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ORIGINAL ARTICLE

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Orthodontic tooth movement causes decreased promoter expression of collagen type 1, bone sialoprotein and alpha-smooth muscle actin in the periodontal ligament

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Structured Abstract

Objective – To evaluate the effects of orthodontic tooth movement on the promoter expression of collagen type 1 (3.6Col1), bone sialoprotein (BSP) and alpha-smooth muscle actin (αSMA) in the periodontal ligament (PDL) using transgenic mice containing transgenes of these promoters fused to green fluorescent proteins (GFP).

Materials and Methods – The maxillary first molars of 10-12 week-old transgenic mice were loaded with 10–12 g of force for 12, 48 h, or 7 days. Mice were transgenic for one of the following GFP-tagged bone markers of osteoblast lineage cells: 3.6-kb fragment of the rat collagen type 1 promoter (3.6Col1), BSP or α-smooth muscle actin (αSMA). Loaded molars under compression and tension were compared with contra-lateral unloaded controls.

Results – On the compression side of the PDL, orthodontic tooth movement caused a significant decrease in GFP expression of all the promoters at each time point. On the tension side, there was a significant increase in BSP–GFP expression, 12 h following loading compared to the contralateral unloaded controls.

Conclusions – An *in vivo* tooth movement model using transgenic mice with promoter–GFP constructs provides an efficient and effective way of investigating the cellular events underlying orthodontic tooth movement. PDL cells may undergo decreased differentiation in response to the compressive force.

Key words: green fluorescent proteins; osteoblast; periodontal ligament; tooth movement; transgenic mice

Introduction

Orthodontic tooth movement is contingent upon the underlying cellular and molecular responses within the periodontal ligament (PDL) to an applied force. This process of mechanotransduction stimulates bone remodeling during which osteoblasts produce bone on the tension side and osteoclasts resorb bone on the compression side of the PDL (1–5). Complex interactions between osteoblasts and osteoclasts involve numerous biologic players including systemic hormones, cytokines and growth factors (6). It has increasingly been recognized that a greater understanding of the cellular determinants and factors regulating the bone remodeling process is necessary to enable future innovations in orthodontic treatment. Consequently, the study of the biology of tooth movement has evolved into an interdisciplinary field, merging the technical expertise and materials science of clinical orthodontics with the molecular investigative acumen of cellular, molecular, and bone biology research.

Orthodontic tooth movement involves the complex interaction of several differentiated populations of cell types within the PDL. Very little is known, however, about how specific cell populations within the PDL respond to the orthodontic forces. New methods have recently been developed to isolate and study defined populations of cells through the use of transgenic mice with green fluorescent proteins (GFP) fused to the promoters of cellular differentiation markers (7). The advantages of using this technology are that it allows for the spatial and temporal visualization of the expression of the promoter on tissue sections, cells can easily be isolated by fluorescence-activated cell sorting (FACS), and one can multiplex different fluorescent reporters (8). These methods have already been successfully used in bone studies to label and isolate cells at distinct stages of osteoblast differentiation (9).

Osteoblast differentiation is characterized by a series of maturational steps during which an osteoprogenitor cell undergoes sequential changes in the expression of bone-associated marker genes. An alpha-smooth muscle actin (αSMA) has been identified as a marker for osteoprogenitor cells prior to entering the osteogenic pathway. In a cellular environment completely devoid of osteoblasts, cells expressing αSMA have been shown to transition to an osteoprogenitor lineage leading to extensive osteogenesis (10). Pre-osteoblasts are characterized by alkaline phosphatase (ALP) and type I collagen (Col1a1) mRNA expression. Early osteoblast stages express bone sialoprotein (BSP) characterized by its ability to bind to hydroxyapatite (11). Mature osteoblasts and osteocytes characteristically express DMP1 (12). The use of the rat Col1a1 promoter as a marker for stages of osteoblast differentiation *in vitro* and *in vivo* has been well established. Interestingly, different rat promoter fragments of Col1a1 label different cells within the osteoblast lineage. For example, the 3.6-kilobase fragment of the Col1a1 promoter (3.6Col1) is expressed predominantly by immature osteoblasts or pre-osteoblasts, whereas the 2.3-kilobase fragment of the Col1a1 promoter (2.3Col1) is characteristic of mature osteoblasts (7).

