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Proliferation and differentiation of periodontal ligament cells following short-term tooth movement in the rat using different regimens of loading

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SUMMARY Previous studies have indicated that periodontal ligament (PDL) cells demonstrate osteogenic potential and osteoblastic differentiation via the extracellular signal-regulated kinase (ERK) pathway under mechanical stress *in vitro* and *in vivo*. This study aimed to further analyse this regulatory process experimentally in the rat.

The right upper first molars of 25 twelve-week-old male Wistar anaesthetized rats were loaded with forces in order to be moved mesially. Constant forces for 4 hours of 0.25 and 0.5 N were applied in five animals each. Furthermore, constant forces for 2 hours of 0.1 N were applied in 10 animals and afterwards, the first and second molars were permanently separated with composite. In these animals, the antagonists were sliced and five rats were killed after 1 day and five after 2 days. As a last experiment, intermittent forces of 0.1 N and 0.25 Hz were applied in five different animals for 4 hours. The untreated contralateral sides served as the control. Paraffin-embedded sections were analysed by immunohistochemistry for proliferating cell nuclear antigen (PCNA), runt-related transcription factor 2 (Runx2/Cbfa1), and phosphorylated extracellular signal-regulated kinase 1/2 (pERK1/2). Statistical analysis to determine differences between the groups was carried out using a Student's *t*-test.

In selected areas under tension, the proportion of pERK1/2-positive cells was increased compared with those in control teeth under all types of loading, whereas these proportions in selected areas under pressure were increased only after the application of intermittent forces. In representative areas, both under tension and pressure, the proportion of Runx2-positive cells decreased after the application of constant forces. After the application of constant forces for 4 hours in representative areas, both under tension and pressure, the proportion of PCNA-positive cells was lower than those in control teeth.

The involvement of pERK1/2, Runx2/cbfa-1, and PCNA in the reaction of PDL cells to different load regimens was verified.

Introduction

Periodontal ligament (PDL) cells are highly specialized cells that reside between tooth and alveolar bone and can differentiate into cementoblasts to synthesize tooth root cementum or osteoblasts to synthesize bone for skeletal support of the tooth. In response to applied mechanical forces, osteoblast-like PDL cells perceive mechanical signals and respond to them via cellular events such as cell proliferation, differentiation, matrix synthesis, and matrix degradation (Davidovitch, 1991; Long *et al.*, 2002). These events, in turn, are controlled by sequential synthesis of neurotransmitters, cytokines, growth factors, and arachidonic acid metabolites that regulate bone resorption at compression sites and bone synthesis at tensions sites (Saito *et al.*, 1991; Krishnan and Davidovitch, 2006).

In vitro studies of cell cultures of different osteoblastic cell lines have demonstrated that the initial reaction of these cells to mechanical stress is partly mediated by deformation of the cytoskeleton (Sandy *et al.*, 1993) via physical interaction of collagen type I and receptors of the integrin family (Calvalho *et al.*, 1996). A key link between these membrane-bound receptors and changes in the pattern of gene expression has been shown to be the mitogen-activated protein kinase (MAPK) pathways (Matsuda *et al.*, 1998; Xiao *et al.*, 2000).

Extracellular signal-regulated kinases (ERKs), members of the MAPK family, have been shown to participate in a diverse array of cellular programmes, including cell differentiation, cell proliferation, and apoptosis in a cell type-specific modus. Mechanical stimuli have been shown to activate ERK1/2 in osteoblastic cells *in vitro*. In detail, the ERK1/2 signal pathway is involved in different cellular responses such as collagen synthesis (Chaudhary and Avioli, 2000), cyclo-oxygenase expression (Wadhwa *et al.*, 2002), and osteopontin production (You *et al.*, 2001). Ziros *et al.* (2002) have shown that mechanical stimuli lead to increased expression of runt-related transcription factor 2 (Runx2/Cbfa1), a transcription factor that is a pivotal regulator of osteoblast differentiation, via the ERK1/2 pathway *in vitro*.

