UCONN LIBRARY

University of Connecticut
DigitalCommons@UConn

SoDM Masters Theses School of Dental Medicine

2010

Effects of Orthodontic Tooth Movement on Osteoblast Differentiation Markers within the Periodontal Ligament

Christopher E. Olson
University of Connecticut Health Center

Follow this and additional works at: http://digitalcommons.uconn.edu/sodm_masters

Part of the Dentistry Commons

Recommended Citation
Olson, Christopher E., "Effects of Orthodontic Tooth Movement on Osteoblast Differentiation Markers within the Periodontal Ligament" (2010). *SoDM Masters Theses*. 176.
http://digitalcommons.uconn.edu/sodm_masters/176

Effects of Orthodontic Tooth Movement on Osteoblast Differentiation Markers within the Periodontal Ligament

Christopher E. Olson

B.A., Stanford University

D.D.S., University of the Pacific School of Dentistry

A Thesis

Submitted in Partial Fulfillment of the

Requirements for the Degree of

Master of Dental Science

At the

University of Connecticut

2010

APPROVAL PAGE

Master of Public Health Thesis

Effects of Orthodontic Tooth Movement on Osteoblast Differentiation Markers within the Periodontal Ligament

Presented by

Christopher E. Olson, D.D.S.

Major Advisor: Flavio Uribe, D.D.S.,M.D.S.

Associate Advisor: Sunil Wadhwa, D.D.S.,Ph.D.

Associate Advisor: Ivo Kalajzic, M.D.,Ph.D.

University of Connecticut

2010

ACKNOWLEDGEMENTS

I would like to thank my team of advisors who supported me during this project. I thank Dr. Uribe for his infectious enthusiasm, optimism and encouragement; his inexorable pursuit of 'the truth'; and his uncompromising adherence to excellence. I thank Dr. Wadhwa for sharing his expansive knowledge and expertise in bone biology, and for his judicious shaping of the scope and design of this project. I appreciate the help I received from Dr. Ivo Kalajzic in providing his technical expertise and laying many of the cornerstones for the use of GFP in transgenic mouse models. Without Zana Kalajzic's incredible patience, generosity, and hard work, this project would not have been completed – she showed me how to roll up my sleeves and perform basic science research. I am grateful to Dr. Nanda for providing me the opportunity and support for this research and to launch my career in orthodontics. This project could not stand on its own merit, but is rather built upon the foundation created by numerous residents to whom I am thankful, including Dr. John Bibko, Dr. Tina Gupta, Dr. Jing Chen and Dr. Elizabeth Blake. A thesis project demands numerous hours of work - time which is dedicated to the research and therefore diverted away from other activities. My family unconditionally supported me throughout this process, and I am eternally grateful for Jill and Jace's love – a love which has empowered me beyond which I can describe.

TABLE OF CONTENTS

TITLE PAGE	
APPROVAL PAGE	ii
ACKNOWLEDGEMENTS	iii
TABLE OF CONTENTS	iv
LIST OF FIGURES	v
CHAPTER I - INTRODUCTION	1
BACKGROUND	1
Mechanotransduction in Orthodontics	1
Tooth Movement Models	3
The Transgenic Mouse Model	5
Molecular Biology Techniques in Tooth Movement Models	7
Markers of Osteoblast Lineage	9
RATIONALE	12
HYPOTHESIS	14
SPECIFIC AIMS	15
CHAPTER II - MANUSCRIPT	16
ABSTRACT	17
INTRODUCTION	18
MATERIALS AND METHODS	20
RESULTS	23
DISCUSSION	25
CONCLUSION	28
REFERENCES	28
CHAPTER III - DISCUSSION	30
Addendum to Chapter II – Aim #3	30
Immunohistochemical Analysis of RANKL Expression	30
CHAPTER IV – CONCLUSION	36
SIGNIFICANCE OF RESULTS	36
FUTURE DIRECTION	36
FIGURES	38
REFERENCES	48

LIST OF FIGURES

Figure 1: Bonding of spring to maxillary first molar, head stabilized with custom 0.032-inch stainless steel mouth prop

Figure 2: Illustration of imaging regions of maxillary first molar

Figure 3: Hematoxylin stained sagittal section

Figure 4: Fluorescent images of sagittal sections of maxillary first molars of transgenic mice containing the 3.6 kb fragment of the rat collagen type 1 (Col3.6) promoter fused to a topaz-fluorescent protein (Col3.6GFP)

Figure 5: Fluorescent images of sagittal sections of maxillary first molars of transgenic mice containing bone sialoprotein (BSP) promoter fused to a topaz-fluorescent protein (BSPGFP)

Figure 6: Fluorescent images of sagittal sections of maxillary first molars of transgenic mice containing α-smooth muscle actin (αSMA) promoter fused to a topaz-fluorescent protein (αSMAGFP).

Figure 7: Comparison of Col3.6-GFP (A, B), αSMA-GFP (C,D), and BSP-GFP (E,F) expression as a ratio of GFP+ cells to total cells in pressure and tension sites of the periodontal ligament in loaded vs. contra-lateral unloaded maxillary first molars at 12 h, 48 h, and 7 days of treatment.

Figure 8: Immunohistochemical analysis of RANKL expression in a sagital section

Figure 9: Colocalization of immunihistochemical analysis of RANKL expression in a sagittal section overlaid with corresponding Col3.6GFP sagital section

Table 1: Mean ratios for GFP positive cells on the pressure side by duration of force and GFP transgene

CHAPTER I - INTRODUCTION

BACKGROUND

Mechanotransduction in Orthodontics

Orthodontic tooth movement is ultimately dependent on the underlying cellular and molecular responses to an applied force. As early as the nascent beginnings of orthodontic research in the early twentieth century, investigators described the bone remodeling process in terms of resorptive osteoclast activity and appositional osteoblast activity. Orthodontic luminaries such as Angle, Sandset and Oppeneim advanced the concept that at the tissue and cellular level, orthodontic tooth movement involves a differential response to tensile and compressive forces within the periodontium and alveolar bone complex [1]. It has been well documented that both soft and mineralized paradental tissues respond to external mechanical stimuli, with bone resorption occurring at sites of pressure and formation in areas of tension [2-5]. This characteristic, therefore, forms the biological basis of orthodontics. While this macro-level understanding of the bone remodeling process has been generally accepted, a well-defined picture of the molecular biology governing orthodontic tooth movement remains obscure. As such, numerous investigations have sought to elucidate the process of transduction of mechanical stimuli, e.g. orthodontic force, into a cellular biological event (mechanotransduction).

Past studies have documented that orthodontic treatment can alter the native pattern of alveolar bone remodeling, which when unperturbed maintains a homeostatic state. Turnover of the alveolar bone surrounding orthodonticaly-treated teeth is not balanced in the short-term, but instead is characterized by periods of activation,

resorption, reversal and formation of new bone [5]. An increase of bone formation rate during orthodontic tooth movement can be attributed to an escalation in proliferation rate and the number of active osteoblasts on bone surface [5, 6]. Strong evidence demonstrates that large numbers of osteoclasts are recruited to the resorptive front during tooth movement [7, 8]. Complex interactions between osteoclasts and osteoblasts involve numerous biologic players, including systemic hormones, cytokines and growth factors. Precise details regarding the development and maturation of osteoclasts (osteoclastogenesis) in areas of orthodontic tooth movement, however, have yet to be fully delineated.

Osteoclastogenesis has been shown to be regulated primarily by the cytokines RANKL (Receptor Activator of Nuclear Factor Kappa B Ligand) and M-CSF (macrophage colony-stimulating factor) [9]. Cytokines are low-molecular weight proteins (mw < 25 kDa) produced by cells that regulate or modify the action of cells in an autocrine (acting on the cell of origin) or paracrine (acting on adjacent cells) manner [1]. RANKL is produced by osteoblast precursors and binds to the RANK receptor on osteoclast progenitors in order to activate them for further differentiation. This coupling can be competitively inhibited by OPG (osteoprotegerin), which binds to RANKL on an osteoblast precursor, thereby preventing the RANKL/RANK activation and mediating the resorptive process [10]. In order for an osteoclast progenitor to differentiate into a mature osteoclast, the osteoclast progenitor must directly contact an activated RANKL-expressing osteoblast [11, 12]. Research by Kanzaki *et al.* demonstrated that cells within the periodontal ligament (PDL), when subjected to a continuous compressive force, can generate osteoclastogenesis-supporting activity [11, 13]. In human studies, RANKL

expression in the crevicular fluid has been shown to increase 28-fold during orthodontic treatment compared to controls [14]. PDL cells are a heterogeneous population of cells predominantly comprised of fibroblasts characterized by high alkaline phosphatase activity [15]. To this day it is unknown which specific cell type is responsible for producing RANKL within the PDL. Shiotoni suggested that RANKL may be produced by osteoblasts/stromal cells in the periodontal tissues [16].

Tooth Movement Models

Beginning with the seminal works of Oppenheim and Sandset, several animal models have been designed to study tissue responses to mechanical loading during orthodontic tooth movement. Primate, dog and cat models have been reported in pioneering histological studies using light microscopy [2, 3] and electron microscopy [2, 17]. Among the first to champion the use of the rat model due to increased levels of experimental control over other animal models, Waldo developed his eponymous technique utilizing an orthodontic elastic placed interproximally between rat molars in 1954. Today, rats are the most commonly used animal models, accounting for over half of all orthodontic tooth movement animal studies [18]. Compared with most other animals, rats offer a relatively low-cost, high-throughput model that facilitates histological preparation and has many commercially available antibodies for molecular techniques [18]. Rat models have enabled a diverse scope of orthodontic research, ranging from measuring proliferation rates of periodontal cells under load to assessing the effects of prostaglandins, bisphosphonates and leukotrienes on tooth movement. Like any animal model, the rat model is not without its drawbacks due to anatomical and physiologic differences with humans, including denser alveolar bone and less osteoid

tissue than humans [4, 18]. Moreover, Ren *et al.*'s systematic review of rat model studies over the past twenty years found that the vast majority of the experimental models utilized poorly designed force systems that lacked control over force levels and constancy over the duration of tooth movement.

Cell culture models are an alternative to *in vivo* studies that can afford an investigator more control over variables such as force magnitude and load deflection, thereby circumventing the force system limitations that Ren noted. However, limited culture time has been one of the major criticisms of *in vitro* culture models – tooth slice cultures have demonstrated successful results for up to several hours [19] to two weeks [20]. Unpublished reports of a mouse mandible culture model by Bibko *et al.* show tissue viability and cellular response to orthodontic force up to 12 hours in culture [21]. Furthermore, cultures of primary cell populations are not homogeneous, and in cases of cloned immortalized or transformed osteoblast lines, cells may be examined at different stages of differentiation. A major concern with any bone culture is that the cells may express an incomplete or altered osteoblast phenotype in a culture condition [22].

While the rat remains the predominant *in vivo* animal model in orthodontic research, advances in molecular biology techniques and recombinant DNA technology have ushered in a promising pool of transgenic animal models. The development of multiple genetically manipulated mice has been particularly promising and facilitates the study of genes and proteins that are involved in orthodontic tooth movement. Pavlin *et al.* were among the first researchers in the bone field to utilize transgenic mice in their studies of bone-specific and hormone-dependent regulation of type I collagen (Col1a1) gene expression. One of the significant outcomes of these studies was the evidence that

the full expression of an osteoblast phenotype requires a native bone environment, and that regulation of osteoblastic genes in cell culture condition is different than that in an intact animal [22]. To date, many applications of transgenic mice have been tested in bone biology. In orthodontics, transgenic mice are beginning to be used to study the mechanical response in bone and the remodeling of the dento-alveolar complex subjected to mechanical stress [22].

