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AAO Foundation Final Report Form
(a/o 1/3/2018)

In an attempt to make things a little easier for the reviewer who will read this report, please consider these two questions before this is sent for review:

- *Is this an example of your very best work, in that it provides sufficient explanation and justification, and is something otherwise worthy of publication? (We do publish the Final Report on our website, so this does need to be complete and polished.)*
- *Does this Final Report provide the level of detail, etc. that you would expect, if you were the reviewer?*

Please prepare a report that addresses the following:

Type of Award

Center Award

Name(s) of Principal Investigator(s)

Reginald W. Taylor, D.M.D., D.M.Sc.

Institution

Texas A&M College of Dentistry

Title of Project

Periodontal Ligament Osteoblast Recruitment in Orthodontic Tooth Movement

Period of AAOF Support

07-01-16 to 12-31-18

Amount of Funding

$25,200

Summary/Abstract

Cell lineage tracing enables the tracking of specific cell populations throughout differentiation *in vivo*. 3.2kbCol1α1 promoter is present early in osteoblastogenesis.

Purpose: The purpose of this study was to orthodontically treat temporally inducible cell lineage tracing mice to study osteoblast response within the PDL.
Materials and methods: 3.2Col1α1/Tomato mice were used in this study. Springs were bonded between the maxillary first molar and incisors to mesialize the first molar. 5 groups of 4 were studied: #1: no induction, no orthodontic treatment; #2: induction, no treatment; #3: induction, 1-day treatment; #4: induction, 2-day treatment; #5: induction, 4-day treatment. Radiograph analysis was performed to determine tooth movement. Tomato signal was characterized. H&E and Ki67 immunofluorescent staining were performed.
Results: Tomato-positive cells were identified within the PDL of moving teeth, and their numbers increased with treatment. Ki67 expression was inversely related to length of treatment.
Conclusions: 3.2kbCol1α1-positive cells are present in the periodontal ligament and increase in response to orthodontic tooth movement.

Detailed results and inferences:

1. If the work has been published please attach a pdf of manuscript OR
2. Describe in detail the results of your study. The intent is to share the knowledge you have generated with the AAOF and orthodontic community specifically and other who may benefit from your study. Table, Figures, Statistical Analysis and interpretation of results should be included.

MATERIALS AND METHODS

Animals

5 groups of mice were evaluated: control, induction only, 1-day orthodontic tooth movement (OTM), 2-day OTM, and 4-day OTM. 3.2kbCol1α1CreER$_{T2}$; R26RtdTomato C57/B6 mice were generated and maintained for use in this study. Genotypes of the mice were determined by PCR analysis of genomic DNA extracted from tissue biopsies before postnatal 10 days of age. Pups were weaned at 21 days of age, when they were separated by sex to prevent both pregnancy and aggression. Mice were kept in their weaning groups for the remainder of the study. Mice at 6 weeks of age were used in the study; tamoxifen was administered via intraperitoneal injection on day 39 and mice were sacrificed on day 45. A one-time 100μL dose of 20 mg/mL tamoxifen/corn oil solution was used for induction. Mice were sacrificed through the use of CO_2 asphyxiation. All animal studies were approved by the Institutional Animal Care and Use Committee of Texas A&M University College of Dentistry.

Experimental Treatment

Three time points for orthodontic treatment were tested, in addition to induction-only and null-control groups. Orthodontic treatment time points were 1-day, 2-days, or 4-days. The total time of reporter gene induction in all time points was 6 days; therefore, in the 1-day treatment group springs were mounted 1-day before sacrifice and five days after induction, in the 2-day treatment group springs were mounted 2 days before sacrifice and 4 days after induction, and in the 4 day treatment group springs were mounted 4 days before sacrifice and 2 days after induction. All animals were sacrificed on the seventh day (see table 1). Mice were anesthetized with a ketamine/xylazine cocktail at a dose of 0.1mL/20g, administered via intraperitoneal injection. Mice were situated in a prone position with custom designed retractors allowing for access to the maxillary areas. The mesial surface of the first molar was prepped through the use of a water wipe, followed by a 70% ethanol wipe, followed by forced air drying. The surface was then treated with Transbond™ Plus Self Etching Primer, applied according to manufacturer's instructions. A 3mm piece of spring was bonded to the prepared surface using Transbond™ XT Light Cure Adhesive and cured for a total of 20 seconds to ensure set. Orthodontic springs were obtained to generate 0.03 N, ~ 3 grams (G&H Orthodontics, Franklin, IN). The same procedure