Runx2 binds to the osteoblast-specific cis-acting element 2 (OSE2), which is found in the promoter regions of many osteoblast-specific genes (i.e. osteocalcin, collagen type I, bone sialoprotein, osteopontin, alkaline phosphatase, and collagenase-3), and controls their expression (Ducy *et al.*, 1997; Harada *et al.*, 1999). Conceivably, Runx2 expression plays a key role during osteoblast differentiation and skeletogenesis (Karsenty *et al.*, 1999). Kawarizadeh *et al.* (2005) have shown that in the rat, a short-term mechanical stimulus up-regulated Runx2 and that this regulation may be achieved via the ERK pathway.

The aim of this study was to expand previous *in vitro* and *in vivo* results by experiments applying various load regimens to the upper first molars of rats and by quantitative analysis of immunohistochemical detection of ERK1/2 and Runx2 in regions representing the classic tension and pressure zones around the mesial root. In addition, the relationship between cell differentiation and cell proliferation in the PDL during the early phase of tooth movement was examined.

Materials and methods

Animals

Twenty-five 12-week-old male Wistar rats, weighing 300–350 g each (Harlan Winkelmann, Borchen, Germany) were used in the experiment. They were provided with food and water *ad libitum*. The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee of the local district government and the Animal Care Commissioner of the University of Bonn, Germany.

Experimental protocol

According to the experimental protocol of Kawarizadeh *et al.* (2005), the rats were anaesthetized with 0.01 ml Rompun (Bayer, Leverkusen, Germany) and 0.24 ml Ketavet (Pharmacia and Upjohn, Erlangen, Germany). The animals were clamped in a head-holding device and the occlusal surface of the maxillary right first molar was prepared by grinding a small hole with a dental diamond bur (Komet H4MC.FG.010/12, Gebr. Brasseler GmbH & Co. KG, Lemgo, Germany). The tooth surface was then treated with self-etching bonding material (Xeno III, Dentsply DeTrey, Konstanz, Germany) for 60 seconds. An orthodontic appliance consisting of a T-loop (0.016 × 0.022 inch stainless steel wire, Ormco Corp., Glendora, California, USA) was placed between the molar and a high-resolution three-dimensional force/torque transducer (ATI, Industrial Automation, Garner, North Carolina, USA), which had a resolution of 0.0125 N for force and 0.0625 Nmm [the Systeme International unit of the Torque (Moment) given in Newton times millimetre] for torque (Figure 1A). The T-loop was fixed to the occlusal surface of the molar with light-curing composite (Tetric, Vivadent, Schaan, Liechtenstein). In a first group of animals, constant forces for 4 hours of 0.25