The Transgenic Mouse Model

In 2000, Pavlin *et al.* developed and characterized a mouse model that allows for a controlled, reproducible tooth movement and an assessment of histomorphometric and genetic responses of periodontal tissues as a function of duration of treatment. Hence, this model is a useful tool for applying transgenic technology to the research of mechanotransduction pathways in bone during orthodontic treatment [22]. The study used an orthodontic coil spring with a low force/deflection rate, producing an average force of 10-12 g. This affords for precision and control over the delivery of a low level of force that does not degrade rapidly over time. The spring was bonded between the maxillary incisors and the first molar; the force system resulted in a predictable tipping movement of the molar with the center of rotation at the root apices. Histological response during tooth movement was consistent with optimal tissue changes for initiation of bone turnover reported in other animal models [5]. Histomorphometric study revealed 14 and 39% increase in the number of osteoblasts on the alveolar bone surface in tension sites between 48 hours and 12 days of treatment, respectively.

Since the advent of Pavlin's characterized model, the transgenic mouse model has been utilized by numerous investigators to examine the roles of key mediators in bone

remodeling under mechanical stresses. In 2006, Yoshimatsu *et al.* modified Pavlin's protocol, using a 0.1 mm stainless-steel ligature wire to ligate a NiTi coil spring between the maxillary molars and incisors [23]. The group, however, did not independently test the load/deflection rate of the spring, but rather assumed that the manufacturer's reported 10g of force was correct. In the study, the authors identified osteoclasts histologically using tartrate-resistant acid phosphatase (TRAP) staining. They found the number of TRAP-positive osteoclasts on the pressure side of the mechanically stressed periodontal ligament significantly increased in a time-dependent manner from day 0 to day 6 of treatment.

Keles *et al.* investigated the relative efficacy of pamidronate vs. osteoprotegerin (OPG) in inhibiting bone resorption and tooth movement in transgenic mice [24]. Rather than a coil spring, the study design utilized a Y-shaped spring appliance to constrict the maxillary first molars palatally. Results demonstrated that osteoclast influx to compression sites initiated on day three of treatment, was maximal on day four, and persisted to day twelve of force application. In 2006, Fujihara *et al.* used the mouse transgenic model to analyze the molecular responses and expression of osteopontin (OPN), a bone matrix glycoprotein, in response to an orthodontic force [25]. Osteopontin has been shown by the same author to act as a chemoattractant of osteoclasts during bone remodeling caused by mechanical stress. Using OPN knockout mice and transgenic mice carrying green fluorescent protein (GFP), they showed two key findings: 1. Bone remodeling in response to mechanical stress was suppressed in OPN knockout mice. 2. The 5.5 kilobase (kb) upstream region of the OPN gene is responsible for the OPN gene expression in osteocytes on pressure force application [25].

Molecular Biology Techniques in Tooth Movement Models

The molecular techniques of *in situ* hybridization to detect gene expression in tissue sections and immunohistochemistry to identify specific proteins and cell types in tissue sections have revolutionized tooth movement studies [1]. Both techniques have been applied to transgenic mouse models. *In situ* hybridization is a method of localizing and detecting specific mRNA sequences in morphologically preserved tissue sections or cell preparations by hybridizing the complementary strand of a nucleotide probe to the sequence of interest [26]. Pavlin *et al* in 2000 utilized single-stranded RNA probes for *in situ* hybridization of alkaline phosphatase (ALP), which is an early marker of osteoblast phenotype that is mechanically upregulated in both osteoblast precursors migrating toward the bone surface and in mature osteoblasts [27]. The results of the *in situ* hybridization experiments demonstrated a cell-specific enhancement of ALP and collagen I gene by a mechanical osteoinductive signal. However, the authors noted these findings do not *per se* exclude the possibility that the hybridization signal could have been present because of the recruitment, proliferation and accumulation of a larger number of mature osteoblasts in the area adjacent to the bone surface [27].

In 2003, Gluhak-Heinrich *et al.* employed Pavlin's transgenic model and utilized immunohistochemistry to detect levels of dentin matrix protein (DMP-1), a glycoprotein which is highly expressed in osteocytes compared to osteoblasts and which may directly modulate mineralization within the osteocyte canalicular and lacunar walls, as suggested in DMP1 knockout models [28, 29]. Using *in situ* hybridization to assess DMP1 mRNA expression, the authors concluded that loading of alveolar bone produced a steady and significant increase in DMP-1 gene expression in osteocytes on both the resorption and

formation sides of the bone [28]. In contrast, immunohistochemistry analysis of DMP-1 protein showed a transient decrease in immunoreactivity after three days of loading on both the formation side and resorption side when compared to contralateral controls. However, by seven days of loading, there was a significant increase in DMP-1 protein immunoreactivity on both sides. The immunohistochemistry result could have been related to the availability of the protein to the antibody and may not accurately reflect the true levels of DMP1-producing osteocytes and osteoblasts [28]. Consequently, even when examining the same tissue specimens, one can see that *in situ* hybridization and immunohistochemistry can yield conflicting results. Although this technology has greatly simplified tooth movement research, one should not forget that the mRNA message is not always translated into protein, and the presence of a protein does not necessarily mean that it is biologically active [1].

The results of these two studies highlight the potential shortcomings of these molecular techniques when relied upon alone. While *in situ* hybridization is undoubtedly a very powerful technique, for the average laboratory it is expensive to undertake, is time consuming, and requires detailed molecular biological knowledge of subcloning, *in vitro* transcription and bacterial expression. The probes most often used (RNA or cDNA) are not generally available commercially and are often obtained on an *ad hoc* basis, laboriously prepared on a case by case basis by the investigator and once purchased often require time-consuming and expensive preparation before use. Furthermore, depending on the type and length of the probe used, tissue penetration and specificity can be altered [26]. Although *in situ* hybridization expression has been widely used in developmental studies, expression of the promoters has been reported to be low and may be affected by

technical problems [30]. Therefore, a need exists for alternative means for visualization and quantification of genetic activity as a means for cell identification within transgenic models of tooth movement. The use of transgenic constructs and fluorescent proteins may overcome these experimental problems and simplify the detection of differentiated bone cells at various stages of development, such as osteoblasts.

Markers of Osteoblast Lineage

Osteoblast differentiation is characterized by a series of maturational steps during which an osteoprogenitor cell proliferates and undergoes sequential changes in morphology and expression of bone-associated marker genes. α-Smooth muscle actin (αSMA) has been identified as a marker specific for osteoprogenitor cells prior to entering the osteogenic pathway; in a cellular environment completely devoid of osteoblast cells, cells expressing αSMA have been shown to transition to an osteoprogenitor lineage leading to extensive osteogenesis [31]. Preosteoblasts are characterized by fibroblastic morphology, alkaline phosphatase (ALP), and type I collagen (Col1a1) messenger RNA (mRNA) expression. Early osteoblast stages are more cuboidal and express bone sialoprotein (BSP). BSP is a highly sulfated, phosphorylated and glycosylated protein that is characterized by its ability to bind to hydroxyapatite [32]. The deposition of BSP into the extracellular matrix and the ability of BSP to nucleate hydroxyapatite crystal formation indicate a potential role for this protein in the initial mineralization of bone [33]. Moreover, BSP has been reported to be mitogenic for pre-osteoblasts and to promote the differentiation of these cells into osteoblasts, thereby stimulating bone calcification [34], and expression of BSP mRNA has been reported to

be increased in the tension area during rodent tooth movement [35] and during *in vitro* compression of Saos-2 human osteoblastic cell lines [32].

Mature osteoblasts and osteocytes characteristically express DMP1 [28]. During mechanical loading using Pavlin's transgenic model, expression of DMP1 mRNA in osteocytes was shown to increase 2-fold as early as six hours after treatment in both bone formation and bone resorption sites, and up to 3.5 fold after four days of loading. In contrast, osteoblast mRNA expression showed a transient 45% decrease in bone formation sites and a constant decrease of DMP1 mRNA during the entire course of treatment in resorption sites [28]. This is in agreement with reports that DMP1 is highly expressed in osteocytes compared to osteoblasts [36]. Terminal differentiation of the osteoprogenitor cell is associated with Osteocalcin (OC) mRNA and mineralization of bone [28].

The use of the rat type I collagen (*Col1a1*) promoter as a marker for stages of osteoblast differentiation *in vitro* and *in vivo* has been well established [30]. Different lengths of collagen promoters (3.6kb and 2.3kb) containing a 13-base pair bone element have demonstrated high level expression in osteoblasts [37]. Transgenic mice have been developed which carry a green fluorescent protein (GFP) tagged to specific promoter fragments. This has enabled investigators to utilize microscopy to visualize the GFP-tagged promoter fragments and to correlate GFP expression with different stages of osteoblast differentiation. Dacic et al. showed that the 3.6 kb rat Col1a1 promoter is expressed in culture during the early post-proliferative stage (day 7-9), and gets stronger when the cell differentiation progresses [38]. In contrast, the 2.3 kb rat Col1a1 promoter is activated at later stages (around day 14), and shows very high expression in

mineralized nodules [38]. This evidence suggests that the 3.6 kb *Col1a1* promoter is a linkage marker for pre-osteoblasts and osteoblasts, while the 2.3 kb *Col1a1* promoter reflects endogenous *Col1a1* expression in differentiating osteoblasts and osteocytes [38].

Transgenic mice containing more than one GFP-labeled promoter construct have recently been developed at the University of Connecticut to advance the detection of bone remodeling cells at various stages of differentiation. Transgenic mice are now available which contain three-color promoter constructs driving distinguishable GFP isomers: Bone Sialoprotein (BSP)- FPtopaz to detect early osteoblasts, Dentin Matrix Protein 1(DMP1)- FPcherry to detect osteocytes, and Tartrate Resistant Acid Phosphatase (TRAP)- FPcyan to detect osteoclasts. These multiplex approaches to the identification and isolation of osteoblast lineage cells should help to define the molecular and cellular determinants that initiate and maintain remodeling during orthodontic treatment [39]. Furthermore, these GFP transgenes offer certain advantages over other molecular biology techniques: retention of their fluorescent property after extensive tissue preparation, visualization in unstained sections that preserve the histological architecture of bone, detection of GFP signals directly through microscopy without depending on the diffusion of a substrate, indefinite stability of prepared specimens. These characteristics of utilizing GFP transgene molecular technology address many of the shortcomings of *in situ* hybridization and immunohistochemistry. When comparing GFP detection results with genetic activity identified through *in situ* hybridization, adjacent tissue sections demonstrated the same expression patterns of transgenes, thereby validating the use of this GFP technique in lieu of *in situ* hybridization [30]. Although *in situ* hybridization and immunological techniques can be used to appreciate the microheterogeneity in a

developing or remodeling tissue, the ease and specificity of detecting a visible marker gene has great experimental appeal. Though no single technique is infallible, any methodology or protocol that accurately streamlines specimen analysis and facilitates data collection may inherently diminish procedural errors and reduce problems with sensitivity, accuracy and precision of measurement. Therefore, GFP, when driven by a promoter that is activated at a particular level of cellular differentiation, may provide a strategy for identifying and isolating subpopulations of cells at increasing levels of osteoblast development.

RATIONALE

Although Pavlin developed a transgenic mouse model to investigate bone remodeling in response to orthodontic force in 2000, few studies have since been documented which employ *in vivo* transgenic mouse models. Furthermore, no *in vivo* orthodontic tooth movement model has utilized visual promoter transgene (GFP) markers for direct microscopic visualization and quantification of osteoprogenitor cells at various stages of maturation. Orthodontic tooth movement involves the complex interaction of several differentiated populations of cell types within the periodontal ligament. Very little is known, however, about how specific cell populations within the PDL respond to orthodontic force. With the development of multi-colored GFP promoter transgenes to detect various stages of cellular differentiation of the osteoblast lineage, we have a powerful marker to efficiently visualize how a homogeneous cell population within the periodontal ligament responds to orthodontic force using GFP transgene technology. Therefore, the goals of this study are to develop an *in vivo* tooth movement model using mice with GFP transgenes and to evaluate the expression and localization of osteoblast lineage cells in periodontal ligament over a time course of orthodontic force application.

