimulation in human PDL osteoblastic cells. The present data demonstrated that mechanical stress increased the mRNA level of CBFA1 in human PDL cells, which is in agreement with the results from an *in vivo* study (Kawarizadeh *et al.*, 2005), and also upregulated the mRNA levels of ALP and OPG, which suggests that mechanical stimulation might promote the differentiation of human PDL cells by activating CBFA1, the crucial transcription factor. CBFA1 can stimulate the transcription of such osteoblast-specific genes through cognate binding sites in their promoter regions (Ducy *et al.*, 1997; Thirunavukkarasu *et al.*, 2000). On the one hand, mechanical stress results in augmentation of CBFA1 levels and, on the other, some osteoblast-specific genes are induced under various conditions of mechanical stress (Thirunavukkarasu *et al.*, 2000; Yang *et al.*, 2006). However, the above results cannot illustrate the up- and downstream

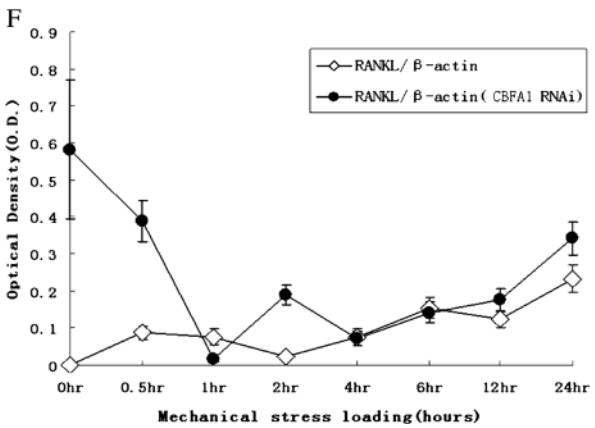

A
No RNAi
CBFA1 RNAi
0h 0.5h 1h 2h 4h 6h 12h 24h
CBFA1
ALP
OPN
OPG
RANKL
β-actin
172bp
372bp
348bp
342bp
351bp
540bp

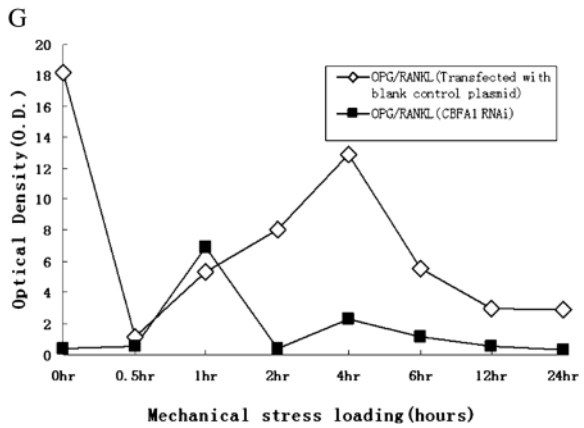

B
CBFA1/β-actin
CBFA1/β-actin(CBFA1 RNAi)
Optical Density(O.D.)
Mechanical stress loading(hours)
0hr 0.5hr 1hr 2hr 4hr 6hr 12hr 24hr


C
ALP/β-actin
ALP/β-actin(CBFA1 RNAi)
Optical Density(O.D.)
0hr 0.5hr 1hr 2hr 4hr 6hr 12hr 24hr


D
OPN/β-actin
OPN/β-actin(CBFA1 RNAi)
Optical Density(O.D.)
Mechanical stress loading(hours)
0hr 0.5hr 1hr 2hr 4hr 6hr 12hr 24hr


E
OPG/β-actin
OPG/β-actin(CBFA1 RNAi)
Optical Density(O.D.)
Mechanical stress loading(hours)
0hr 0.5hr 1hr 2hr 4hr 6hr 12hr 24hr


F
RANKL/β-actin
RANKL/β-actin(CBFA1 RNAi)
Optical Density(O.D.)
Mechanical stress loading(hours)
0hr 0.5hr 1hr 2hr 4hr 6hr 12hr 24hr


G
OPG/RANKL(Transfected with blank control plasmid)
OPG/RANKL(CBFA1 RNAi)
Optical Density(O.D.)
Mechanical stress loading(hours)
0hr 0.5hr 1hr 2hr 4hr 6hr 12hr 24hr