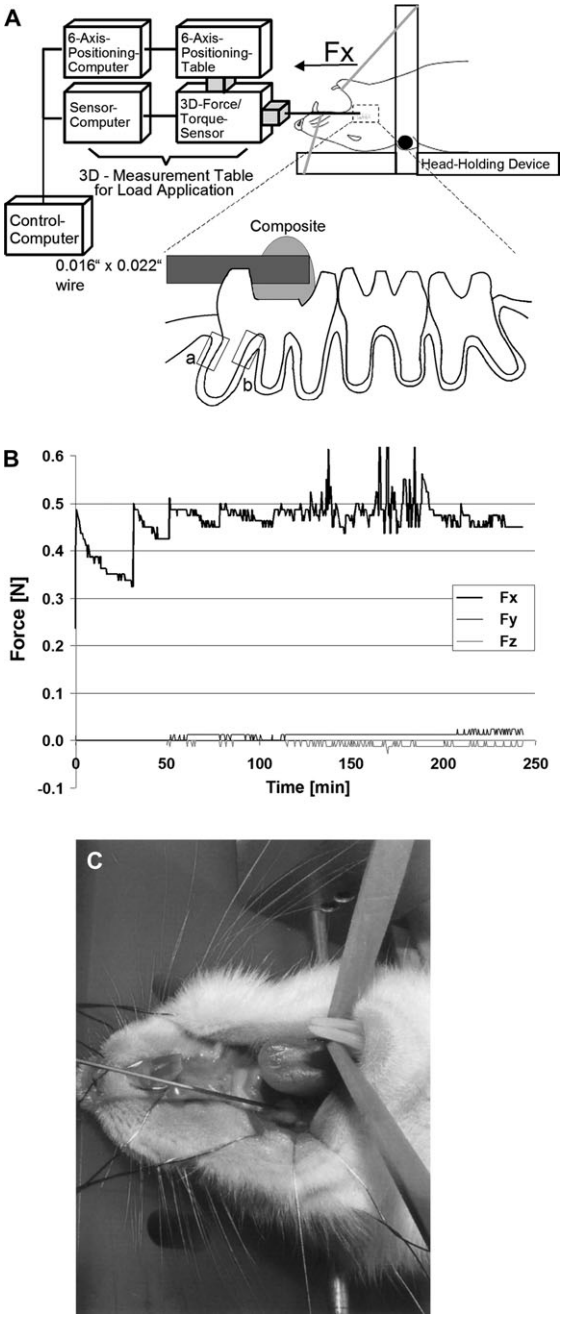

Figure 1 (A) Diagrammatic representation of the experimental procedure for application of the orthodontic force system. The force was applied and measured using a three-dimensional force/torque transducer and mounted onto a six-axis-positioning table. By moving the transducer into the corresponding direction, loads were applied to the first molar. Areas under investigation in which immunopositive cells were counted: (a) mesiocoronal and (b) distocoronal. (B) Force diagram: The measured force curves in the *x* direction indicate a constant loading of the rat molar over the time of the experiment. The forces in other directions were close to zero (Fx, mesial force; Fy, transversal force; Fz, vertical force). (C) Placement of the appliance in the oral cavity of the rat.

and 0.5 N were applied in five animals in order to move the molars mesially. In the second group, constant forces for 2 hours of 0.1 N were applied in 10 animals. Afterwards, the first and second molars were passively separated with

composite and the antagonists were sliced. Five rats were killed after 1 day and five after 2 days. Finally, intermittent forces of 0.1 N and 0.25 Hz were applied for 4 hours in five animals. The applied forces were recorded continuously for the time of the experiment (Figure 1B). The untreated contralateral molars in five rats served as the controls.

Histology

On completion of the experiments, the anaesthetized animals were killed by an intravenous injection of 2 ml T61 (embutramid mebezonium iodide; Intervet, Unterschleissheim, Germany) and decapitated. The maxillae were removed and dissected into right and left halves. The soft tissues around the jaw bone, except for the gingiva, were removed. The specimens were fixed in 4 per cent paraformaldehyde in 0.1 M phosphate buffer for 24 hours and decalcified in 10 per cent ethylene diamine tetraacetic acid at room temperature for 6 weeks. After being dehydrated in ascending grades of alcohol, cleaned in xylene, and paraffin embedded, 5 μm serial sections were cut parasagittally on a microtome (HM 355s; Microm Int., Walldorf, Germany) and mounted on glass slides (K. Roth, Karlsruhe, Germany). Selected sections were stained with haematoxylin–eosin.