HYPOTHESIS

Using an *in vivo* transgenic mouse model, our project aims to characterize the localization of osteoblast precursor cells within the periodontal ligament over a time course of orthodontic tooth movement. We will specifically analyze the furcation area of the maxillary first molar, which includes areas of compression and tension, based on the direction of the applied force. To localize cells within the osteoblast lineage, the model will be applied to mice transgenic for early osteoblast differentiation markers, specifically transgenic mice containing α-smooth muscle actin GFP-fused promoter (αSMAGFP), transgenic mice containing the 3.6 kb fragment of the rat collagen type 1 promoter fused to a Topaz-fluorescent protein (Col3.6GFP), and transgenic mice containing a bone sialoprotein GFP-fused promoter (BSPGFP). Using these mice, we hypothesize that there will be an increase in expression of αSMA, Col3.6, and BSP GFP positive cells on the tension side of loaded specimens compared to unloaded controls in the furcation of the maxillary first molar from zero to seven days *in vivo*.

Null hypotheses:

1. There will be no increase in expression of αSMA, Col3.6, or BSP GFP positive osteoblast lineage cells post application of orthodontic force on the tension side compared to the control side from zero to seven days *in vivo*.

SPECIFIC AIMS

Aim #1: Develop an *in vivo* orthodontic tooth movement mouse model

Pavlin et al. developed and characterized an *in vivo* mouse tooth movement model to analyze histomorphometric and genetic responses of periodontal tissues to orthodontic force. Using similar materials as well as adapting unpublished techniques from an *in vitro* mouse mandible organ culture tooth movement model developed by Bibko et al., we will develop an *in vivo* orthodontic tooth movement model in mice.

Aim #2: Apply the model to mice transgenic for fluorescent protein (GFP) tagged promoters which identify various stages of osteoblast maturation.

Using the transgenic mice, we will characterize the differential expression of αSMA, Col3.6, and BSP GFP within the PDL in the *in vivo* orthodontic tooth movement model.

Aim #3: Examine if αSMA, Col3.6, or BSP GFP expressing cells also express RANKL within the PDL in an *in vivo* orthodontic tooth movement model.

RANKL will be localized in the periodontal area of the maxillary first molar using immunohistochemistry. The immunohistochemistry images will be overlaid with the GFP fluorescence images to identify if a specific population of osteoblast precursor cells is co-localized with the presence of RANKL in the PDL.

CHAPTER II - MANUSCRIPT

(for submission to a peer-reviewed journal, covering Aim #1 and Aim #2, with Aim #3 covered in Chapter III)

Localization of Osteoblast Precursor Cells in the Periodontal Ligament Using a Novel *In Vivo* Orthodontic Tooth Movement

Christopher E. Olson,[a] Zana Kalajzic,[b] Sunil Wadhwa,[c] Flavio Uribe[d]
Farmington, CT

[a] Former Resident, Division of Orthodontics, Department of Craniofacial Sciences, Health Center, University of Connecticut, Farmington.
[b] Laboratory Technician, Department of Craniofacial Sciences, Health Center, University of Connecticut, Farmington
[c] Assistant Professor, Department of Craniofacial Sciences, Division of Orthodontics, Health Center, University of Connecticut, Farmington
[d] Associate Professor and Program Director, Department of Craniofacial Sciences, Division of Orthodontics, Health Center, University of Connecticut, Farmington

The authors report no commercial, proprietary, or financial interest in the products or companies described in this article.

Reprint requests to:
Dr. Flavio Uribe
Department of Craniofacial Sciences
Division of Orthodontics
University of Connecticut Health Center
263 Farmington Ave
Farmington, CT 06030-1725
Phone: (860) 679-3656
Fax: (860) 679-1920
e-mail: FUribe@uchc.edu

Localization of Osteoblast Precursor Cells in the Periodontal Ligament Using an *In vivo* Orthodontic Tooth Movement Model

ABSTRACT

Objective: To evaluate the effects of orthodontic tooth movement on cells of the osteoblast lineage in the periodontal ligament model using transgenic mice containing transgenes of promoters of osteoblast difffferntiation fused to green fluorescent proteins (GFP).

Materials and Methods: The maxillary first molar was loaded with 10-12 grams of force for 12 hr, 48 hr, or 7 d in transgenic mice 10-12 weeks of age. Mice were transgenic for one of the following GFP-tagged bone markers of osteoblast lineage cells: α-smooth muscle actin (αSMA), 3.6 kb fragment of the rat collagen type 1 promoter (Col3.6), or Bone Sialoprotein (BSP). Loaded sites of pressure and tension were compared with contra-lateral unloaded controls.

Results: Frozen sections of the maxillary first molar showed a significant decrease in GFP expression for all osteoblast bone markers in the PDL at all time points when comparing the pressure side of control sites to the pressure side of loaded sites. The tension side of loaded sites predominantly demonstrated a slight, but not significant, increase in GFP expression compared to controls.

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

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 pressure side of the PDL.[1-5] Complex interactions between osteoclasts and osteoblasts involve numerous biologic players, including systemic hormones, cytokines and growth factors.[6] Increasingly, it has been recognized that a greater understanding of the cellular determinants and the 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 periodontal ligament. Very little is known, however, about how specific cell populations within the PDL respond to orthodontic force. New methods have recently been developed to isolate and study defined populations of cells through the use of transgenic mice with green fluorescent protein (GFP) reporters fused to the promoter of 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 Fluorescent 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 expression of bone-associated marker genes. α-Smooth muscle actin (αSMA) has been identified as a marker specific for osteoprogenitor cells prior to entering the osteogenic pathway; in a cellular environment completely devoid of osteoblast cells, cells expressing αSMA have been shown to transition to an osteoprogenitor lineage leading to extensive osteogenesis.[10] Preosteoblasts 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.[7] Transgenic mice have been developed which carry GFP tagged to specific promoter fragments. This has enabled investigators to utilize microscopy to visualize the GFP-tagged promoter fragments and to correlate GFP expression with different stages of osteoblast differentiation.

No *in vivo* orthodontic tooth movement model has been reported in the literature that utilized visual promoter transgene markers (GFP) for direct microscopic visualization and quantification of osteoprogenitor cells at various stages of maturation. With the development of multi-colored GFP promoter transgenes to detect various stages of cellular differentiation of the osteoblast lineage, we have a powerful marker to efficiently visualize how a homogeneous cell population within the periodontal ligament

responds to orthodontic force using GFP transgene technology. Therefore, the purpose of this study was to develop an *in vivo* tooth movement model using mice with GFP transgenes and to evaluate the expression and localization of osteoblast lineage cells in periodontal ligament over a time course of orthodontic force application.

MATERIALS AND METHODS

All experiments were performed under an institutionally approved protocol for the use of animals in research (University of Connecticut Health Center #2008-432). Thirty-six transgenic mice 10-12 weeks of age weighing 20-25 g were used for the study. Mice were weighed daily, and any mouse that lost more than 20 % of its body weight was sacrificed and excluded from the study. Twelve mice (n=12) were transgenic for α-smooth muscle actin GFP-fused promoter (αSMA), twelve mice (n=12) were transgenic for 3.6 kb fragment of the rat collagen type 1 GFP-fused promoter (Col3.6), and twelve mice (n=12) were transgenic for bone sialoprotein GFP-fused promoter (BSP). The animals were housed under normal laboratory conditions, fed transgenic soft dough diet (Bio-Serv, Frenchtown, NJ) and water *ad libitum,* and acclimated for 2 weeks under experimental conditions.

Mice were anesthetized with intramuscular injections of ketamine (6μg/g body weight) and fitted with a custom mouth prop formed from 0.032" round stainless steel wire for appliance placement (Figure 1). A custom-made 0.006" x 0.030", closed, nickel-titanium coil spring (Ultimate Wireforms, Inc., Bristol, CT) was used to deliver orthodontic force. The force/deflection rate (F/Δ) for the spring was determined to be 10 to 12 g over a range of 0.5 to 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) 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) and cured with a curing light (Flashlite 1401, Discus Dental© Culver City, CA). 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 – the 2 mm discrepancy accounting 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 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 activated by pulling it toward the left central incisor with the wire. Activation distance was calibrated with a Michigan-O periodontal probe with Williams markings 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 grams. 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). The mandibular incisors were reduced to prevent appliance damage. Only the left side of the maxilla was mechanically loaded; the contralateral right side served as control.

Each group of 12 GFP transgenic mice was equally divided into three time intervals of force duration: 12 hrs, 48 hrs, and 7 days. After completion of the time

course, mice were euthanized with CO_2 followed by cervical dislocation. The mice were decapitated and the maxillae were removed and cleaned of soft tissues and muscles. The hemisected maxillae were placed in 10% formalin for five days at 4° C, washed in phosphate buffered saline, and placed in 30% sucrose for 12 hrs. The maxillae were immersed in individual disposable base molds containing frozen embedding medium (Shandon M-1, Thermo Scientific, Waltham, MA). The embedding media was flash frozen in a chilled solution of 2-methylbutane over dry ice. Sagittal sections 5-µm thick were cut of the loaded left and control right sides using a Leica CM1900 Cryostat (D-69226; Leica, Inc., Nussloch, Germany). Sections were oriented to visualize the mesial-buccal and distal-buccal roots of the maxillary first molars, including the interradicular bone and the coronal 1/3rd of the radicular pulp. Four tissue sections were cut for each the left and right side.

Digital images of each section were captured using a Zeiss Axiovert 200 M microscope equipped with a GFP FITC/Texas Red dual filter cube, a motorized stage, and digital camera. Images were taken at 20x 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 (pressure side) was imaged. Conversely, the distal aspect of the mesial-buccal root (tension side) was imaged. For comparison, the same pressure 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 in order to capture the region of the PDL in closest proximity to the furcation (Figure 2).

Following GFP imaging, sections were stained with hematoxylin (Invitrogen, Carlsbad, CA) according to the manufacturer's directions.

To quantify the number of osteoblast lineage cells, images were viewed in Adobe Photoshop (Adobe Systems Inc., San Jose, CA) 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 for the pressure and tension sides in both the mechanically-loaded left side and the unloaded right side were counted in identical fashion. The same imaging protocol was used to capture images and count the total cells in the corresponding hematoxylin 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 = (# GFP positive cells / # all cells). Images of the pressure and tension sites of the first molar were taken and counted from four tissue sections per side (loaded left and control right) per mouse. The average GFP labeling index of the pressure and tension sites of the four sections was calculated for each side (left vs. right) for each mouse. For each of the GFP transgenes and time points, 4 mice were used, and the mean GFP labeling index for each group was calculated. The means for the GFP labeling index of the pressure and tension sites for the loaded left molar and unloaded right molar at each time point for each GFP transgene were compared using student t-tests. Significance was accepted when $P<.05$. Statistical analyses were carried out with GraphPad Prism (GraphPad Software, Inc., La Jolla, CA).

RESULTS

During the duration of the experiment, animals typically lost weight on the first day, returned to their original weight after days 2 to 3, and continued to gain weight

through day 7. No animal lost any body weight after 1 week compared to day 0 (data not shown). Qualitatively, both the GFP images and hematoxylin images demonstrated that the applied force consistently produced a narrowing, or compression, of the PDL space on the mesial surface of the distal-buccal root. Conversely, the distal surface of the mesial-buccal root displayed a widening of the PDL space in response to the tensile force. These morphologic changes were visible even in the groups loaded for only 12 hours (Figure 3).

After 12 hours of mechanical loading, a significant decrease in fluorescent protein expression for all three osteoblast differentiation markers was observed in the pressure side of the furcation area of loaded first molars compared to unloaded controls (Table 1). Figures 4, 5, and 6 A-D show sagittal sections of fluorescent images after 12 hours of loading in Col3.6, BSP, and αSMA mice, respectively. Arrows signify direction of force application. For the tension side of the furcation of the first molar at 12 hours of loading, the mechanically loaded BSP group demonstrated a significant increase ($P<0.05$) in GFP expression compared to the unloaded BSP group (Fig. 5 C, D). In the Col3.6 and αSMA 12 hour mice, a non-significant increase in GFP expression was observed on the loaded tension sides when compared to the unloaded tension controls (Fig. 7).