Transgenic mice carrying GFP tagged with αSMA, 3.6Col1, and BSP promoter fragments have been developed. In the PDL, αSMA–GFP has been shown to label cells associated within the vasculature and may label a potential osteoprogenitor population (13). In addition, we have previously shown that 3.6Col1–GFP is localized throughout the PDL, whereas BSP–GFP is predominantly localized adjacent to bone and cementum surfaces (14). We also examined GFP expression in an orthodontic tooth movement *ex vivo* organ culture model, using mandibles from transgenic mice containing promoters of BSP and 3.6Col1 fused to GFP transgenes. We found that 6 h of orthodontic tooth movement caused a significant decrease in 3.6Col1 and BSP promoter activity on the compression side compared to unloaded controls (14). However, the major shortcoming of this study was that we were not able to extend the observational period beyond 6 h because of a lack of viability of the mandibular organ culture. Therefore, the goal of the current study was to develop an *in vivo* mouse orthodontic tooth movement model in transgenic mice containing promoters of αSMA, 3.6Col1, and BSP fused with GFP. We hypothesized that orthodontic tooth movement would cause a decrease in 3.6Col1 and

BSP promoter activity on the compression side and increase in αSMA, 3.6Col1, and BSP promoter activity on the tension side over the 7-day tooth movement period.

Materials and methods

All experiments were performed under an institutionally approved protocol for the use of animals in research and in accordance with the National Guiding Principles for Animal Research. Thirty-six transgenic mice (10–12 weeks old) weighing 20–25 g were used in this study. Mice were weighed daily. Twelve mice (n = 12) were transgenic for α-smooth muscle actin GFP-fused promoter (αSMA), twelve mice (n = 12) were transgenic for the 3.6-kb fragment of the rat collagen type 1 GFP-fused promoter (3.6Col1), and twelve mice (n = 12) were transgenic for bone sialoprotein GFP-fused promoter (BSP). The animals were housed under normal laboratory conditions and fed a soft dough diet (Bio-Serv, Frenchtown, NJ, USA) and water *ad libitum*.

Mice were anesthetized with an intraperitoneal injection of ketamine (87 mg/kg) and xylazine (13 mg/kg). A custom mouth prop formed from 0.032″ round stainless steel wire was inserted into the animal's mouth to aid in appliance placement (Fig. 1). A custom-made 0.006″ × 0.030″ nickel–titanium closed-coil spring (Ultimate Wireforms, Inc., Bristol, CT, USA) was used to deliver orthodontic force. The force/deflection rate (F/Δ) for the spring was determined to be 10–12 g over a range of 0.5–1.5 mm activation (data not shown).

Appliance delivery was performed under a dissecting microscope. A 0.008″ stainless steel wire was threaded through the contact between the first and second left maxillary molars. Self-etching primer (Transbond Plus self-etching primer, 3M Unitek, Monrovia, CA, USA) was applied to the lingual surface of the first molar, and the wire was bonded to the tooth with light-cured dental adhesive glass-ionomer cement (GC Fuji Ortho LC, GC America) that was cured with a curing light (Flashlite 1401; Discus Dental©, Culver City, CA, USA). The distance between the maxillary first molar and the left incisor was measured to the nearest 0.5 mm with a conventional Michigan-O periodontal probe with Williams markings. A segment of the spring was cut to measure 2 mm less than the molar–incisor distance. This 2-mm discrepancy accounted for up to 1.5 mm of activation plus 0.5 mm of space occupied by the 0.008″ wire between the first molar and the spring. The spring was then ligated to the wire around the first molar. A second 0.008″ stainless steel wire was inserted through the mesial end of the spring. The spring was then activated at the time of insertion itself by pulling it toward the left central incisor with the wire. Activation distance was calibrated with the Michigan-O probe by measuring the distance from the incisor to the mesial end of the passively ligated spring. With the probe in place, the spring was activated 1.5 mm to deliver a force of 10–12 g. The wire on the mesial end of the spring was ligated around the left incisor and bonded in place with light-cured dental adhesive resin (Transbond XT; 3M Unitek, Monrovia, CA, USA). The mandibular incisors were reduced to prevent appliance damage. Only the left side of the maxilla was mechanically loaded; the contra-lateral right side served as the control.

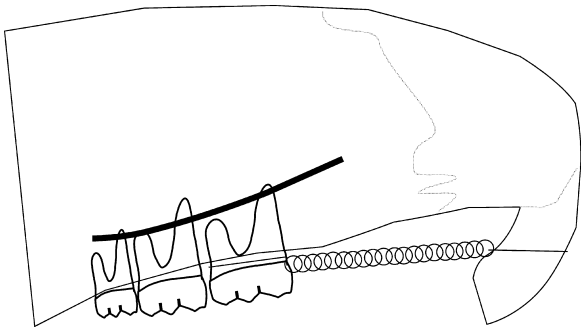

Fig. 1. Bonding of the spring to the maxillary first molar.