Immunohistochemistry

Immunohistochemical staining was carried out with anti-proliferating cell nuclear antigen (PCNA) mouse monoclonal antibody (diluted at 1:500, Zymed Laboratories, South San Francisco, California, USA), Runx2 goat polyclonal antibody (diluted at 1:50, Santa Cruz Biotechnology, Santa Cruz, California, USA), and phosphorylated extracellular signal-regulated kinase 1/2 (pERK1/2) mouse monoclonal antibody (diluted at 1:50, US Biological, Swampscott, Massachusetts, USA). The sections were deparaffinized and rehydrated, rinsed with tris–hydroxymethyl aminomethane-buffered saline solution (TBS) at pH 7.4 for 10 minutes, and then soaked in methanol/H_2O_2 for 20 minutes in the dark to block endogenous peroxidase activity. Anti-PCNA, Runx2, and pERK1/2 antibodies were used in TBS–bovine serum albumin (BSA; 4 per cent) at 4°C overnight and diluted in a humidity chamber. Subsequently, sections were washed in TBS and incubated with Envision+/horse radish peroxidase (HRP) anti-mouse or anti-goat immune globulin/HRP (DakoCytomation, Hamburg, Germany), as secondary antibodies for 30 minutes in a humidity chamber at room temperature. Antibody complexes were visualized using diaminobenzidine for 10 minutes resulting in brown staining. Thereafter, the slides were rinsed, counterstained with Mayer's haematoxylin for 5 seconds, rinsed again, and mounted. Negative controls were prepared by omission of the primary antibodies from the staining procedures. The specificity of the used antibodies was confirmed before use by immunoblotting analysis (Ogata *et al.*, 1985) or by the manufacturer (US Biological).

Morphometry

To establish representative regions of the periodontium and the adjacent alveolar bone, those sections were chosen that showed the third root of the right upper first molar in maximal length and with complete radicular pulp. From these, three sections at 30 μm intervals were taken for each quantitative analysis. Sections were scanned by means of a camera (AxioCam MRC; Zeiss, Göttingen, Germany) mounted on a light microscope (Axiophot 2; Zeiss) and viewed with imaging software (Axiovision) on a personal computer. Counting of the percentage of immunohistochemically positive PDL cells was performed in two separate predefined areas of 750 × 375 μm each in every selected section. These areas were located mesio- and distocoronally to the mesial root (Figure 1A). Counts were performed at a magnification of ×400. Means and standard deviations were calculated for each group of five animals.

Statistical analysis

Student's *t*-tests to determine differences between groups and with regard to the localization of counted positive cells were performed. The level of significance was set at $P < 0.05$. To evaluate the accuracy of the method, one author (DP) double counted 30 randomly chosen sections. The intraobserver error was 3.9 per cent.

Results

Histology

Orthodontic loading of the upper first molars resulted in stretching of the periodontal fibres at the distocoronal aspects of the mesial root (Figure 1Ab) and compression of the ligament on the mesiocoronal side of the same root (Figure 1Aa). Hence, zones of pressure and tension were formed (Ogata *et al.*, 1985; Kawarizadeh *et al.*, 2004) and could be clearly identified. In the pressure zone, obvious structural disturbance of PDL fibres was observed indicating hyalinization. Many fibroblasts showed pyknosis and also vessel damage and extravasation. In the areas under tension, the typical morphology of stretched PDL fibres was visible. Apposition of bone on the mesial surfaces of the alveolar septa and cementum on the distal root surfaces was seen. These surfaces were mostly covered by osteoblasts or cementoblasts, respectively.

Immunohistochemistry and morphometry

In the selected areas under tension, the proportion of pERK1/2-positive cells was higher than for the control teeth under all types of loading, whereas these proportions in selected areas under pressure were higher only after the application of intermittent forces ($P < 0.05$; Figures 2A–D and 3A,B). In