Among the mice in the 48 hour group, both the αSMA and BSP mice showed a non-significant increase in GFP expression on the loaded tension sides compared with the unloaded control tension sides. The Col3.6 48 hour group, however, demonstrated a non-significant decrease in GFP expression on the loaded tension side when compared to the unloaded tension controls. GFP expression decreased significantly in the pressure side of

the furcation area of loaded first molars compared to unloaded controls for the Col3.6, BSP, and αSMA 48 hour groups (Table 1; Figs. 4, 5, 6).

In the 7 day group, all three osteoblast differentiation markers demonstrated a significant decrease in GFP expression when comparing loaded pressure sides to unloaded pressure sides (Table 1; Figs. 4, 5, 6). On the experimental tension sides of the 7 day groups, GFP expression in the Col3.6 and αSMA mice increased, but not significantly, when compared to the control tension sides. For the BSP 7 day group, a non-significant decrease in GFP expression was observed in the loaded tension vs. unloaded tension sites.

Comparison of the pressure sides to the tension sides of the controls showed no significant difference at any time point for any GFP transgene.

DISCUSSION

The focus of this study was on the response of osteoblast lineage cells to orthodontic force in an *in vivo* murine model. In order to localize osteoblast cells within the periodontal ligament, the model was used in transgenic mice with GFP markers for different stages of osteoblast differentiation. Cells expressing proteins such as type I collagen and bone sialoprotein have been shown to be representative of the osteoblastic stage of differentiation. Different length collagen promoter fragments containing a 13-base pair element, including the 3.6kb and 2.3kb fragments, have demonstrated high levels of expression in osteoblasts.[13] Dacic et al. showed that the 3.6 kb rat Col1a1 promoter is expressed in culture during the early post-proliferative stage, and gets stronger when the cell differentiation progresses.[14] By illuminating cells which express these various promoters with GFP markers, we now have a powerful tool to efficiently

visualize populations of homogeneous osteoblast cells at known stages of differentiation in a tooth movement model. Use of these GFP transgenes offers certain advantages over other molecular biology techniques such as *in situ* hybridization and immunohistochemistry: retention of their fluorescent property after extensive tissue preparation, visualization in unstained sections that preserve the histological architecture of bone, detection of GFP signals directly through microscopy without depending on the diffusion of a substrate, and indefinite stability of prepared specimens. [8]

Application of orthodontic force to the maxillary first molar in this study resulted in two distinct patterns of response from osteoblast lineage cells on the tension and pressure sides. On the tension side, the ratio of cells expressing GFP markers of osteoblast lineage typically increased, though not significantly, compared to controls. The earliest time point registered in this study was at 12 hours, and the longest was 7 days; for these times, as well as for the 48 hour groups, the majority of the GFP markers showed a modest increase in expression. These data are consistent with Pavlin's findings that the number of osteoblasts on the alveolar bone surface in tension sites of the periodontal ligament showed a non-significant increase between 48 hours and 6 days of orthodontic loading. In Pavlin's studies, however, no difference between loaded sites of tension and controls was seen in the first 24 hours, and significant increases were observed from days 6 through day 12 of observation.[15, 16] The contrast in significance in the present study with Pavlin's may be attributed to terminating the time course at 7 days; perhaps had the force application been extended longer, more significant expression would have been seen in the tension side, similar to Pavlin's data. Differences in results may also be attributed to the materials and methods used to detect osteoblasts; GFP

detection utilized in this study measures promoter activity, whereas Pavlin determined Col1a1 mRNA activity using *in situ* hybridization.

In contrast to the increase observed on the tension side, GFP expression was significantly reduced on the compression sites when compared to controls at the same time points. This result has not previously been reported in the literature in an *in vivo* study. BSP mRNA has been reported to be increased during *in vitro* compression of Saos-2 human osteoblastic cell lines.[11]

One interpretation of the significant decrease in GFP expression on the pressure side is that the osteoblast cells lose their phenotype due to de-differentiation 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.[1,2,4] Though not confirmed in this study, the light force range of 10-12 grams used has previously been shown in mouse models to promote physiologic tooth movement without inducing areas of hyalinization.[15] Definitive light microscopy analysis would be needed to confirm the absence of hyalinization in this study.

In order 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 that can express multiple markers of sequential stages of osteoblast maturation simultaneously and distinctly within the same animal would be of great benefit. Such mice could be used to definitively show how osteoblast 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.

CONCLUSION

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

REFERENCES

1. Meikle M. The tissue, cellular, and molecular regulation of orthodontic tooth movement: 100 years after Carl Sandstedt. European Jour of Orth 2006; 28:221-240
2. Rygh, P., Ultrastructural changes in tension zones of rat molar periodontium incident to orthodontic tooth movement. Am J Orthod, 1976. 70(3): p. 269-81.
3. Storey, E., The nature of tooth movement. Am J Orthod, 1973. 63(3): p. 292-314.
4. Reitan, K. Clinical and histologic observations on tooth movement during and after orthodontic treatment. Am J Orthod 1967 Oct;53(10):721-45.
5. King GJ, Keeling SD, Wronksi TJ. Histomorphometric study of alveolar bone turnover in orthodontic tooth movement. Bone 1991; 12; 401-409.
6. Krishnan V, Davidovitch Z. Cellular, molecular, and tissue-level reactions to orthodontic force. Am J Orthod 2006 Apr;129(4): 469.e1-32.
7. Kalajzic I, Kalajzic Z, Kaliterna M, Gronowicz G, Clark SH, Lichtler AC, Rowe D. Use of Type I collagen green fluorescent protein transgenes to identify subpopulations of cells at different stages of the osteoblast lineage. J of Bone and Miner Res 2002; 17:15-25.
8. Xi Jiang, Zana Kalajzic, Peter Maye, Alen Braut, Justin Bellizzi, Mina Mina and David W. Rowe Histological Analysis of GFP Expression in Murine Bone. Journal of Histochemistry and Cytochemistry Volume 53 (5): 593-602, 2005
9. Bilic-Curcic, I., et al., Visualizing levels of osteoblast differentiation by a two-color promoter-GFP strategy: Type I collagen-GFPcyan and osteocalcin-GFPtpz. Genesis, 2005. 43(2): p. 87-98.
10. Kalajzic Z, Li H, Wan LP, Jian X, Lamothe K, Adams DJ, Aguila HL, Rowe DW, Kalajzic I. Use of an alpha-smooth muscle actin GFP reporter to identify an osteoprogenitor population. Bone 2008; 43:5101-510.
11. Mitsui N, Suzuki N, Maeno M, Mayahara K, Yanagisawa M, Otsuka K, Shimizu N. Optimal compressive force induces bone formation via increasing bone sialoprotein and prostaglandin E2 production appropriately. Life Scie 2005; 77:3168-3182
12. Gluhak-Heinrich J, Ye L, Bonewald LF, Feng JQ, MacDougall M, Harris SE, Pavlin D. Mechanical loading stimulates dentin matrix protein 1 (DMP1) expression in osteocytes *in vivo*. J Bone Miner Res 2003; 18:807-817.

13. Bedalov A, et al., Regulation of Col1A1 expression in type I collagen producing tissues: identification of a 49 base pair region which is required for transgene expression in bone of transgenic mice. J Bone Miner Res. 1995 Oct; 10(10): 1443-51.
14. Dacic, S., et al., Col1a1-driven transgenic markers of osteoblast lineage progression. J Bone Miner Res, 2001. 16(7): p. 1228-36.
15. Pavlin D, Magness M, Zadro R, Goldman ED, Gluhak-Heinrich J. Orthodontically stressed periodontium of transgenic mouse as a model for studying mechanical response in bone: The effect on the number of osteoblasts. Clin. Orthod. Res. 3, 2000; 55-66
16. Pavlin D, Dove SB, Zadro R, Gluhak-Heinrich J. Mechanical loading stimulates differentiation of periodontal osteoblasts in a mouse osteoinduction model: Effect on Type I Collagen and Alkaline Phosphatase genes. Calcif Tissue Int 2000; 67:163-172

CHAPTER III - DISCUSSION

Addendum to Chapter II – Aim #3

In addition to the materials and methods described in Chapter II which relate to Aim #1 and Aim #2 of this thesis, experimental design and results for Aim #3 (RANKL Localization) were obtained but not included in the manuscript for journal submission. The materials and methods and results of the RANKL experimentation are presented below, followed by comprehensive discussion and conclusion chapters inclusive of all Aims.

Immunohistochemical Analysis of RANKL Expression

Materials and Methods

After GFP imaging, the same sections were used for detection of RANKL expression. After washing in PBS for 5 minutes three times, endogenous peroxidase activity in the fixed sections was prevented by treating them with 3% hydrogen peroxide in PBS for 25 min, followed by washing in PBS. Non-specific reactivity was blocked with 10% rabbit serum in PBS for 1 hour and 30 minutes at room temperature. Anti-RANKL (goat polyclonal anti-mouse RANKL antibody, BD Biosciences, San Jose, CA) was then added at a dilution of 1:200 in 2% rabbit serum for 24h at 4°C. The sections were washed with PBS and incubated with biotinylated rabbit anti-goat secondary antibody diluted 1:200 in 2% rabbit serum for one hour at room temperature. A negative control was used in the immunostaining procedure with non-immune goat serum and addition of secondary antibody. The immunocomplexes were visualized in brown with a DBA substrate kit (VECTASTAIN Elite ABC) following the manufacturer's directions under a light microscope, followed by counterstaining with hematoxylin. Sections were

dehydrated in ascending alcohol solutions (50%, 70%, 80%, 95% x2, and 100% x2) and finally cleared with xylene two times prior to mounting with Permount.

Images of the RANKL stained sections were captured using the same procedure described previously in Chapter II for the GFP images. Using Adobe Photoshop, images of the RANKL stained sections were superimposed over the corresponding images of GFP promoter expression. The number of RANKL positive cells overlapping a GFP positive cell was counted.

Results

Representative hematoxylin and RANKL-stained images from the frozen sections are shown in Figure 8. RANKL positive cells were seldom detected within the periodontal ligament of the frozen sections. Though negative controls obtained during the RANKL staining process did confirm the success of the staining via evidence of brown cells observed in the interradicular marrow spaces under low magnification light microscopy, the PDL space under high magnification consistently showed little or no RANKL staining in both loaded and unloaded specimens. RANKL positive cells were more frequently observed in peri-vascular areas within the alveolar bone. The limited number of RANKL positive sections revealed no pattern or correlation related to type of force (pressure vs. tension), GFP promoter expression, or duration of force.
A representative image of the RANKL stained section overlaid with its corresponding Col3.6 GFP image is shown in Figure 9. The results of RANKL staining, therefore, could not be used for meaningful statistical analysis.

DISCUSSION

In the present study, an *in vivo* tooth movement model using mice with GFP transgenes was developed to evaluate the localization of osteoblast lineage cells in the periodontal ligament over a time course of orthodontic force application. The biomechanical force system was adapted from the model described by Pavlin et al [22]. Using a super-elastic nickel-titanium coil ligated from the maxillary first molar to the incisors, a force of 10-12 grams was delivered to groups of transgenic mice. Based on the application of force delivery, the resulting vector of force had a mesial and extrusive direction. Therefore, the furcation of the molar presented an ideal area for analysis since the mesial-buccal and distal-buccal roots in this location can portray areas of mesial and extrusive movement. The force system was applied for a period of 12 hours, 48 hours, or 7 days. This time course was consistent with previous murine models of orthodontic tooth movement, and represented a manageable investment in time allocation to initially develop an *in vivo* model while minimizing the likelihood of appliance failure over a longer time interval. Hematoxylin stained frozen sections showed demonstrable areas of compression and tension in the periodontal ligament for specimens subjected to force. Sections were not stained with eosin because hematoxylin staining was performed after RANKL staining.