was then repeated on the lingual surfaces of both incisors, resulting in the spring bonded to the incisors as a unit (see figure 4.).

After treatment, mice were kept warm on a surgical warming mat until conscious. Mice were transferred back to home cages, were they were maintained on DietGel® 31M (ClearH2O, Westbrook, Maine).

Tissue Preparation

Immediately following sacrifice, tissues were harvested and immersion fixed in 4% paraformaldehyde at 4°C, overnight, and decalcified in 10% EDTA. After radiological imaging and in anticipation of frozen sectioning, samples were transferred to 30% sucrose solution overnight. Next, samples were embedded in Tissue-Tek® O.C.T. Compound, (Sakura® Finetek), and sectioned with a Leica CM1850 Cryostat, (Leica Biosystems Inc., Buffalo Grove, IL 60089). 10μm sections were obtained at -26°C on charged slides. Samples were stored at -20°C until use.

Radiographs

Radiological ratios were used to quantify tooth movement. Maxilla were removed from samples and soft tissue was dissected away and hemisected. Samples were then arranged onto the flat surface of a Faxitron model MX-20 Specimen Radiography System (Faxitron X-Ray Corp., Lincolnshire, IL, USA). Analysis of radiographs was performed using ImageJ software. The angle of the first molar was measured, using the buccal cusp, the apex of the mesial root and the apex of the distal root to form the angle. This measurement of tooth movement indicates change from the control angle as a signal in tooth movement, as we know that bodily movement is not possible with this method of force application. This form of measurement also reduces error which is produced as a result of angle irregularities during image capture.

Histology

Samples were stained with hematoxylin and eosin, and alcian blue with nuclear fast red counterstain, separately. Frozen sections stored at -20C were brought to rest at room temperature for 20 minutes before beginning the staining protocols, Samples were mounted with Permount™ mounting medium (Electron Microscopy Sciences, Hatfield, PA) and stored in the dark at room temperature.

Immunofluorescence

Immunostaining was performed as previously described[1], using 5% BSA in PBS as blocking solution and 1% BSA in PBS for antibody dilution. Samples were stained with rabbit anti-collagen I antibody (Abcam; 1:100), rabbit anti-DMP1 antibody (provided by Dr. Chunlin Qin at Texas A&M University, 1:400), rabbit anti-Cathepsin K (Abcam, 1:100) and Ki67(Invitrogen, 1:100). Corresponding AlexaFlour-488 secondary antibodies (Thermofisher, 1:1000) were used to enable fluorescent detection.

Microscopy

Scanning light microscopy was utilized to image histological samples. Confocal microscopy was used to capture fluorescent images with a SP5 Leica microscope. Red color was detected at 581nm, green at 488nm, and blue at 405nm (UV). Images were taken using the stack function at the frequency of 100Hz with a resolution of 1024x1024.

Sample Analysis

All quantitative analyses of images, including radiographs, was conducted using ImageJ software[2]. To calibrate the scale of radiological images, an image was taken of a 6mm OrthoEasy® pin (Pforzheim,Germany) shooting from the apex of the pin. This end-on

radiograph allowed for the widest part of the pin to be imaged accurately. Angles of the first molar were measured on radiographs using the mesio-buccal crown cusp point, the mesio-superior point of the mesial rootof the first molar, and the mesio-superior point of the mesial root of the first molar The apex of the angle is formed at the mesio-superior point of the mesial root of the first molar (see figure 7). This angle was measure on 4 samples each from control, tamoxifen, 1-day, 2-day, and 4-day groups. Width of the periodontal ligament (PDL) space was measured by obtaining measurements from histological slides. Images of the mesial root of the first molar were oriented in a way the expected quadrants from tipping forces (see figure 2.) could be superimposed over the root, with the y-axis parallel and through the dental pulp and the x-axis bisecting the root from furcation to apex. 6 measurements of width, alveolar-PDL junction to PDL-cementum junction were obtained in each quadrant. 4 samples each from control, tamoxifen, 1-day, 2-day, and 4-day groups were measured.