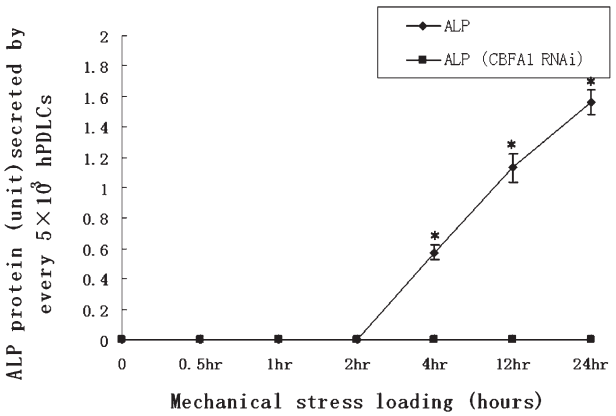


Figure 3 Secretion of alkaline phosphatase (ALP) protein in anti-core binding factor a1 (CBFA1) small hairpin RNAs (shRNA)-transfected human periodontal ligament (PDL) cells under intermittent mechanical stress. After 24 hours of transfection, mechanical force was applied to the transfected human PDL cells and the culture medium was harvested at 0, 0.5, 1, 2, 4, 12, and 24 hours. The non-transfected human PDL cells were used as the control. Both the transfected cells (test group) and the non-transfected cells (control group) were seeded onto the silicone membranes at 3×10^5 cells per membrane and exposed to intermittent stretching force by a cycle of 3 minutes. ALP protein in the culture medium secreted by non-transfected human PDL cells under intermittent mechanical stress was detected after 2 hours of loading and then accumulated until 24 hours. However, the secreted ALP became undetectable in the culture medium for anti-CBFA1 shRNA-transfected human PDL cells under intermittent mechanical stress, even after 24 hours accumulation. The asterisks represents significant difference between the two groups ($P < 0.05$). Experiments were repeated three times with similar results ($n = 3$).

relationship between CBFA1 and the other osteoblast-specific genes, although the change of the other genes occurred slightly later compared with that of CBFA1.

RNA interference (RNAi), a widely used tool for analysis of gene function in invertebrates and plants (Hammond *et al.*, 2001; Caudy *et al.*, 2003), can efficiently knock-down specific gene expression. Thus, the up- and downstream relationship among genes can be observed from a negative point of view by knock-down of the suspected upstream gene by RNAi. A modified U6 anti-CBFA1 shRNA plasmid was constructed and transfected to human PDL cells in the present study. The results demonstrated a 71.9 per cent reduction of CBFA1 expression. It also demonstrated that after knocking down CBFA1 expression using the RNAi technique, the expressions of ALP mRNA and OPG mRNA were also significantly reduced under mechanical force application. The expression of ALP and the downregulation of CBFA1 were undetectable by RT-PCR. Since the change in the expression of ALP mRNA was significant, the secreted ALP in culture medium was tested in order to evaluate the change of ALP expression at the protein level. The result of the ALP protein test confirmed that ALP expression was almost completely inhibited in anti-CBFA1 shRNA-transfected human PDL cells. The above results suggest that CBFA1 is located upstream of ALP and OPG. 'Mechanical stress–CBFA1 activation–change of ALP and OPG' may be one of the signalling pathways in human PDL cell differentiation induced by mechanical force without exclusion of other regulating pathways.

In the present study, mechanical stress induced CBFA1 augmentation in a time-dependent manner in human PDL cells. It reached its peak at 1 hour and then declined. It can be assumed that some unknown mechanism resulted in the shut down since the PDL maintains its biological width under constant biting force. It has been suggested that the only factor to control CBFA1 expression directly is CBFA1 itself, and there should be an autoregulation mechanism for CBFA1 (Ducy, 2000; Ziros *et al.*, 2002). This may contribute to maintaining physiological PDL width under mechanical loading due to occlusion or orthodontic tooth movement.