Though much information has been learned through the use of animal models in orthodontics over the past century, more information is still needed about the biological responses of the paradental tissues to mechanical stimuli, especially in the initial phases of orthodontic treatment. An increase in the expression of cytokines, such as interleukin-1α and tumor necrosis factor-α, has been seen within three hours in sites of compression

and tension during tooth movement studies in rats [40]. As early as four hours, cAMP and insulin-like growth factor-I levels have been shown to increase, and cells within the periodontal ligament demonstrate evidence of differentiation [41]. The bone transcription factor Runx2 and extracellular signal-regulated kinases have been shown to be up-regulated in response to orthodontic force; proportions of Runx2-positive cells and pERK1/2-positive cells significantly increase after only eight hours of loading in rats [42].

The first part of this study focused on the response of osteoblast lineage cells to orthodontic force in an *in vivo* murine model. In order to localize osteoblast cells within the periodontal ligament, the model was used in transgenic mice with GFP markers of different stages of osteoblast differentiation. Cells expressing proteins such as type I collagen and bone sialoprotein have been shown to be representative of the osteoblastic stage of differentiation. Different length collagen promoter fragments containing a 13-base pair element, including the 3.6kb and 2.3kb fragments, have demonstrated high levels of expression in osteoblasts [37]. Dacic et al. showed that the 3.6 kb rat Col1a1 promoter is expressed in culture during the early post-proliferative stage, and gets stronger when the cell differentiation progresses [38]. By illuminating cells which express these various promoters with GFP markers, we now have a powerful tool to efficiently visualize osteoblast cells at known stages of differentiation in a tooth movement model. Use of these GFP transgenes offer certain advantages over other molecular biology techniques such as *in situ* hybridization and immunohistochemistry: retention of their fluorescent property after extensive tissue preparation, visualization in unstained sections that preserve the histological architecture of bone, detection of GFP

signals directly through microscopy without depending on the diffusion of a substrate, and indefinite stability of prepared specimens.

Application of orthodontic force to the maxillary first molar in this study resulted in two distinct and contradicting patterns of response from osteoblast lineage cells. On the tension side, the ratio of cells expressing GFP markers of osteoblast lineage typically increased, though not significantly, compared to controls. The earliest time point registered in this study was at 12 hours, and the longest was 7 days; for these times, as well as for the 48 hour groups, the majority of the GFP markers showed this modest increase in expression. These data are consistent with Pavlin's findings that the number of osteoblasts on the alveolar bone surface in tension sites of the periodontal ligament showed a moderate but non-significant increase between 48 hours and 6 days of orthodontic loading. In Pavlin's study, however, no difference between loaded sites of tension and controls was seen in the first 24 hours, and significant increases were observed from days 6 through day 12 of observation [22]. The lack of significance in this study may therefore be attributed to terminating the time course at 7 days; perhaps had the force application been extended longer, more significant expression would have been seen in the tension side, similar to Pavlin's data.

In contrast to the increase observed on the tension side, GFP expression was significantly reduced on the compression sites when compared to controls at the same time points. This unexpected result has not previously been reported in the literature in an *in vivo* study. BSP mRNA has been reported to be increased during *in vitro* compression of Saos-2 human osteoblastic cell lines [32]. Cultures of primary cell populations, however, are not homogeneous, and in cases of cloned immortalized or

transformed osteoblast lines, cells may be examined at different stages of differentiation. A major concern with any bone culture is that the cells may express an incomplete or altered osteoblast phenotype in a culture condition [22].

One interpretation of this significant decrease is that the osteoblast cells lose their phenotype due to de-differentiation 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 [1,2,4]. Though not confirmed in this study, the light force range of 10-12 grams used has previously been shown in mouse models to promote physiologic tooth movement without inducing areas of hyalinization [22]. Definitive light microscopy analysis would be needed to confirm the absence of hyalinization in this study. Due to the use of frozen sectioning in the present study, detection of hyalinization was not possible. While frozen sectioning did preserve the overall morphology of the native histological environment, the fine details and micro-architecture of the sections was not well maintained using this technique.

In the second part of the study, the frozen sections were stained to detect the presence of RANKL within the paradental tissues. Frozen sectioning was utilized in order to best preserve GFP expression. Previous work has shown that when compared to paraffin processing of transgenic GFP mice, frozen sectioning yields superior retention of GFP expression and stronger GFP signaling [43]. The quality of histological images obtained from frozen sectioning, however, has widely been acknowledged to be inferior to paraffin sectioning. In this regard, using frozen sectioning represents a trade off between GFP detection and histological analysis. For this study, frozen sectioning was

elected in order to first meet the objective of characterizing a baseline of data for localization of osteoblast lineage GFP expression. As a result, the quality of histological sections was handicapped, and RANKL staining was most likely impeded.

CHAPTER IV – CONCLUSION

SIGNIFICANCE OF RESULTS

An *in vivo* tooth movement model using transgenic mice with GFP bone markers provides an efficient and effective model to investigate the cellular events of orthodontic tooth movement. Using this model, consistent results were observed that revealed possible trends in osteoblast lineage cells during the initial stages of orthodontic tooth movement. In particular, osteoblast lineage cells may lose their osteoblast phenotype within the first day of compressive force application. This trend is sustained through seven days of tooth movement.

FUTURE DIRECTION

In order 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 that can express multiple markers of sequential stages of osteoblast maturation simultaneously and distinctly within the same animal would be of great benefit. A single mouse that could express αSMA, Col3.6, Col2.3, and DMP1 transgenes would greatly amplify the investigative potential. Such mice could be used to definitively show how osteoblast 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. Finally, paraffin processing and longer decalcification times for tissue specimens would best be applied in cases where immunohistological staining is anticipated.

FIGURES

Figure 1: Bonding of spring to maxillary first molar, head stabilized with custom 0.032" stainless steel mouth prop

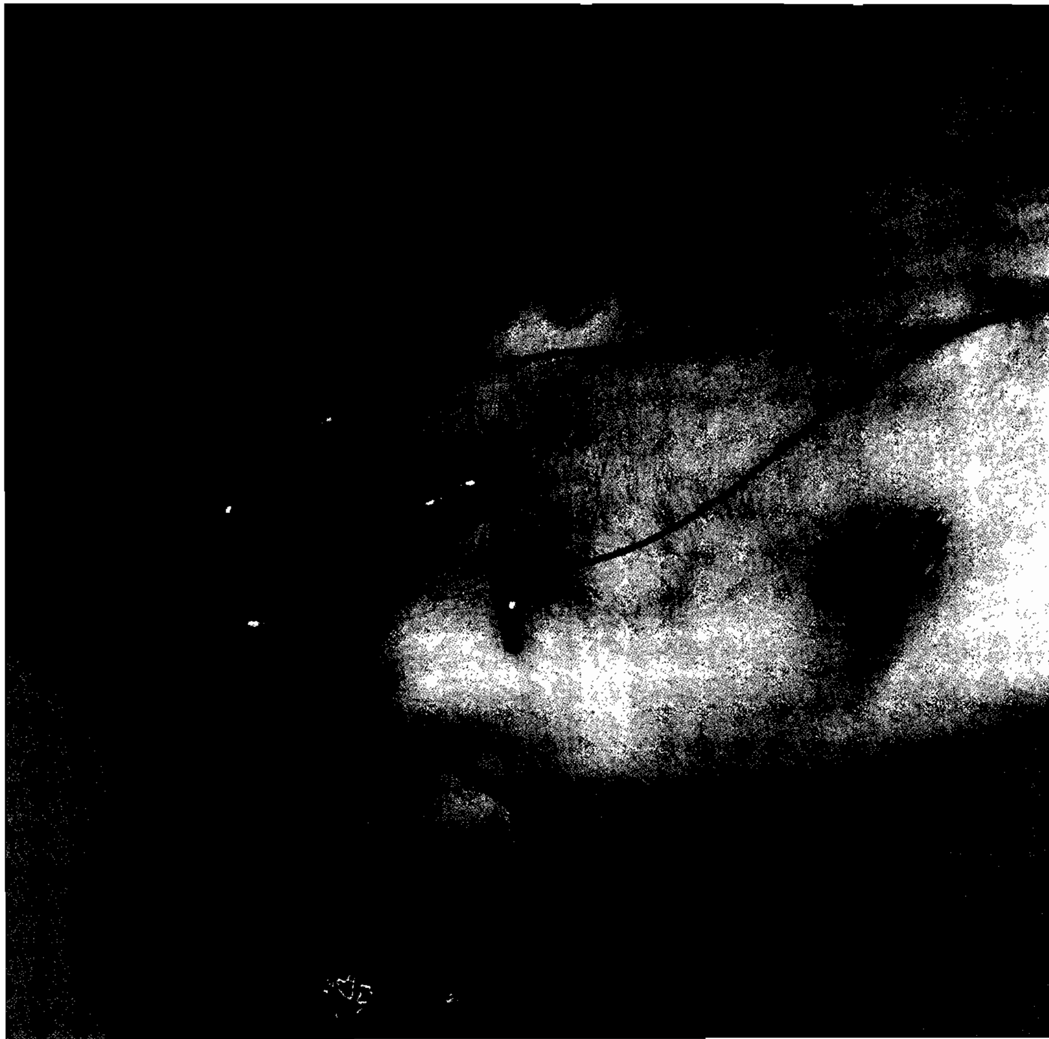

Figure 2: Illustration of imaging regions of maxillary first molar. Images were taken at 20x magnification in the furcation area of both the mesial-buccal and distal-buccal roots. Based on the mesial direction of the force (arrow), the mesial surface of the distal-buccal root (pressure side) was imaged. Conversely, the distal aspect of the mesial-buccal root (tension side) was imaged. For comparison, the same pressure 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 in order to capture the region of the PDL in closest proximity to the furcation

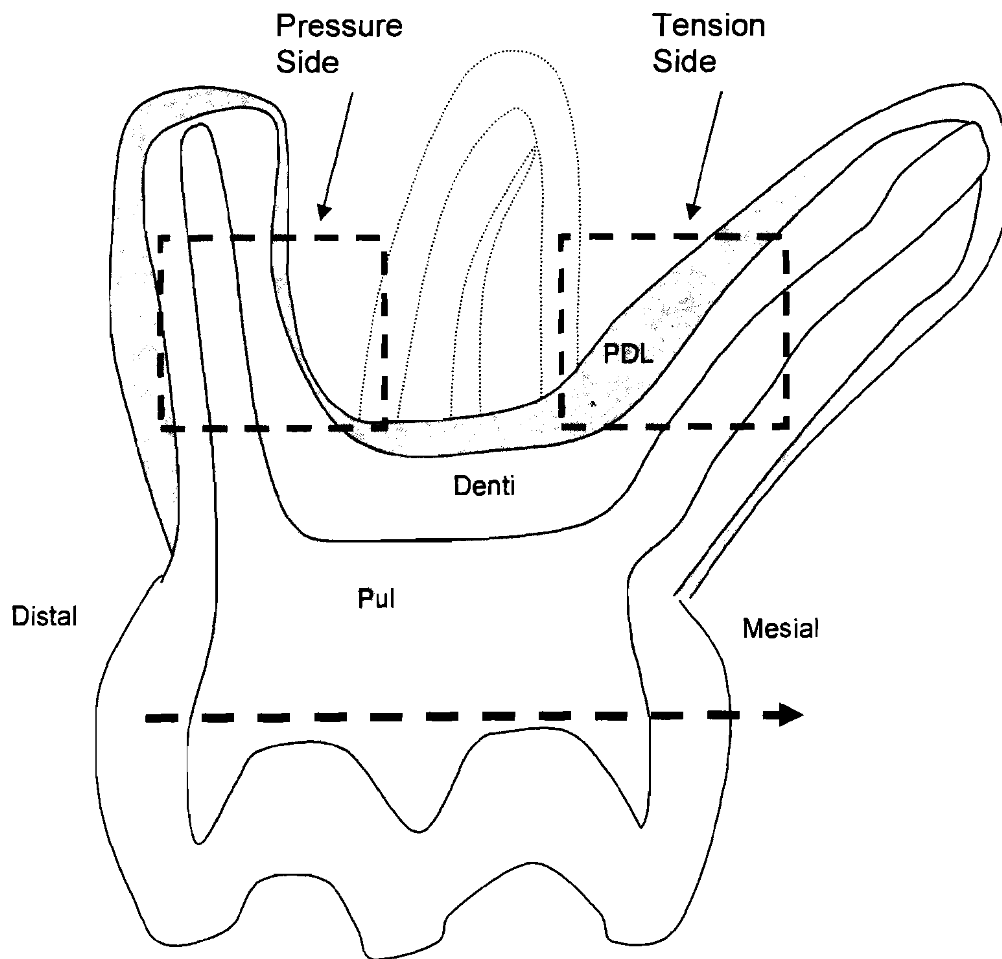

Figure 3: 5x hematoxylin stained sagittal section of the maxillary first molar. Note the compressed PDL on the mesial aspect of the DB root (C) and the stretched PDL on the distal aspect of the MB root (T)

Figure 4. 20x Fluorescent images of sagittal sections of the distal-buccal (pressure side) and mesial-buccal (tension side) roots of maxillary first molars of transgenic mice containing the 3.6 kb fragment of the rat collagen type 1 promoter fused to a topaz-fluorescent protein (Col3.6GFP). Note the significant decrease in expression of GFP in the PDL in images B, F, J (with force) relative to images A, E, I (without force), respectively.