Each group of 12 GFP transgenic mice was equally divided into three time intervals of force duration: 12, 48 h, and 7 days. After completion of the time course, the mice were euthanized with CO_2 followed by cervical dislocation. The mice were decapitated, and the maxillae were removed and cleaned off soft tissues and muscles. The hemisected maxillae were placed in 10% formalin for 5 days at 4°C, washed in phosphate buffered saline, and placed in 30% sucrose overnight. The maxillae were then placed in individual dispos-

able base molds containing frozen embedding medium (Shandon M-1; Thermo Scientific, Waltham, MA, USA). The embedding medium was flash-frozen in a chilled solution of 2-methylbutane over dry ice. Five-micrometer-thick sagittal sections of the loaded (left) and control (right) sides were cut using a Leica CM1900 Cryostat (D69226; Leica, Inc., Nussloch, Germany). Sections were oriented to visualize the mesial-buccal and distal-buccal roots of the maxillary first molars, including the inter-radicular bone.

Fluorescent digital images of each section were captured using a Zeiss Axiovert 200 M microscope equipped with a GFP fluorescein isothiocyanate (FITC)/Texas Red dual filter cube, a motorized stage, and a digital camera. Images were taken at 20× magnification in the furcation area of both the mesial-buccal and distal-buccal roots. Based on the mesial direction of the force, the mesial surface of the distal-buccal root (compression side) was imaged. Conversely, the distal aspect of the mesial-buccal root (tension side) was imaged. For comparison, the same compression and tension locations of the furcation area were imaged on both the mechanically loaded left first molar and the unloaded right first molar. The inferior border of the image area was aligned at the most coronal portion of the respective root surface to capture the region of the PDL in closest proximity to the furcation (Fig. 2). Following GFP imaging, sections were stained with hematoxylin and eosin (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's directions and imaged using a bright-field filter under the same microscope.

To quantify the number of osteoblast lineage cells, the images were viewed in Adobe Photoshop (Adobe Systems Inc., San Jose, CA, USA) and cells expressing GFP fluorescence within the boundaries of the PDL space were counted in a blinded fashion by a calibrated investigator who did not know which tissue samples were being counted. Images of both compression and tension sides from the mechanically loaded left first molar and the unloaded right first molar were counted in identical fashion. The same imaging protocol was used to capture images and count the total number of cells in the corresponding hematoxylin and eosin images. A GFP labeling index (number of GFP-positive cells/total number of cells) was calculated according to the following formula: Ratio of GFP-positive cells = (number of GFP-positive cells/number of total cells). Images of the compression and tension sides of the first molar were taken and counted from four tissue sections per molar (loaded left and control right) per mouse. The average GFP labeling index of the compression and tension sides was thus calculated for each mouse. For each of the GFP transgenes and time points, four mice were used and the mean GFP labeling index for each group was calculated. The means of the GFP labeling indices for each transgene on the compression and tension sides of the loaded left molar and unloaded right molar at each time point were compared using Student's t-tests. Significance was accepted at $p < 0.05$. Statistical analyses were carried out using the GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA).

The PDL thickness was measured using the OsteoMeasure computerized image analysis system

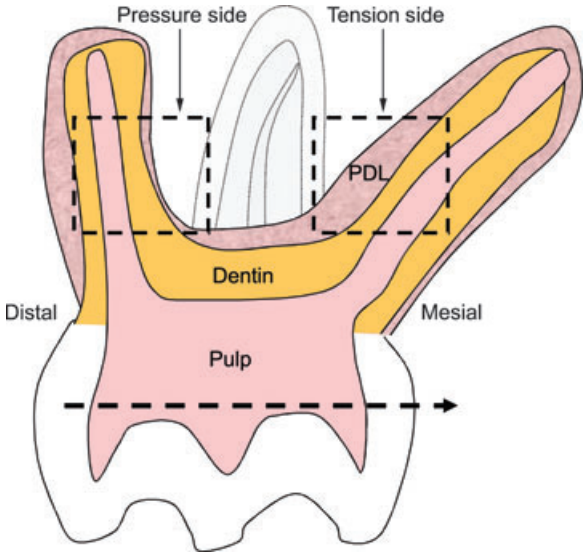

Fig. 2. Illustration of imaging regions of the maxillary first molar. Images were taken at 20× magnification in the furcation area of both the mesial-buccal and distal-buccal roots. Based on the mesial direction of the force (arrow), the mesial surface of the distal-buccal root (compression side) was imaged. Conversely, the distal aspect of the mesial-buccal root (tension side) was imaged. For comparison, the same compression and tension locations of the furcation area were imaged for both the mechanically loaded left side and the unloaded right side. The inferior border of the image area was aligned at the most coronal portion of the respective root surface to capture the region of the periodontal ligament in closest proximity to the furcation.