Statistical Evaluation

Student's t-tests were used to compare means of angles. Mean
and standard deviation were used to characterize mean width data.
Because distances from histologically processed samples should only be
considered semi-quantitative, statistical test for differences was not performed.

References

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CHAPTER IV

RESULTS
Radiological Analysis

Qualitative analysis indicates that there is no perceptible tooth movement or difference between control or tamoxifen samples (see figure6). There is space observed in the 1-day, 2-day, and 4-day orthodontic molar results in areas of radiolucency on the crown on the first molar on some samples. Comparing the orthodontically treated samples to the control samples, there is space between the second molar mesial surface and the first molar distal surface that is not observed in the controls. It is not apparent between orthodontic time points if the distance of orthodontic movement is significantly different. Quantitative analysis results are displayed in figures 8-9. Using student's t-test to compare the means of control and tamoxifen first molar angles, the resulting p-value of 0.402 indicates that there is no significant difference between these two groups. A one sample t-test was utilized to compare the orthodontic treatment group means to the tamoxifen control value of 45.62 (mean in degrees) individually. Each p value was <0.05.

Histological Evaluation

Qualitative analysis of H&E stained samples showed: 1) periodontal ligament fiber reorganization when comparing orthodontic treated samples to the control, 2) increased secretion of acidic matrix with increased length of orthodontic treatment and more matrix when compared to the control, 3) presence of osteoclasts with large resorption bays in the 4-day treatment group (see figures 10, 11). Quantitative results of mean periodontal space width in four quadrants are illustrated in figure 12. No statistical analysis was performed. In the pressure root area, there was a sharp decrease in the mean width at 1-day of treatment, with an increase in width nearing control at 4 days of treatment. In the tension root area, there was little overall change in the mean

PDL mean width between control and any treatment groups. In the pressure crown area, the 4 day group showed a noticeable decrease in mean PDL width. Finally, the tension crown quadrant mean PDL width steadily decreased as treatment time increased.

Cell Lineage Tracing

3.2Col1α1 Signal

3.2Col1α1-positive cells were detected in the dental pulp, cementoblasts, Tomes' fibers, odontoblasts, and osteoblasts (see figure 13). Qualitative analysis of the difference in signal between the control and orthodontically treated samples shows a noticeable increase in the number of labeled cells. Very noticeable is the increased labeling in the odontoblast processes in response to orthodontic movement (figures 14, 15). The number of labeled cells in the PDL space is markedly increased in all orthodontically treated samples when compared to the control, but it is not apparent if there is a difference between orthodontically treated samples in number of positive cells.

Patterning

3.2Col1α1-positive labeled cells are not evenly distributed throughout the PDL space in orthodontically treated samples. Overall, there is a greater concentration of cells in the mesial PDL space when compared to the distal PDL. There is an increase in positive cells in the PDL in the crown pressure area of orthodontic treated samples when compared to the control. Additionally, the 4-day sample exhibited a striking labeling of the odontoblast processes that clearly extends from the odontoblasts lining the dental pulp space to the dento-cemental junction. When comparing the root sections of samples (see figure 16), there was increased presence of positive cells in the cellular cementum when comparing the control to 1-day. In the 1-day sample, there were labeled cells present lining the cementum that was not observed in the control. Comparing the 1 to 2-day sample, there was a drastic increase in labeling in the day 2 sample. Between the day 2 and day 4 samples, the labeling increased further. Positively labeled cells were present in the cellular cementum, the dento-cemental junction, the PDL space, the PDL-alveolar junction, alveolar bone, and the bone marrow spaces, in addition to the aforementioned dental pulp and odontoblasts. The shape of the PDL space in the 4 day sample is similar to the shape of the PDL in the control, but the width of the PDL is different: wider on the tension side and thinner on the pressure side. Examining the crown tension PDL space of samples do not show a large difference in the labeling between the control, 1-day, 2-day, or 4-day groups (figure 15).