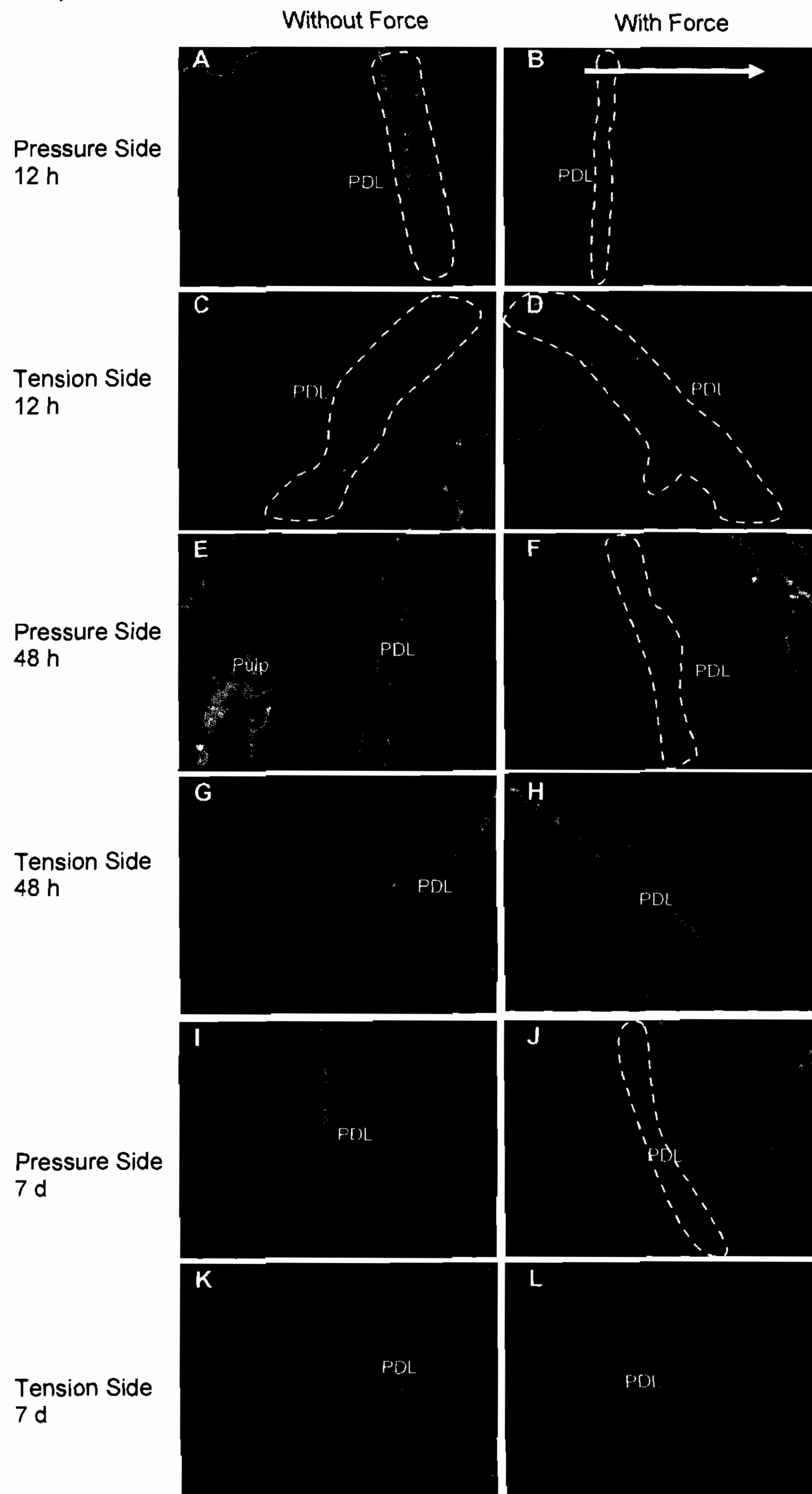

Figure 5: 20x Fluorescent images of sagittal sections of the distal-buccal (pressure side) and mesial-buccal (tension side) roots of maxillary first molars of transgenic mice containing bone sialoprotein (BSP) promoter fused to a topaz-fluorescent protein (BSPGFP). Note the significant decrease in expression of GFP in the PDL in images B, F, J (with force) relative to images A, E, I (without force), respectively.

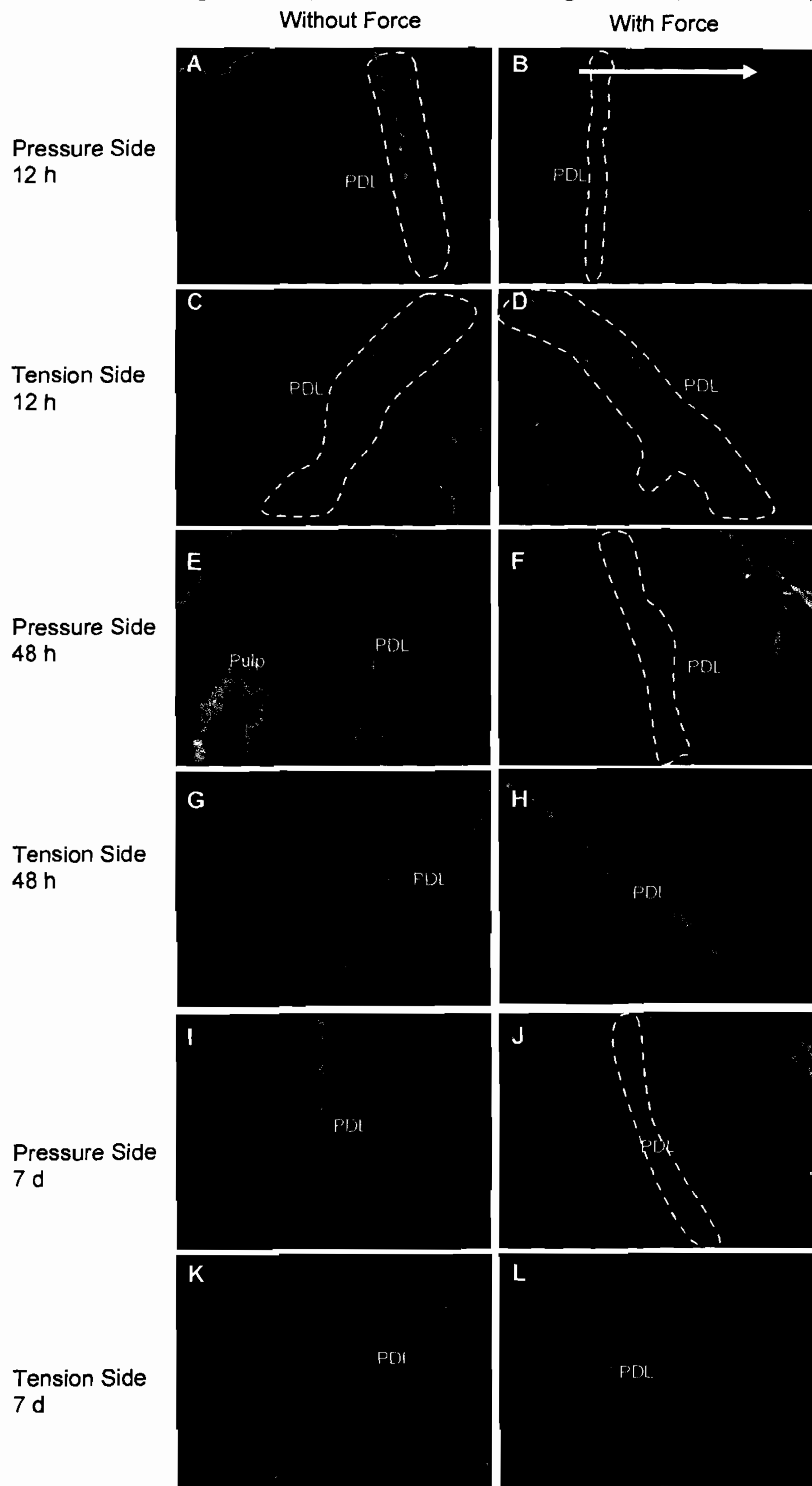

Figure 6: 20x Fluorescent images of sagittal sections of the distal-buccal (pressure side) and mesial-buccal (tension side) roots of maxillary first molars of transgenic mice containing α-smooth muscle actin (αSMA) promoter fused to a topaz-fluorescent protein (αSMAGFPP). Note the significant decrease in expression of GFP in the PDL in images B, F, J (with force) relative to images A, E, I (without force), respectively.