(OsteoMetrics, Atlanta, GA, USA) interfaced with an Optiphot microscope (Nikon, Melville, NY, USA). Measurements were taken at 20× magnification in the furcation area of both the mesial-buccal and distal-buccal roots. Three sections were analyzed from three different mice corresponding to each time point. Statistical analyses were carried out using the GraphPad Prism software (GraphPad Software, Inc.). The differences between means were determined by independent t-tests with the level of statistical significance set at $p < 0.05$.

To detect apoptosis on the compression side of the PDL, the DeadEnd™ Fluorometric TUNEL (TdT-mediated dUTP Nick-End Labeling) assay kit (Promega, Madison, WI, USA) was used according to the manufacturer's directions. Apoptotic cells were detected by the tetramethyl rhodamine iso-thiocyanate (TRITC) fluorescent cube, while total cells were detected by the 4′,6-diamidino-2-phenylindole (DAPI) fluorescent cube of the Zeiss Axiovert 200 M microscope.

Results

During the experiment, animals typically lost weight on the first day but returned to their original weight on day 2–3 and continued to gain weight through day 7. No animal lost any body weight at the end of the experiment (after 1 week) compared to day 0 (data not shown). Comparison of the compression sides to the tension sides of the controls showed no significant difference at any time point for any GFP transgene (Fig. 4). The width of the PDL on the compression side of the experimental group showed a statistically significant decrease in thickness at 12 and 48 h and a significant increase at 7 days compared to the control group (Figs 3 and 4). In the area under tension side, PDL thickness was significantly increased in the experimental group compared to the control group at all time points (Fig. 4).

After 12 h of mechanical loading, a significant decrease in fluorescent protein expression of all three promoter markers was observed on the compression side of the furcation area of loaded first molars compared to unloaded controls

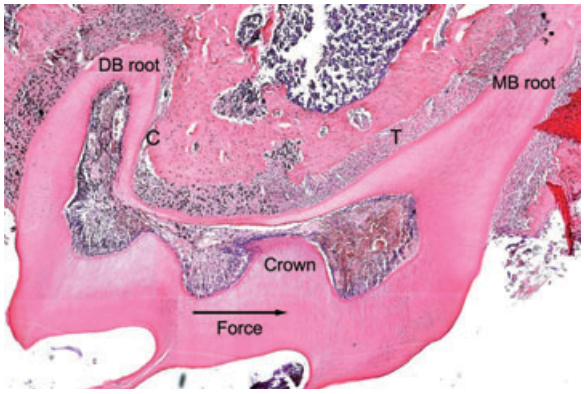

Fig. 3. 5× hematoxylin and eosin–stained sagittal section of the maxillary first molar. Note the compressed periodontal ligament on the mesial aspect of the distal-buccal (DB) root (C) and the stretched PDL on the distal aspect of the mesial-buccal (MB) root (T).

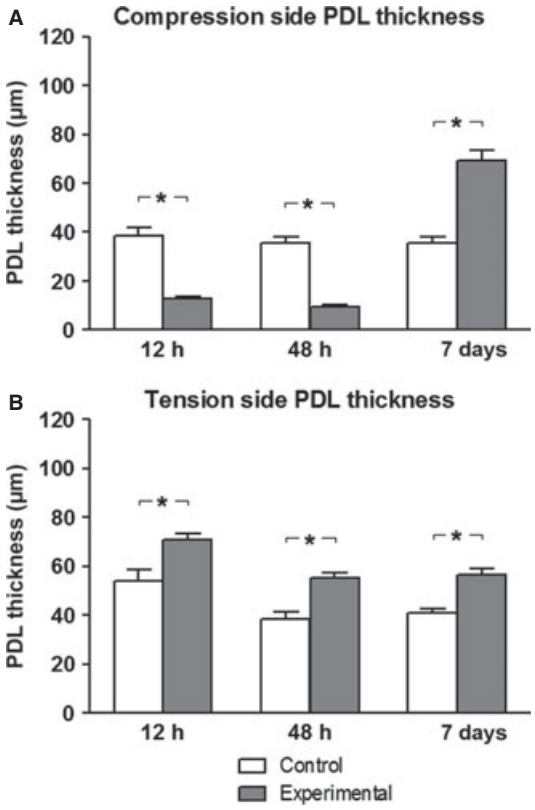

Fig. 4. Comparison of periodontal ligament thickness measurements between control (non-loaded) and experimental (loaded) sides. * denotes statistical significance ($p < 0.05$).