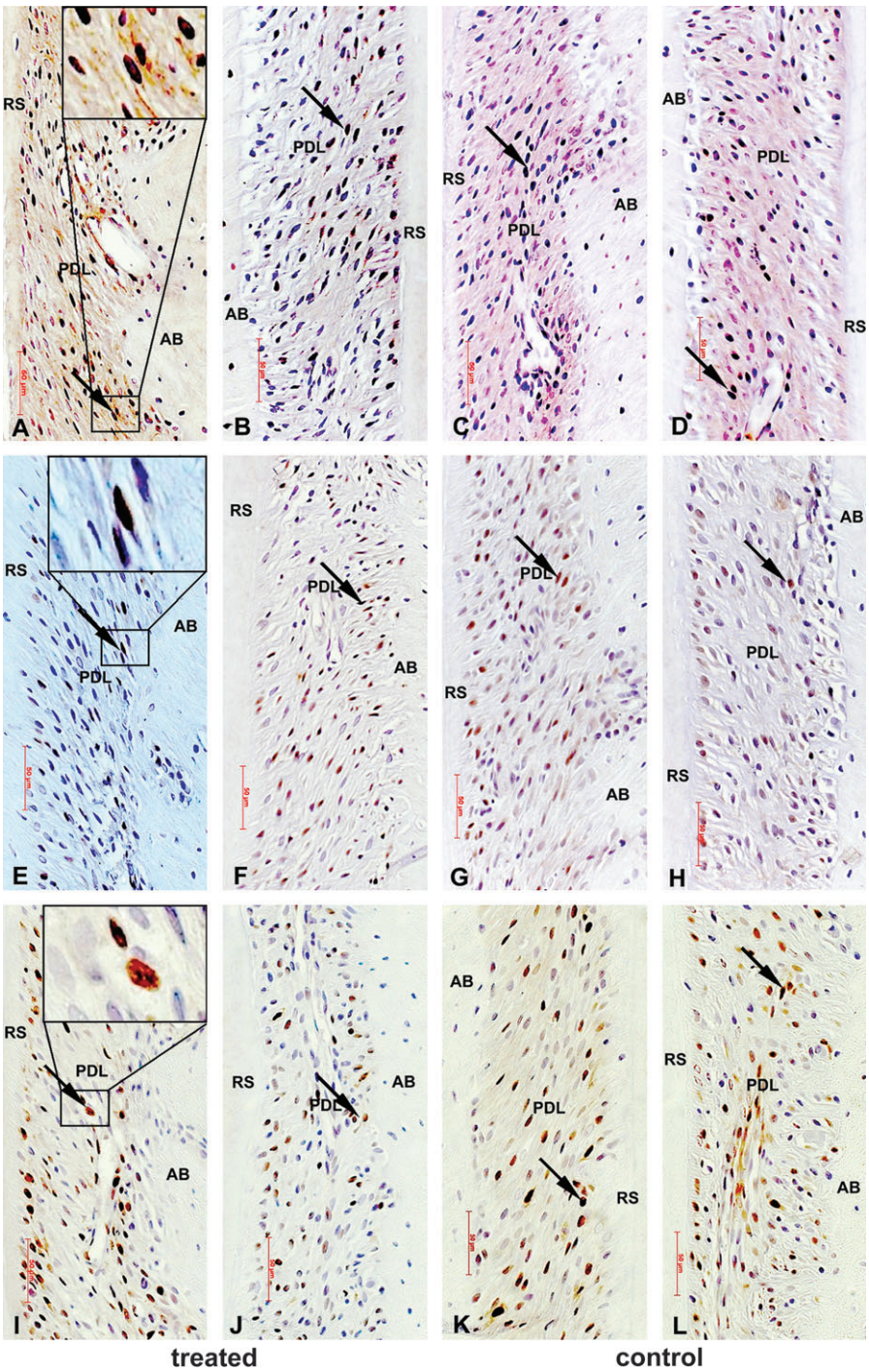

Figure 2 Immunolocalization of phosphorylated extracellular signal-regulated kinases 1/2 (PERK 1/2) (A–D), runt-related transcription factor 2 (Runx2) (E–H), and proliferating cell nuclear antigen (PCNA) (I–L) on the distocoronal (A, C, E, G, I, and K) (tension side) and mesiocoronal (B, D, F, H, J, and L) (pressure side) of the mesial root in the periodontal ligament (PDL). Representative views of immunolabelling in the control of pERK1/2 (C and D) after 4 hours with application of a force of 0.5 N (A and B). The positive cells are immunostained and appear brown. Representative immunostaining of Runx2 and PCNA in the control (G and H) and (K and L) after 4 hours with application of a force of 0.5 N (E and F) and (I and J). The labelled cells are regularly distributed in the PDL. Magnification ×400; The insets in (A, E and I) are presented with a higher magnification of ×1200. scale bars = 50 μm; AB, alveolar bone; RS, root surface; arrows, immunopositive cells.

addition, only in the tension zones was a higher number of pERK1/2-positive cells within the same time interval after the application of higher constant forces (0.5 N) compared with the lower force (0.25 N) ($P < 0.01$; Figures 2A and 3A) observed. There was no statistical difference concerning pERK1/2-positive cells between the tension and pressure zones except for the third group, where constant forces for 2 hours of 0.1 N were applied and the animals were killed after 1 day ($P < 0.05$).