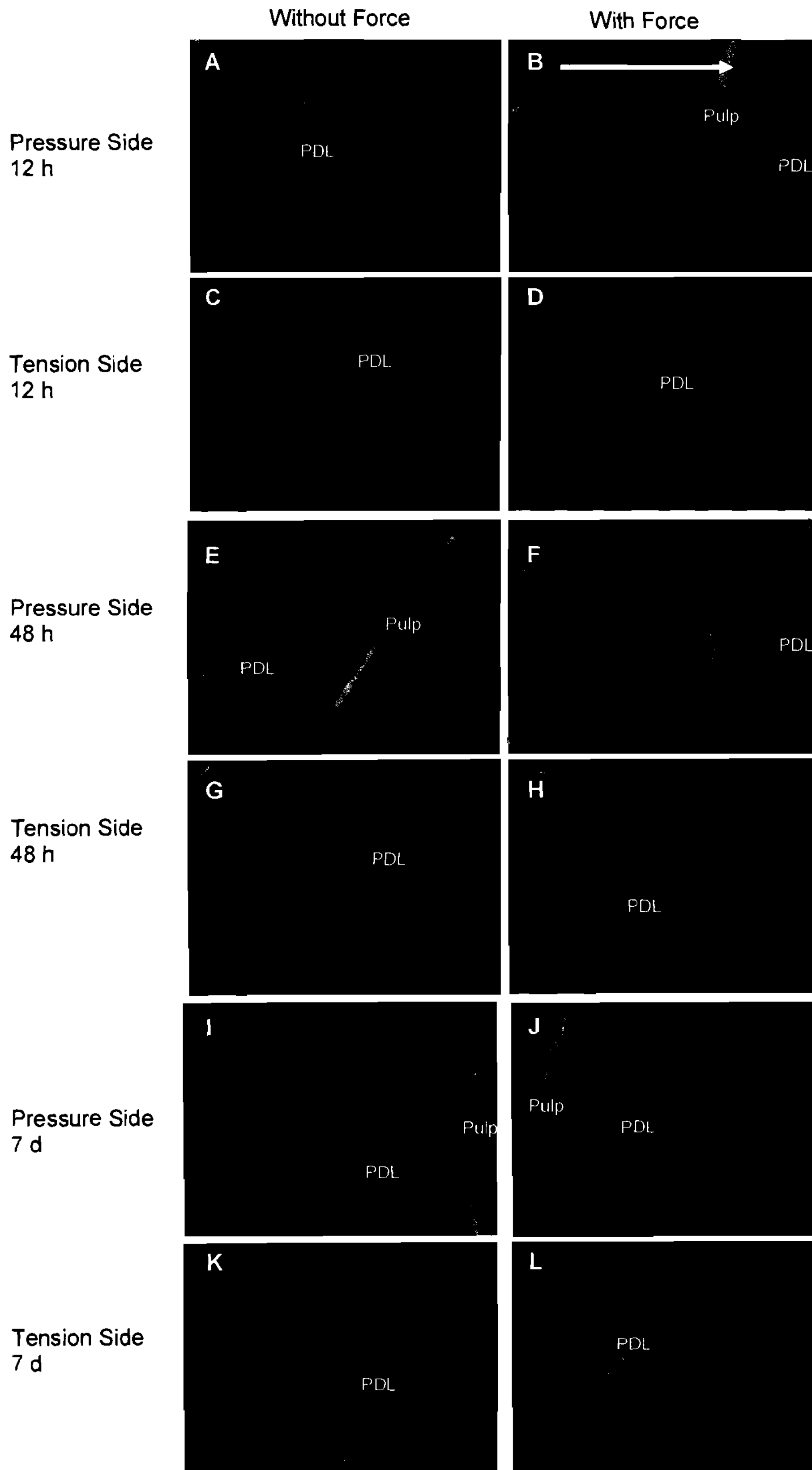

Figure 7: Comparison of Col3.6-GFP (A, B), αSMA-GFP (C,D), and BSP-GFP (E,F) expression as a ratio of GFP+ cells to total cells in pressure and tension sites of the periodontal ligament in loaded vs. contra-lateral unloaded maxillary first molars at 12 h, 48 h, and 7 days of treatment.

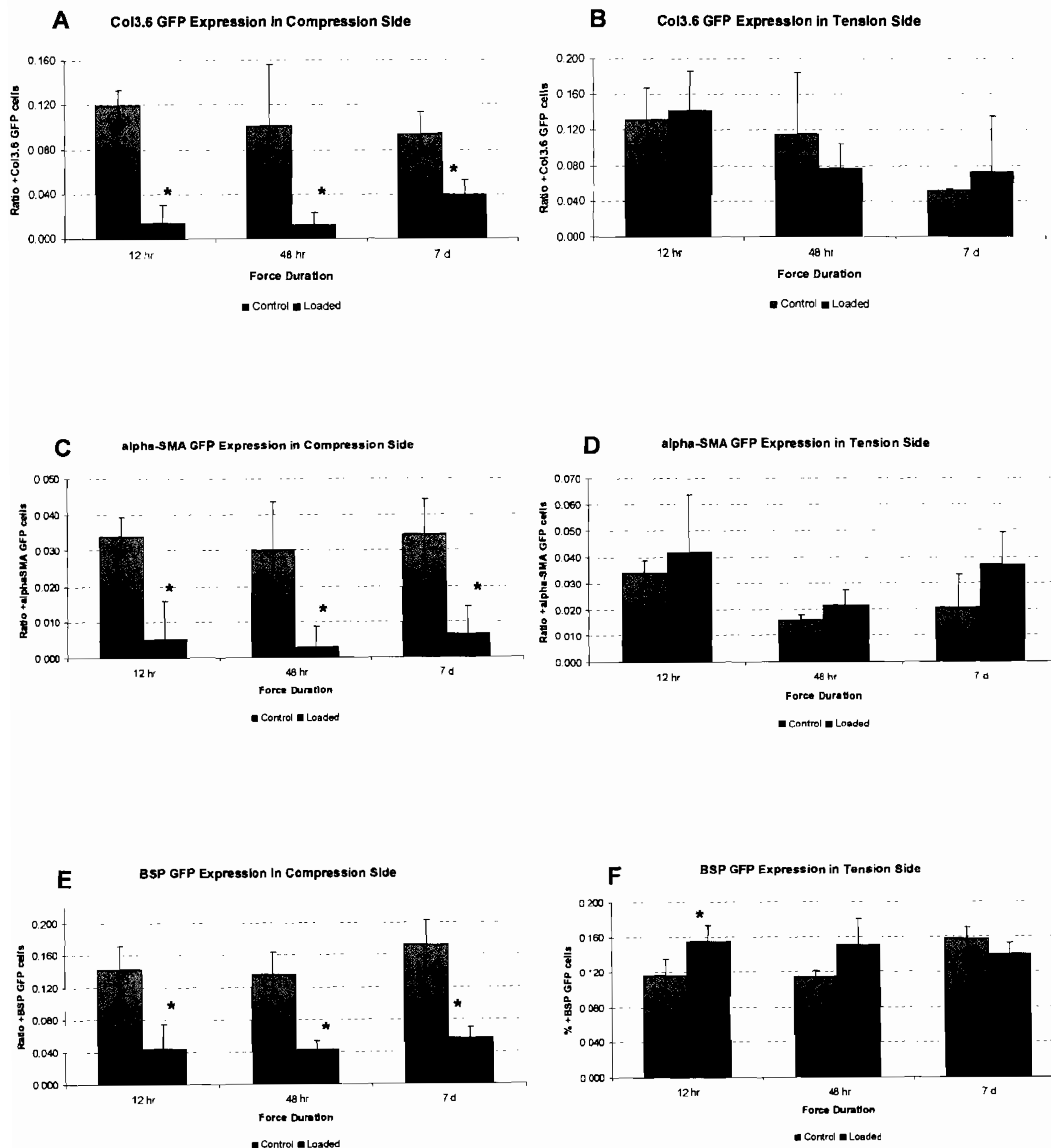

Figure 8: 20x Immunohistochemical analysis of RANKL expression in a sagittal section of the distal-buccal root of the maxillary first molar. Note poor quality of anatomy due to frozen section. R+ indicates RANKL positive cells localized around a blood vessel in the alveolar bone. Note lack of RANKL staining within PDL.

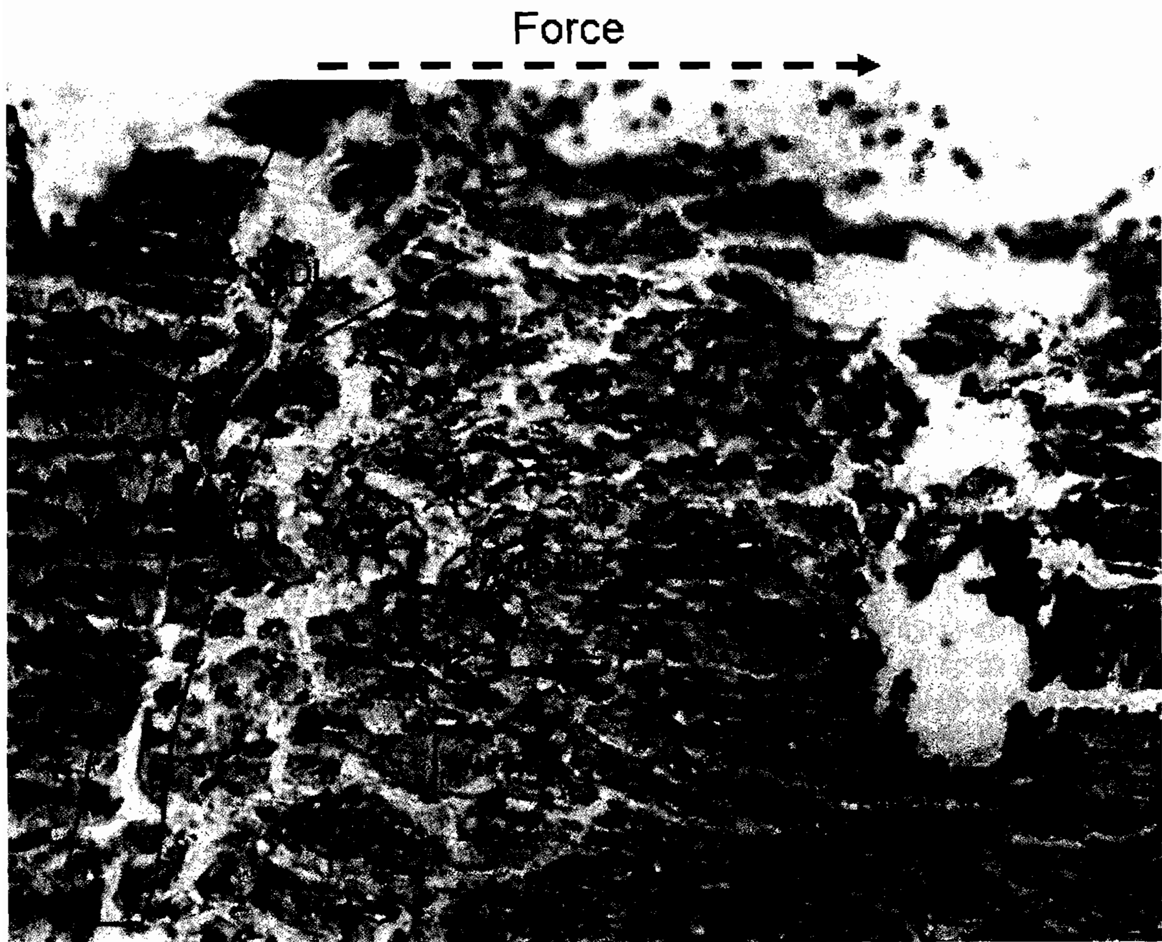

Figure 9: Co-localization of immunohistochemical analysis of RANKL expression in a sagittal section overlaid with corresponding Col3.6GFP sagittal section

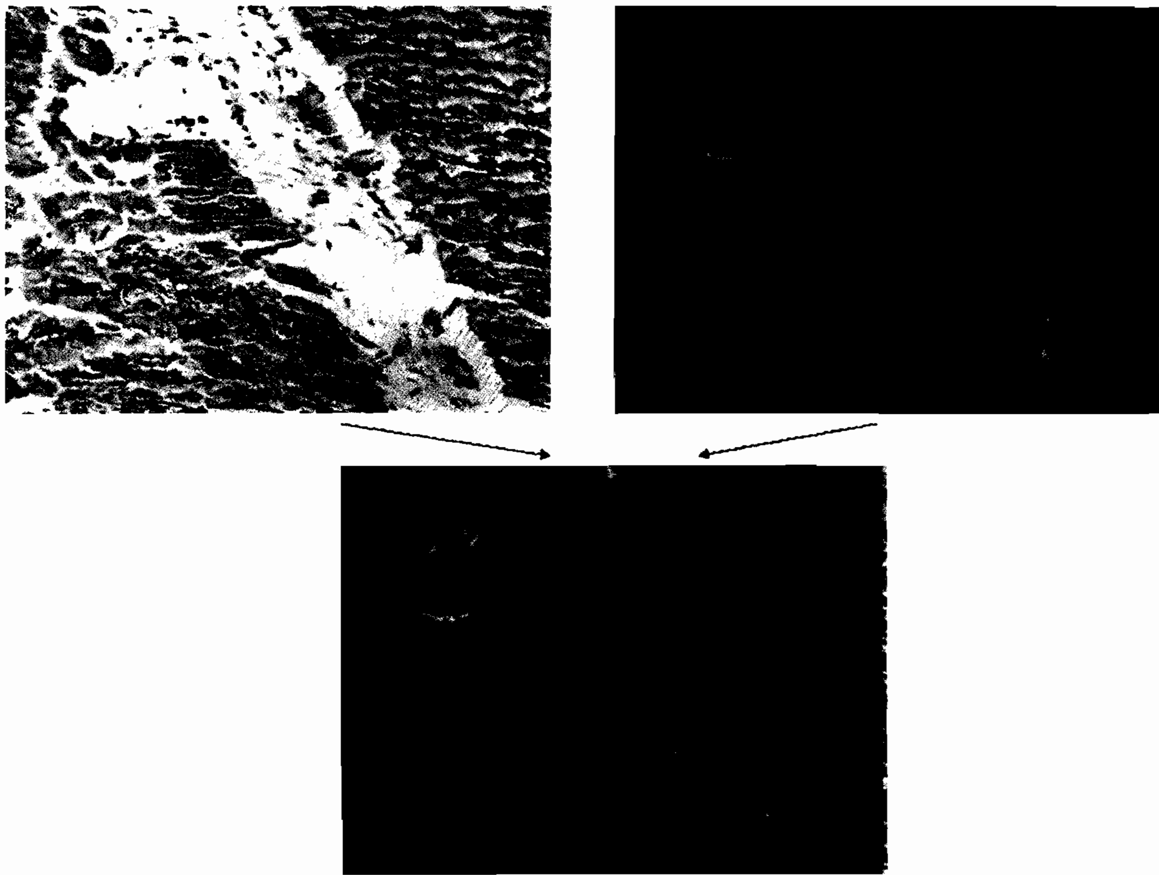

Table 1. Mean ratios for GFP positive cells on the pressure side by duration of force and transgene