(Figs 5A–B, 6A–B, 7A–B and 8). Also, at this time point, orthodontic tooth movement induced a significant increase in BSP–GFP expression on the tension side compared to controls (Fig. 8). Figures 5, 6 and 7A–D show fluorescent images of sagittal sections after 12 h of loading in 3.6Col1, BSP, and αSMA mice, respectively.

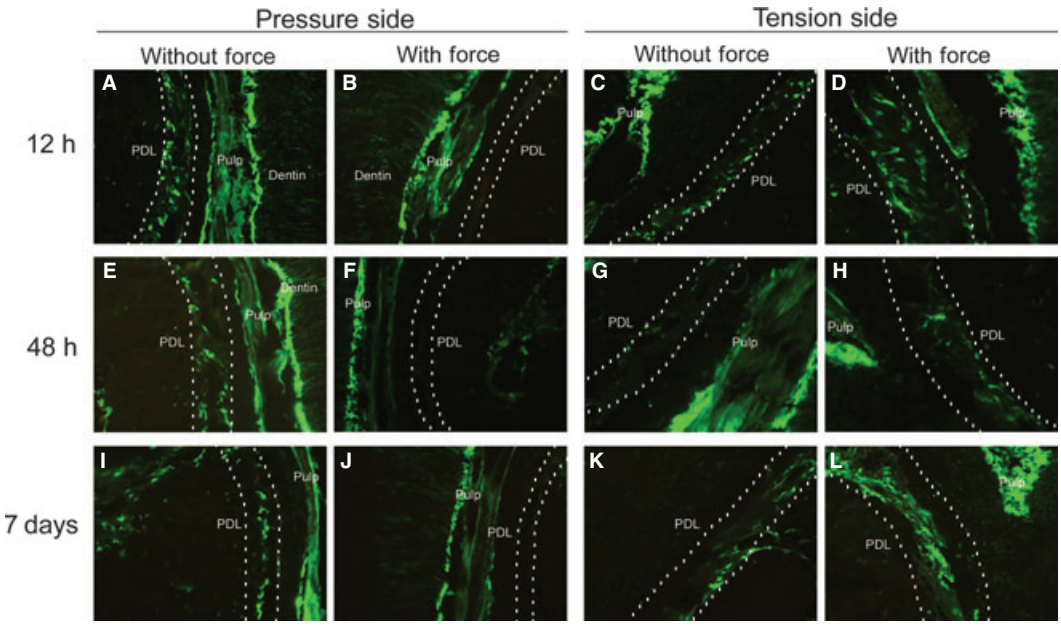

Fig. 5. 20× fluorescent images of sagittal sections of the distal-buccal (compression side) and mesial-buccal (tension side) roots of maxillary first molars of transgenic mice harboring the 3.6-kb fragment of the rat collagen type 1 promoter fused to a topaz-fluorescent protein (3.6Col1-GFP). Note the decrease in green fluorescent proteins expression in the periodontal ligament in images B, F, and J (with force) relative to images A, E, and I (without force).