In the representative areas both under tension and pressure, the proportion of Runx2-positive cells decreased after the application of constant forces ($P < 0.05$; Figures 2E,F and 3C,D). Interestingly, in the same way as with pERK1/2, there was no statistical difference in Runx2-positive cells between the tension and the pressure zones.

In the selected areas, both under tension and pressure, the proportion of PCNA-positive cells after the application of constant forces for 4 hours was lower than in the control teeth ($P < 0.01$; Figures 2I–L and 3E,F). In the fourth group, where constant forces for 2 hours of 0.1 N were applied and the animals were killed after 2 days, the proportion of PCNA-positive cells under pressure was lower than on the tension side ($P < 0.01$) and also lower than in the control teeth ($P < 0.001$; Figure 3F).

Discussion

Orthodontic tooth movement is induced by mechanical stimuli and realized by remodelling of the alveolar bone. Specifically, PDL cells bear the phenotypic characteristics of osteoblast-like cells and play a pivotal role in remodelling and repair (Meikle, 2006), but the exact sequence of events that take place during these processes remains unresolved.

The present study was performed to investigate the responses of PDL cells to various mechanical loading. Constant forces of varying amounts were applied for different time periods. Moreover, it is the first time that intermittent forces have been applied in a well-controlled animal model. It was demonstrated that application of precise loading modifies the transcription of ERK1/2, Runx2, and PCNA.

Continuous mechanical stretching of human PDL cells has been shown to enhance ERK activity and increase the activator protein 1 (AP-1), a transcription factor that plays a pivotal role in controlling gene expression in osteoblast differentiation (Peverali *et al.*, 2001). There are complex mechanisms for the interaction between AP-1 and Runx-2, including direct physical interactions (Franceschi and Xiao, 2003). In accordance with that, Ziros *et al.*, 2002 demonstrated that after mechanical stretching of human PDL cells, Runx2 was activated by means of ERK phosphorylation. These findings strongly implicate the ERK cascades in the stretch-elicited up-regulation of Runx2-binding activity.

Kawarizadeh *et al.* (2005) investigated short-term orthodontic loading in the same animal model. Those authors demonstrated that application of precise short-term loading activates ERK1/2 and Runx2. They showed that in representative areas under tension, the proportions of Runx2-positive and pERK1/2-positive cells increased within 8 hours of loading, whereas these proportions in representative areas under pressure were significantly reduced in comparison with those in control teeth. These findings suggest that PDL cells undergo osteoblastic differentiation via the ERK pathway in the mentioned zones.

In this research, rat molars were loaded orthodontically in the same way as in the study of Kawarizadeh *et al.* (2005) with a high-resolution three-dimensional force/torque transducer for exact periods of time, while the animals were under anaesthesia. Different force levels were applied and the animals were killed after 2 hours, 4 hours, 1 day, and 2 days. By fixing the separated teeth in their forced position, the observation period was prolonged for the last two groups for 1 and 2 days, respectively. An increased proportion of pERK1/2-positive cells in comparison with the control teeth in the tension zones were found. This increase occurred in a force-dependent manner. In the pressure zones, only the intermittent forces produced a higher proportion of pERK1/2-positive cells in comparison with the controls. On the other hand, differences between the tension and the pressure sides were not significant, which could be explained by the fact that there was already an obvious difference in the number of positive cells between the pressure and the tension zone in the control teeth.