Osteoclasts are the only cells involved in bone resorption. Direct contact with the body of osteoblasts/stromal cells is necessary for osteoclastogenesis. The combination of RANKL on osteoblasts with RANK on pre-osteoclasts activates osteoclastogenesis. As a non-functional receptor of RANKL, OPG, which is produced by osteoblasts, competitively binds with RANK. It can prevent bone resorption by restraining osteoclastogenesis. OPG and RANKL are the terminal points of osteoclastogenesis induced by various stimuli (Akatsu *et al.*, 1998; Yasuda *et al.*, 1999; Yang *et al.*, 2006). It has been reported that CBFA1 can upregulate the expression of RANKL at mRNA level (Gao *et al.*, 1998), while O'Brien *et al.* (2002) considered CBFA1 had no distinct effects on RANKL. In

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Figure 2 The expressions of core binding factor a1 (CBFA1), alkaline phosphatase (ALP), osteopontin (OPN), osteoprotegrin (OPG), and receptor activator nuclear factor kappa B ligand (RANKL) in anti-CBFA1 small hairpin RNA (shRNA)-transfected human PDL cells under intermittent mechanical stress estimated by reverse transcription–polymerase chain reaction (PCR). Human PDL cells were harvested at 0, 0.5, 1, 2, 4, 6, 12, and 24 hours, respectively, after mechanical stress loading. (A) The sizes of PCR products were compared with a 100 bp DNA ladder (left lane). Expression of a, CBFA1 messenger mRNA (mRNA) in no-RNA interference (RNAi) group; b, CBFA1 mRNA in RNAi group; c, ALP mRNA in no-RNAi group; d, ALP mRNA in RNAi group; e, OPN mRNA in no-RNAi group; f, OPN mRNA in RNAi group; g, OPG mRNA in no-RNAi group; h, OPG mRNA in RNAi group; i, RANKL mRNA in no-RNAi group; j, RANKL mRNA in RNAi group; k, β-actin mRNA in no-RNAi group; l, β-actin mRNA in RNAi group. Experiments were repeated three times with similar results. Each product was normalized with that of β-actin from the same template and shown as a ratio. (B) The expression of CBFA1 in human PDL cells transfected with anti-CBFA1 shRNA was significantly downregulated to 71.9 per cent of the control. At the expression peak of CBFA1, after 1 hour of mechanical stress loading, the expression of CBFA1 was reduced to 87.0 per cent of the control. (C and E) Along with the downregulation of CBFA1, the expression of ALP became undetectable by real-time PCR and OPG fell to a low level. (D) Compared with the control, the first expression peak of OPN disappeared. At 6–12 hours, OPN increased more than in the control. (F) The expression of RANKL declined early under mechanical stress, then increased to the control level, and higher than the control. (G) The alteration of OPG:RANKL ratio in anti-CBFA1 shRNA-transfected human PDL cells was opposite to the control in the early stage. After 6 hours, the ratio became smooth and was slightly lower than the control. The experiments were repeated three times with similar results ($n = 3$).

the present study, RANKL changed almost simultaneously with CBFA1 under mechanical stimulus. After the expression of CBFA1 was knocked down, the expression of RANKL decreased at the initial mechanical loading stage but increased soon after. This indicates that although the regulation of CBFA1 to RANKL cannot be excluded, it is indirect or there are other regulating pathways.

The ratio of OPG:RANKL reflects that osteoclast activities are regulated by osteoblasts. When the ratio increases, it is favourable for bone formation, and when it declines, it is good for bone resorption (Akatsu *et al.*, 1998; Yasuda *et al.*, 1999; Yang *et al.*, 2006). The present data showed that when mechanical force was applied to human PDL cells, the ratio of OPG:RANKL declined rapidly in the initial hour, which is favourable for the activation of osteoclasts. After downregulation of CBFA1, the ratio increased with the application of mechanical force, which indicates that CBFA1 to some degree regulates the ratio of OPG:RANKL. With the prolongation of mechanical force application, the ratio became stable at a relatively low level, which is beneficial for the balance of bone formation and bone resorption, i.e. CBFA1 is a closely related biological signal transduction for bone remodelling in mechanical stimulated human PDL cells.