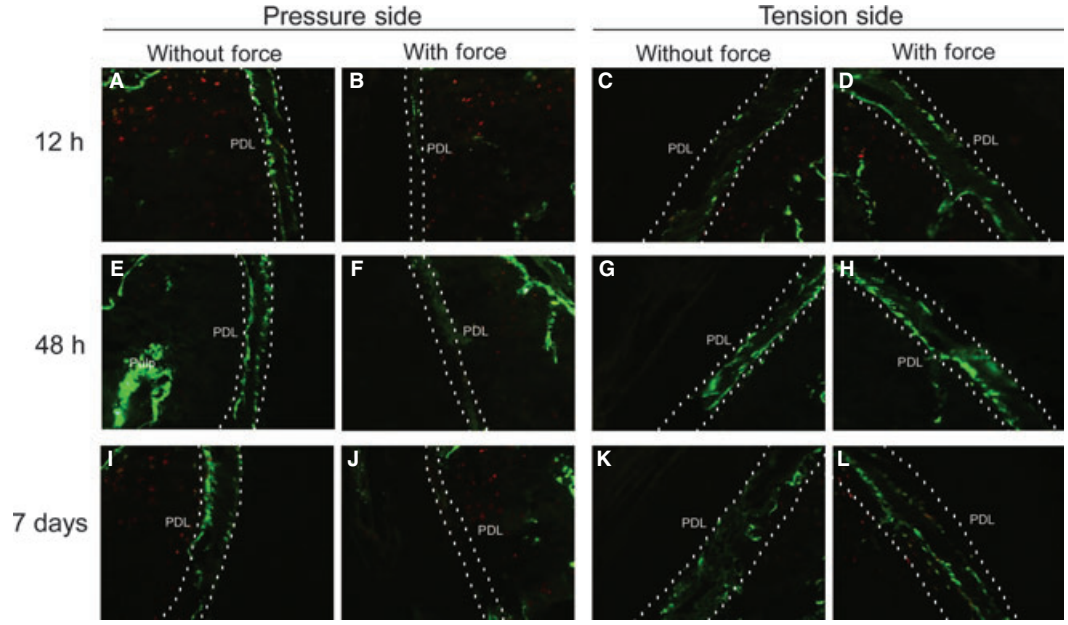

Fig. 6. 20× fluorescent images of sagittal sections of the distal-buccal (compression side) and mesial-buccal (tension side) roots of maxillary first molars of transgenic mice harboring the bone sialoprotein (BSP) promoter fused to a topaz-fluorescent protein (BSP–GFP). Note the decrease in green fluorescent proteins expression in the periodontal ligament in images B, F, and J (with force) relative to images A, E, and I (without force).

After 48 h and 7 days of loading, 3.6Col1, BSP, and αSMA promoter activity decreased significantly on the compression side of the furcation area of loaded first molars compared to unloaded controls (Figs 5E–J, 6E–J, 7E–J and 8).

TdT-mediated dUTP Nick-End Labeling (TUNEL) staining showed only a few if any apoptotic cells on the compression side of the PDL of the loaded molars; however, 4′,6-diamidino-2-phenylindole (DAPI)-stained nuclei were well demarcated

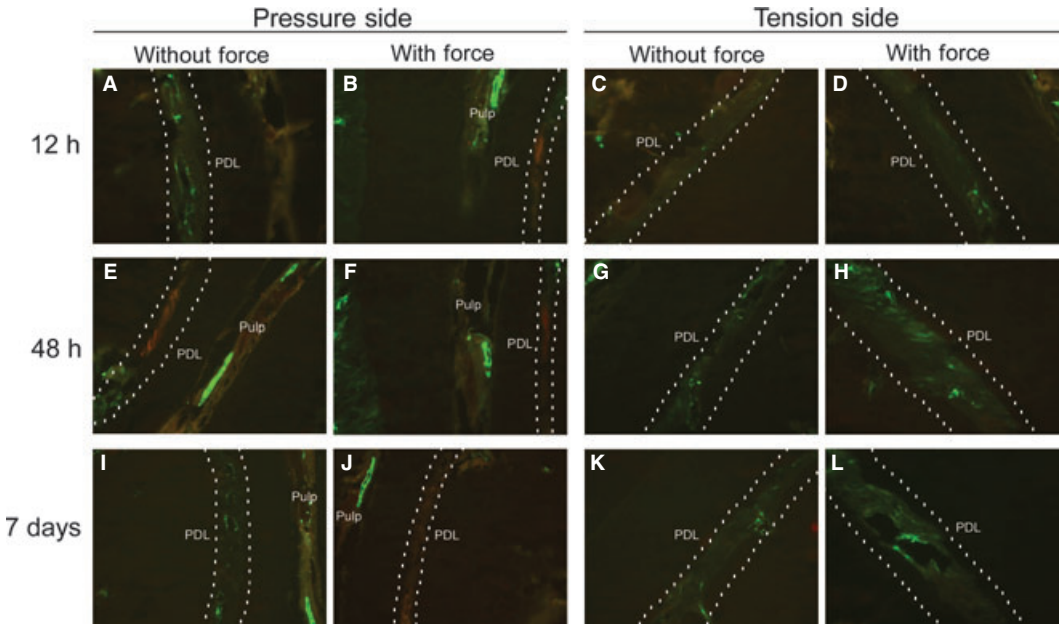

Fig. 7. 20× fluorescent images of sagittal sections of the distal-buccal (compression side) and mesial-buccal (tension side) roots of maxillary first molars of transgenic mice harboring the α-smooth muscle actin (αSMA) promoter fused to a topaz-fluorescent protein (αSMA–GFP). Note the decrease in green fluorescent proteins expression in the periodontal ligament in images B, F, and J (with force) relative to images A, E, and I (without force).