Immunoflourescence

Ki67

(See figures 17-19). Comparison of 1-day, 2-day, and 4-day samples show overall decreased expression with increased length of orthodontic treatment. This decrease in Ki67 was observed in the PDL space, the alveolar bone, and the bone marrow. Comparing quadrants of the same sample showed a difference in expression in the PDL depending on the area. Areas expected to experience tension exhibited more Ki67 activity, while pressure areas exhibited markedly less.

CathepsinK

(See figure 20). CathepsinK expression strongly decreased in response to length of orthodontic treatment. Expression was detection in the alveolar bone, the dental pulp, and the PDL space. While osteoclast activity was determined to be the strongest at day 4 in HE stained sections, CathepsinK activity in the day 4 samples was not as strong as in day 1 samples.

CHAPTER V
DISCUSSION

Tamoxifen

In cre-lox constructs, tamoxifen is commonly referred to as an estrogen receptor (ER) antagonist[1,2,3]. This is incorrect and is fundamental flaw in the understanding of tamoxifen.

Tamoxifen is a selective estrogen receptor modulator (SERM). SERMs are able to act as both ER agonists and antagonists, dependent on tissue type. Of particular importance to this research, SERMs, especially tamoxifen, are known to interfere with normal bone homeostasis[4]. Tamoxifen is commonly administered in conjunction with chemotherapy regimens in humans[5]. In addition, SERMs have been approved for the treatment of postmenopausal women[6,7]. It is from these types of studies that it is well known that SERMs inhibit osteoclast differentiation, and even increasing osteoblast activity. While the mechanism of this class of drugs has been a breakthrough for the treatment of osteoporosis and osteoarthiritis, it is detrimental to orthopedic research depending upon tamoxifen-inducible cre-lox models. In this study, if the physiological response of bone remodeling is inhibited, the premise of the study is invalid. Using imperfect transgenic models may be necessary currently, but researchers should be aware of the risks—rigorous dose-response preliminary studies should be performed to ensure that a phenotype is not produced from tamoxifen induction alone. While this study attempted to control for this mechanism of tamoxifen, using an SERM in orthopedic research at all severely limits the validity and applicability of this research. Doxycycline-inducible models are not a solution, but may be a viable alternative depending on the target cell population. For osteoblast specific targeting, there is an Osx/Sp7 doxycycline inducible model available from Jackson Laboratories (stock number 006361, Bar Harbor, ME).

CathepsinK

CathepsinK results were anomalous in that expression was noted in soft tissue. CathepsinK has previously been reported to be a highly specific osteoclast marker. Upon further research, I found that CathepsinK is present in many craniofacial tissues, including the dental pulp and periodontal ligament[8]. Upon the realization of this finding, it is apparent that CathepsinK is a poor choice for an osteoclast-specific marker in the study of tooth movement. In the future, the use of antitartrate-resistant acid phosphatase (TRAP) antibody would prevent similar results.

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CHAPTER VI
CONCLUSIONS

1. The methods and techniques described in this study produce a valid and replicable murine model of orthodontic tooth movement.

2. 3.2Col1α1 positive cells are present in the periodontal ligament and respond to orthodontic treatment.

3. 3.2Col1α1 positive cells proliferate in response to orthodontic treatment.