Time	*Trans-gene*	*Control Mean Ratio +/- S.D.*	*Loaded Mean Ratio +/- S.D.*	*Difference**
12 hr	Col3.6	0.120 +/- 0.014	0.013 +/- 0.017	0.00009*
	BSP	0.143 +/- 0.029	0.043 +/- 0.031	0.00317*
	αSMA	0.034 +/- 0.005	0.005 +/- 0.010	0.00627*
48 hr	Col3.6	0.101 +/- 0.055	0.012 +/- 0.011	0.04526*
	BSP	0.136 +/- 0.026	0.043 +/- 0.011	0.00281*
	αSMA	0.030 +/- 0.013	0.003 +/- 0.006	0.02029*
7d	Col3.6	0.093 +/- 0.019	0.039 +/- 0.013	0.00406*
	BSP	0.171 +/- 0.031	0.056 +/- 0.013	0.00237*
	αSMA	0.034 +/- 0.010	0.007 +/- 0.008	0.00459*

*Levels of significance for Student t-test between control and loaded:
$P > .05$ = no significant difference

REFERENCES

1. Meikle M. The tissue, cellular, and molecular regulation of orthodontic tooth movement: 100 years after Carl Sandstedt. European Jour of Orth 2006; 28:221-240
2. Rygh, P., Ultrastructural changes in tension zones of rat molar periodontium incident to orthodontic tooth movement. Am J Orthod, 1976. 70(3): p. 269-81.
3. Storey, E., The nature of tooth movement. Am J Orthod, 1973. 63(3): p. 292-314.
4. Reitan, K. Clinical and histologic observations on tooth movement during and after orthodontic treatment. Am J Orthod 1967 Oct;53(10):721-45.
5. King GJ, Keeling SD, Wronksi TJ. Histomorphometric study of alveolar bone turnover in orthodontic tooth movement. Bone 1991; 12; 401-409.
6. Roberts WE, Chase DC. Kinetics of cell proliferation and migration associated with orthodontically-induced osteogenesis. J Dent Res. 1981 Feb;60(2):174-81.
7. Rygh, P. Elimination of hyalinized periodontal tissues associated with orthodontic tooth movement. Sand J Dent Res 1974; 82:57-73.
8. Rody Jr WJ, King GJ, Gu Gaoman. Osteoclast recruitment to sites of compression in orthodontic tooth movement. Am J Orthod Dentofacial Orthop 2001; 120:477-489.
9. Udagawa N. Mechanisms involved in bone resorption. Biogerontology 2002; 3:79-83.
10. Kalajzic I, Staal A, Yang W, Wu Y, Johnson S, Feyen JHM, Krueger W, Maye P, Yu F, Zhao Y, Kuo L, Gupta R, Achenie L, Wang H, Shin D, Rowe D. Expression profile of osteoblast lineage at defined stages of differentiation. Journal of Biological Chemistry 2005; 280: 24618-24626
11. Kanzaki H, Chiba M, Shimizu Y, Mitani H. Periodontal ligament cells under mechanical stress induce osteoclastogenesis by receptor activator of nuclear factor kappaB ligand up-regulation via prostaglandin E2 synthesis. J Bone Miner Res 2002; 17:210-220.
12. Kanzaki H, Chiba M, Shimizu Y, Mitani H. Dual regulation of osteoclast differentiation by periodontal ligament cells through RANKL stimulation and OPG inhibition. J Dent Res 2001; 80:887-891.
13. Kanzaki H, Chiba M, Sato A, Miyagawa A, Arai K, Nukatsuka S et al Cyclical tensile force on periodontal ligament cells inhibits osteoclastogenesis through OPG induction. J Dent Res 2006; 85:457-462.
14. Nishijima Y, Yamaguchi M, Kojima T, Aihara N, Nakajima R, Kasai K. Levels of RANKL and OPG in gingival crevicular fluid during orthodontic tooth movement and effect of compression force on releases from periodontal ligament cells in vitro. Orthod Craniofac Res 2006; 8:63-70.
15. Lekic P, McCulloch CA. Periodontal ligament cell population: the central role of fibroblasts in creating a unique tissue. Anat Rec 1966; 245:327-341.
16. Shiotani A, Shibasaki Y, Sasaki T. Localization of receptor activator of NFkappB ligand, RANKL, in periodontal tissues during experimental movement of rat molars. J Electron Microsc (Tokyo) 2001;50:365-369.
17. Roberts WE, Chamberlain JG. Scanning electron microscopy of the cellular elements of rat periodontal ligament. Arch Oral Biol 1978;23:587-589.
18. Ren Y, Maltha JC and Kuijpers-Jagtman AM. The rat as a model for orthodontic tooth movement – a critical review and a proposed solution. European Journal of Orthodontics 2004; 26:483-490.

19. Hasegawa, Effects of various culture conditions on matrix formative functions of rat incisor odontoblasts in a pulp-dentine slice culture system. Jpn. J. Oral Bio., 1989. 31: p. 392-403.
20. Sloan, A.J., et al., An in vitro approach for the study of dentinogenesis by organ culture of the dentine-pulp complex from rat incisor teeth. Arch Oral Biol, 1998. 43(6): p. 421-30.
21. Bibko J. A novel mouse orthodontic tooth movement organ culture model. School of Dental Medicine Master's Thesis, The University of Connecticut Health Center. 2008.
22. Pavlin D, Magness M, Zadro R, Goldman ED, Gluhak-Heinrich J. Orthodontically stressed periodontium of transgenic mouse as a model for studying mechanical response in bone: The effect on the number of osteoblasts. Clin. Orthod. Res. 3, 2000; 55-66
23. Yoshimatsu M, Shibata Y, Kitaura H, Chang X, Morisshi T, Hashimoto F, Yoshida N, Yamaguchi A. Experimental model of tooth movement by orthodontic force in mice and its application to tumor necrosis factor receptor-deficient mice. J Bone Miner Metab 2006; 24:20-27
24. Keles A, Grunes B, DiFuria C, Gagari E, Srinivasan V, Darendeliler MA, Muller R, Kent Jr R, Stashenko P. Inhibition of tooth movement by osteoprotegerin vs. pamidronate under conditions of constant orthodontic force. Eur J Oral Sci 2007; 115:131-136.
25. Fujihara S, Yokozeki M, Oba Y, Higashibata Y, Nomura S, Moriyama K. Function and Regulation of Osteopontin in Response to Mechanical Stress. J Bone Min. Res. 2006; 21 956-964.
26. Jin L, Lloyd RV. In situ hybridization: methods and applications. J Clin Lab Anal. 11(1):2-9, 1997
27. Pavlin D, Dove SB, Zadro R, Gluhak-Heinrich J. Mechanical loading stimulates differentiation of periodontal osteoblasts in a mouse osteoinduction model: Effect on Type I Collagen and Alkaline Phosphatase genes. Calcif Tissue Int 2000; 67:163-172
28. Gluhak-Heinrich J, Ye L, Bonewald LF, Feng JQ, MacDougall M, Harris SE, Pavlin D. Mechanical loading stimulates dentin matrix protein 1 (DMP1) expression in osteocytes *in vivo*. J Bone Miner Res 2003; 18:807-817.
29. Gluha-Heinrich J, Pavlin D, Yang W, MacDougall M, Harris SE. MEPE expression in osteocytes during orthodontic tooth movement. Arch of Oral Bio 2007; 52:684-690.
30. Kalajzic I, Kalajzic Z, Kaliterna M, Gronowicz G, Clark SH, Lichtler AC, Rowe D. Use of Type I collagen green fluorescent protein transgenes to identify subpopulations of cells at different stages of the osteoblast lineage. J of Bone and Miner Res 2002; 17:15-25.
31. Kalajzic Z, Li H, Wan LP, Jian X, Lamothe K, Adams DJ, Aguila HL, Rowe DW, Kalajzic I. Use of an alpha-smooth muscle actin GFP reporter to identify an osteoprogenitor population. Bone 2008; 43:5101-510.
32. Mitsui N, Suzuki N, Maeno M, Mayahara K, Yanagisawa M, Otsuka K, Shimizu N. Optimal compressive force induces bone formation via increasing bone sialoprotein and prostaglandin E2 production appropriately. Life Scie 2005; 77:3168-3182
33. Hunter GK, Goldberg HA. Nucleation of hydroxyapatite by bone sialoprotein. Proceedings of the National Academy of Sciences of the United States of America 1993; 90:8562-8565.

34. Zhou HY, Takita H, Fujisawa R, Mizuno M, Kuboki Y. Stimulation by bone sialoprotein of calcification in osteoblast-like MC3T3-E1 cells. Calcified Tis Intl 1995; 56:403-407.
35. Domon S, Shimokawa H, Yamaguchi S, Soma K. Temporal and spatial mRNA expression of bone sialoprotein and type I collagen during rodent tooth movement. Eur J of Ortho 2001; 23:339-348.
36. Igarashi M, Kamiya N, Ito K, Takagi M. In situ localization and in vitro expression of osteoblast/osteocyte factor 45 mRNA during bone cell differentiation. Histochem J. 2002 May;34(5):255-63.
37. Bedalov A, et al., Regulation of Col1A1 expression in type I collagen producing tissues: identification of a 49 base pair region which is required for transgene expression in bone of transgenic mice. J Bone Miner Res. 1995 Oct; 10(10): 1443-51.
38. Dacic, S., et al., Col1a1-driven transgenic markers of osteoblast lineage progression. J Bone Miner Res, 2001. 16(7): p. 1228-36.
39. Bilic-Curcic, I., et al., Visualizing levels of osteoblast differentiation by a two-color promoter-GFP strategy: Type I collagen-GFPcyan and osteocalcin-GFPtpz. Genesis, 2005. 43(2): p. 87-98.
40. Bletsa, A., E. Berggreen, and P. Brudvik, Interleukin-1alpha and tumor necrosis factor-alpha expression during the early phases of orthodontic tooth movement in rats. Eur J Oral Sci, 2006. 114(5): p. 423-9.
41. Proffit, W. Contemporary Orthodontics 2007(4th Edition).
42. Kawarizadeh, A., et al., Early responses of periodontal ligament cells to mechanical stimulus *in vivo*. J Dent Res, 2005. 84(10): p. 902-6.
43. Xi Jiang, Zana Kalajzic, Peter Maye, Alen Braut, Justin Bellizzi, Mina Mina and David W. Rowe Histological Analysis of GFP Expression in Murine Bone. Journal of Histochemistry and Cytochemistry Volume 53 (5): 593-602, 2005