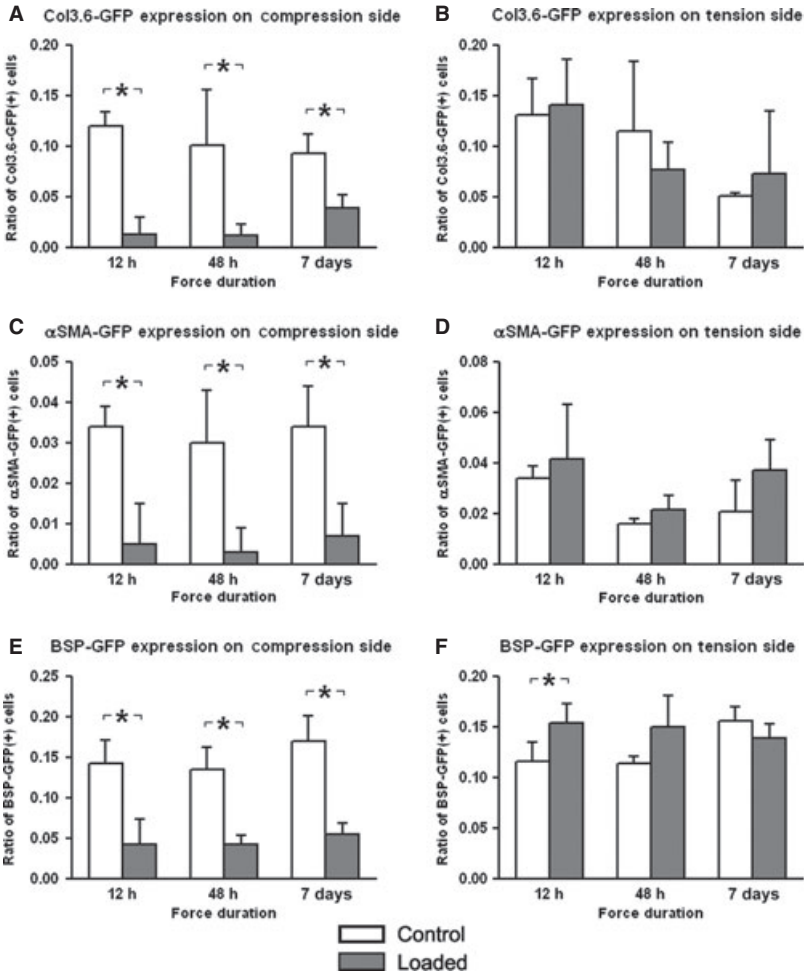

Fig. 8. Comparison of 3.6Col1–GFP (A–B), αSMA–GFP (C–D), and bone sialoprotein–GFP (E–F) expression as a ratio of GFP(+) cells to total cells on compression (A, C, and E) and tension (B, D, and F) sides of the periodontal ligament in unloaded control and contra-lateral loaded experimental maxillary first molars following 12 h, 48 h, or 7 days of orthodontic force application. * denotes statistical significance ($p < 0.05$).

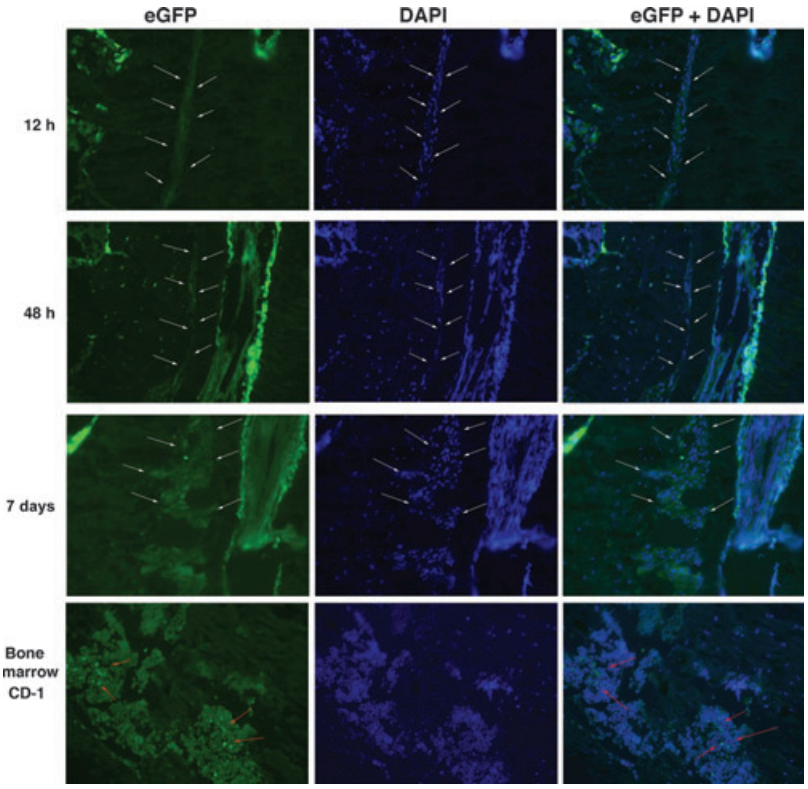

Fig. 9. 20× fluorescent images of sagittal sections of the distal-buccal (compression side) roots of maxillary first molars stained for apoptotic cells using the TUNEL assay. Nuclei were counterstained with DAPI.

and showed no cell abnormality, indicating that the cells were viable (Fig. 9).