The proportion of Runx2-positive cells decreased after the application of constant forces. This decrease on the pressure as well as on the tension side occurred in a time- and force-dependent manner. It could also been seen that on both sides, the numbers of Runx2-positive cells in animals with intermittent forces were comparable with those of animals receiving 0.1 N force for 2 days and also with those animals receiving 0.5 N for 4 hours, showing that an intense stimulus down-regulated the expression of Runx2.

Finally, both under tension and pressure, the proportion of PCNA-positive cells after the application of constant forces for 4 hours was lower than in the controls.

This study, in comparison with that of Kawarizadeh *et al.* (2005), provides novel information concerning tooth movement. It was shown that the expression of the involved factors was dependent on the amount of force applied. Thus, while there was a force-dependent increase of the expression of pERK1/2 on the tension side, the opposite was demonstrated for the expression of Runx2 on the pressure side. Kawarizadeh *et al.* (2005) showed similar results but in a time-dependent manner. The reason for this discrepancy might be differences in the experimental protocol. In the present research, the same amount of force was used but for a much longer period of time and also a higher amount of force was used for the same period of time. This might also explain the insignificant differences between the tension and pressure sides, which are in contrast with the findings of Kawarizadeh *et al.* (2005).

A time-dependent decrease was observed for the expression of Runx2 on both sides as well as for the PCNA on the pressure side. Regarding PCNA, *in vitro* studies have