One point of view is that the chosen mechanical *in vitro* model generating tensional force is different from the *in vitro* force on cells and *in vivo* force on teeth. The former is a microcosmic force, while the latter is a macroscopic force. In other words, *in vitro* tension force should not be regarded to be equivalent to *in vivo* tension. Clinically, orthodontically induced tooth movement produces tension and compression in certain regions of the PDL. At the tension sides, more bone and cementum is deposited, whereas at the compression sites there is a net loss of bone (and cementum) due to osteoclastic resorption. The *in vitro* model of mechanical stress stimulation cannot be equated with the *in vivo* clinical force. *In vivo* studies do not always provide an ideal model for examining the effects of mechanical force due to the complex internal environment. Tensile mechanical strain has been widely used to study the effects of mechanical stress on *in vitro* cultured cells since it is claimed that strain is one of the basic types of microcosmic force to which cells react (Yang *et al.*, 2006; Wescott *et al.*, 2007). Uniaxial strain was chosen in the present research to represent more closely the deformation to which PDL cells are exposed *in vivo* during occlusal loading and orthodontic tooth movement. The strain value of 12 per cent was based on numerical data derived from a finite element model (Natali *et al.*, 2004). This suggested that maximal PDL strains for horizontal displacements of a human maxillary central incisor under physiological loading conditions lie in the vicinity of 8–25 per cent, depending on the apico-crestal position; a value of 12 per cent correlates well with strain conditions predicted at the mid-root.

Bone remodelling is a combination of bone formation and bone resorption. What is significant in the present study is the observation that CBFA1 and bone formation markers were upregulated under mechanical stress at the same time as the OPG:RANKL ratio indicated a shift towards increased osteoclastogenesis and/or osteoclast activity. These data clearly demonstrate that force application leads to the expression of regulatory factors that are responsible for both bone formation and bone resorption, i.e. bone remodelling. Later, the OPG:RANKL ratio became relatively stable rather than continuously decreasing, which indicates that mechanical force induces bone resorption within physiological limits. Orthodontic tooth movement is a physiological condition, and bone formation and bone resorption are balanced under the influence of the above-mentioned factors. In this process, CBFA1 is closely related to important biological signal transduction, but other regulating tracks cannot be excluded.

OPN is an early marker of osteoblast differentiation and plays an important role in bone formation, resorption, and remodelling (Butler, 1989; Denhardt and Guo, 1993; McKee *et al.*, 1993). OPN is thought to promote or regulate the adhesion, attachment, and spreading of osteoclasts to the bone surface during bone resorption (Standal *et al.*, 2004). Previous studies have demonstrated that mechanical stress induced expression of OPN is mostly composed of, or is present in, almost all osteocytes and in some osteoblasts and bone-lining cells at the resorption site in the early stage of experimental tooth movement (Ikeda *et al.*, 1992; Terai *et al.*, 1999). After downregulation of CBFA1, the expression of OPN was reduced, then increased, and finally decreased. These data raise the question of the regulatory relationship of CBFA1 to OPN.

Although semi-quantitative RT-PCR is not as robust as quantitative RT-PCR for quantification, it is simple and economical. In the present study, the dynamics test and cycle determination of RT-PCR were carried out to find the linear range and to determine the amount of RNA, which ensured the creditability and accuracy of the semi-quantitative analysis. The electrophoregram showed changes of gene expression.

The results of the present study suggest the up- and downstream relationship among the mechanoresponsive factors—CBFA1, ALP, OPN, and OPG at mRNA level, and reveal the relationship between CBFA1 and ALP at the protein level. Analysis of these data should facilitate future studies of the involvement of these factors in remodelling the periodontium *in vivo* to further reveal the mechanism of orthodontic tooth movement.

Conclusions

From the positive and negative points of view, the findings of the present study indicate that:

1. Mechanical stress initially activates CBFA1 in human PDL cells *in vitro*, followed by the expression changes of ALP and OPG. CBFA1 locates upstream of ALP

and OPG, and regulates their expressions. OPN and RANKL expressions were also regulated by a change of CBFA1.

2. CBFA1 may, at the same time, take part in osteoclastogenesis through regulation of OPG:RANKL ratio. Mechanical stress: CBFA1—ALP and OPG—PDL homeostasis may be one of the signal transduction pathways of human PDL cell differentiation under mechanical stress without exclusion of the involvement of other pathways.
3. CBFA1 may play an important role in PDL-mediated bone remodelling in response to mechanical stimulation. It was found that CBFA1 is involved in the expression of bone remodelling factors, which are related to both osteoblast differentiation and osteoclastogenesis.

Funding

Natural Scientific Foundation of China (30340063); Natural Scientific Foundation of Beijing (7042034) and Seed Funding Programme for Basic Research, University of Hong Kong (200803159006).

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