Discussion

The focus of this study was to examine the effects of orthodontic tooth movement on αSMA, 3.6Col1, and BSP promoter-GFP expression in the PDL in an *in vivo* murine model. Use of these promoter-GFP transgenes offers certain advantages over other molecular biology techniques such as *in situ* hybridization and immunohistochemistry, which include: 1) retention of their fluorescent property after extensive tissue preparation, 2) visualization in unstained sections that preserve the histological architecture of bone, 3) detection of GFP signals directly through microscopy without depending on the diffusion of a substrate, and 4) indefinite stability of prepared specimens (8). Taken together, we now have a powerful tool to efficiently visualize populations of homogeneous PDL cells at various stages of differentiation in a tooth movement model.

Application of orthodontic force to the maxillary first molar in this study resulted in two distinct patterns of response from PDL cells. On the tension side, the earliest time point registered in this study was 12 h, and the longest was 7 days. We only saw a significant increase in BSP–GFP expression on the tension side after 12 h. In another study examining osteoblast differentiation during tooth movement, it was found that orthodontic tooth movement induced a significant increase in osteocalcin, ALP, and Col1a1 mRNA gene expression from day 2 through day 6 of observation (15, 16). Differences in our results compared to this may be attributed to different differentiation markers being examined and the fact that we examined promoter activity as opposed to mRNA expression. The lack of orthodontic tooth movement-induced increase in GFP expression from promoters that are found within the PDL (αSMA and 3.6Col1), and the increase in BSP–GFP expression of the PDL-bone interface cells may suggest that new bone formation on the tension side is more because of the activation of osteoblast cells rather than migration and differentiation of PDL osteoprogenitor cells.

αSMA, BSP, and 3.6Col1–GFP expression was significantly reduced on the compression side of loaded molars compared to unloaded controls at

all time points. This result has not previously been reported in the literature in an *in vivo* study. One interpretation of the significant decrease in GFP expression on the compression side is that PDL cells undergo de-differentiation to the cartilage phenotype in response to the orthodontic force. Alternatively, the loss of GFP expression may be due to the fact that the cells have undergone necrosis or apoptosis, processes which have been implicated in the formation of regions of hyalinization within the PDL (1, 2, and 4). Surprisingly, we did not see any evidence of increased apoptosis within the PDL during orthodontic tooth movement. This may be due to the fact that apoptosis is occurring at time points that we did not examine or because the cells are undergoing a different death pathway. The lack of fragmented DAPI-stained nuclei suggests that the cells are not necrotic at the time points we examined. Although not confirmed, the light force range of 10–12 g used in this study has previously been shown in mouse models to cause physiologic tooth movement without inducing areas of hyalinization (16). Definitive transmission electron microscopy analysis would be needed to confirm the type of cell death in this study.

To better interpret the results of this study, future study designs would benefit from further development of the GFP model. In particular, the creation of transgenic mice expressing multiple markers of sequential stages of PDL maturation simultaneously and distinctly within the same animal would be of great benefit. Such mice could be used to definitively show how PDL lineage cells continue, cease, or regress in differentiation in response to mechanical loading by analyzing how the relative proportion of each stage of differentiation varies with force application. Furthermore, a longer time course of orthodontic tooth movement would provide greater insight into the underlying biology.

Conclusions

An *in vivo* tooth movement model using transgenic mice with GFP PDL markers provides an efficient and effective model to investigate the cellular events of orthodontic tooth movement. PDL cells may lose their phenotype in response to compressive force.

Clinical relevance

Modulation of orthodontic tooth movement has many clinical applications such as anchorage enhancement and increasing the velocity of tooth movement. Little information is available on how different cell populations in the PDL respond to orthodontic forces. PDL differentiation plays a major role in the remodeling process that occurs during orthodontic tooth movement. We show here that transgenic mouse technology facilitates the monitoring of these cellular processes in real time. Understanding the effect of forces on the PDL differentiation process may facilitate the modulation of orthodontic tooth movement by finding new therapeutic targets.

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