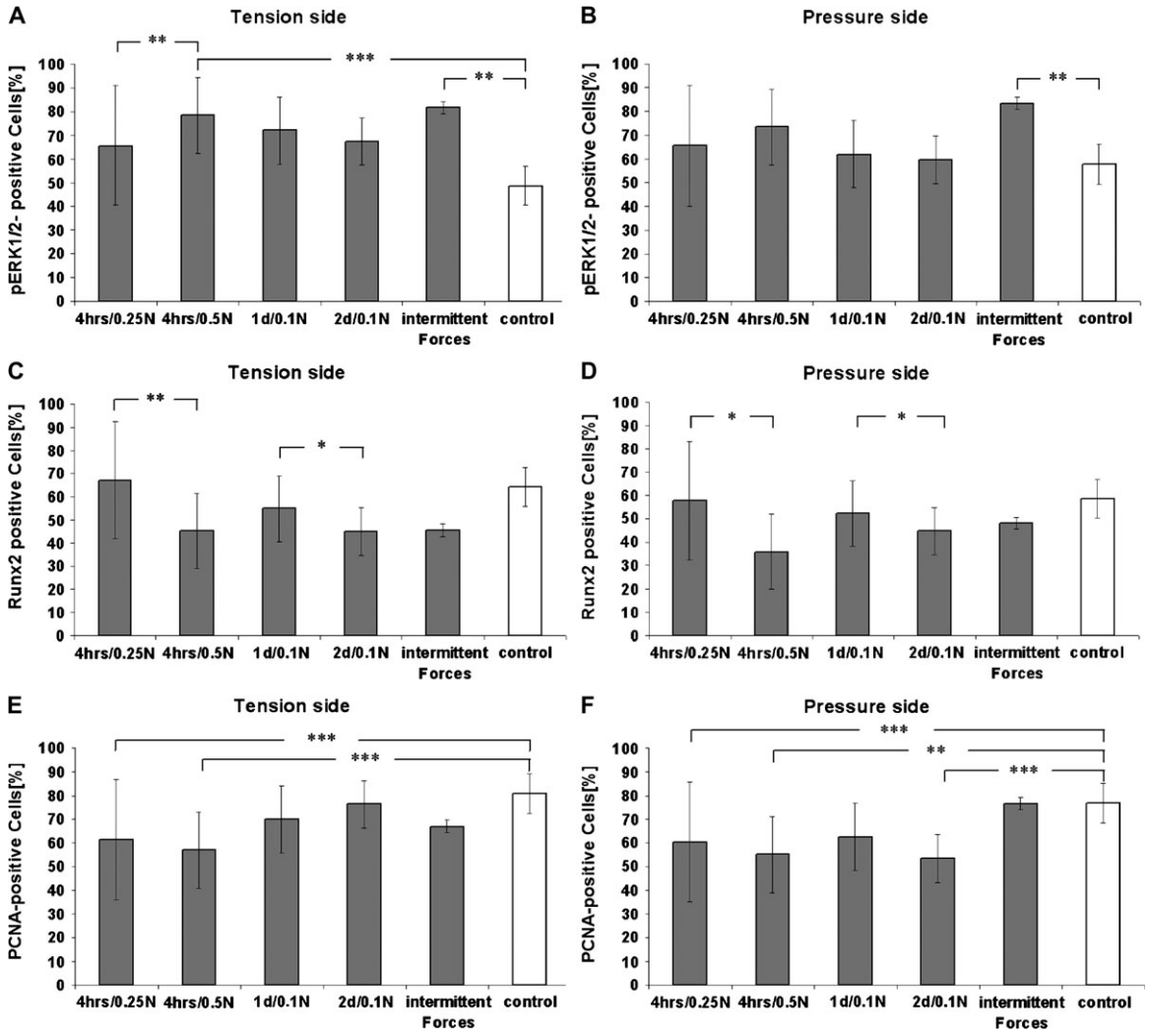

Figure 3 Quantification of pERK1/2-positive cells (A and B), Runx-2-positive cells (C and D), and PCNA-positive cells (E and F) in the areas of interest (A, C, and E distocoronal; B, D, and F mesiocoronal). The immunopositive cells were counted as percentages of the total number of cells. Results are representative of three sections of each animal. For each of the five specimens, a total of two fields in three sections were analysed. The results are expressed as means and standard deviations. The asterisks indicate significant differences (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$).

shown that mechanical stress induces DNA synthesis in human PDL cells following 6 hours of stretching (Kletsas *et al.*, 1998). In the current study, in accordance with Kawarizadeh *et al.* (2005), no obvious increase in proliferation was observed. Thus, *in vitro* and *in vivo* findings should be compared with caution. Intermittent forces resulted in a clear up-regulation of pERK1/2, showing that this stimulus can initiate differentiation of the PDL cells.

Altogether, the findings show that mechanical stimulus induces differentiation of PDL cells towards osteoblasts via the ERK cascade, verifying the hypothesis of Roberts *et al.* (1982), that new osteoblasts are derived from PDL cells during orthodontically induced osteogenesis, and also the report of Camilleri and McDonald (2006), that the *Runx2* gene is involved in the remodelling process of alveolar bone.

A number of studies have identified other agents that affect bone remodelling and tooth movement. Increased immunoreactivity of substance P has been demonstrated in the PDL in the early phases of tooth movement (Davidovitch *et al.*, 1988). Cytokines and especially interleukin-1β were found to affect bone metabolism through stimulation of osteoclast development and activity (Jäger *et al.*, 2005). In addition, the role of components of the RANKL/RANK/OPG system and that of nitric oxide in inducing bone remodelling was recently demonstrated (Shirazi *et al.*, 2002; Ogasawara *et al.*, 2004). A similar study is on going in order to further analyse the regulatory factors mentioned above for elucidating understanding of the re-modelling processes following orthodontic tooth movement.

Conclusions

1. The study proved that new osteoblasts are derived from PDL cells during orthodontically induced osteogenesis

and that the *Runx2* gene is involved in the remodelling process of alveolar bone.
2. Differentiation of PDL cells towards osteoblasts by mechanical stimulus is induced via the ERK cascade.
3. An increased proportion of pERK1/2-positive cells in comparison with the control teeth in the tension zones was found.
4. A time-dependent decrease was observed for the expression of Runx2 on both sides as well as for the PCNA on the pressure side. No obvious increase in proliferation was observed.
5. Intermittent forces resulted in a clear up-regulation of pERK1/2, showing that this stimulus can initiate differentiation of the PDL cells.

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