4. Dentin responds to orthodontic force, evidenced by increased 3.2Col1α1 labeling in odontoblasts.

FIGURES

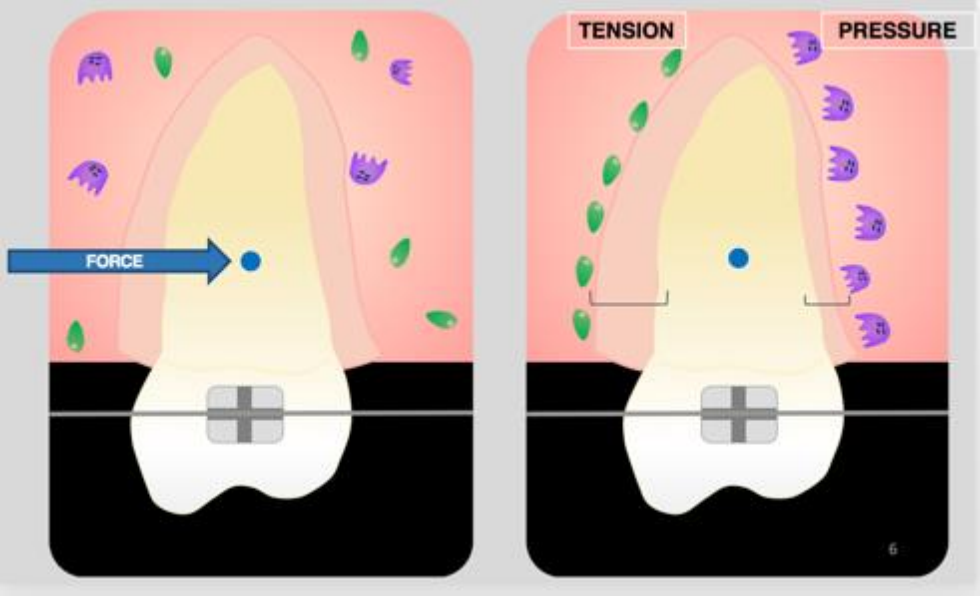

Figure 1. Bodily movement. Blue circle- center of resistance. Green cellsosteoblasts. Purple cells- osteoclasts. Left panel indicates homeostasis; right panel indicates bone remodeling.

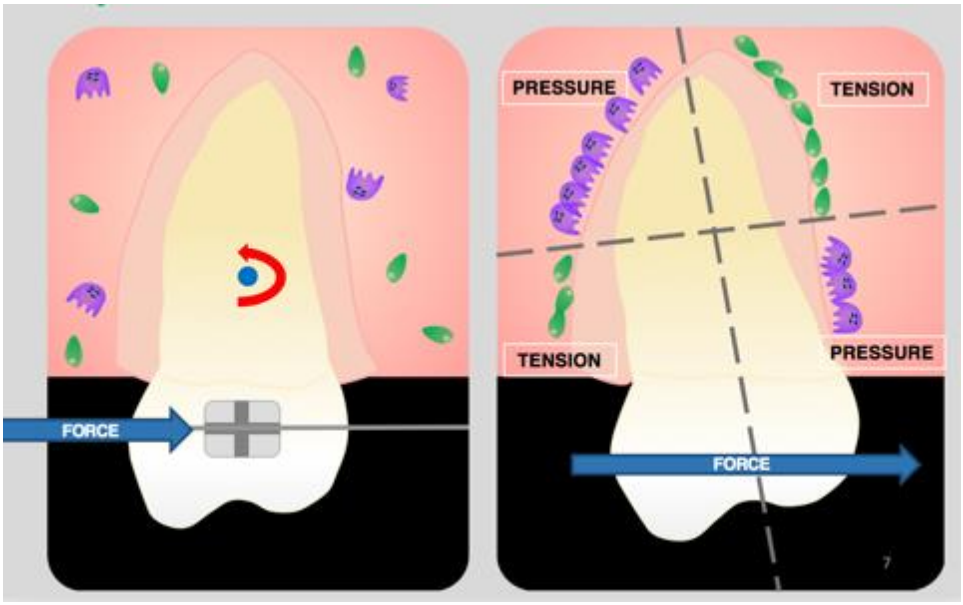

Figure 2. Tipping. Blue circle- center of resistance. Red arrow- torque. Green cellsosteoblasts. Purple cells- osteoclasts. Left panel indicates homeostasis. Right panel indicates bone

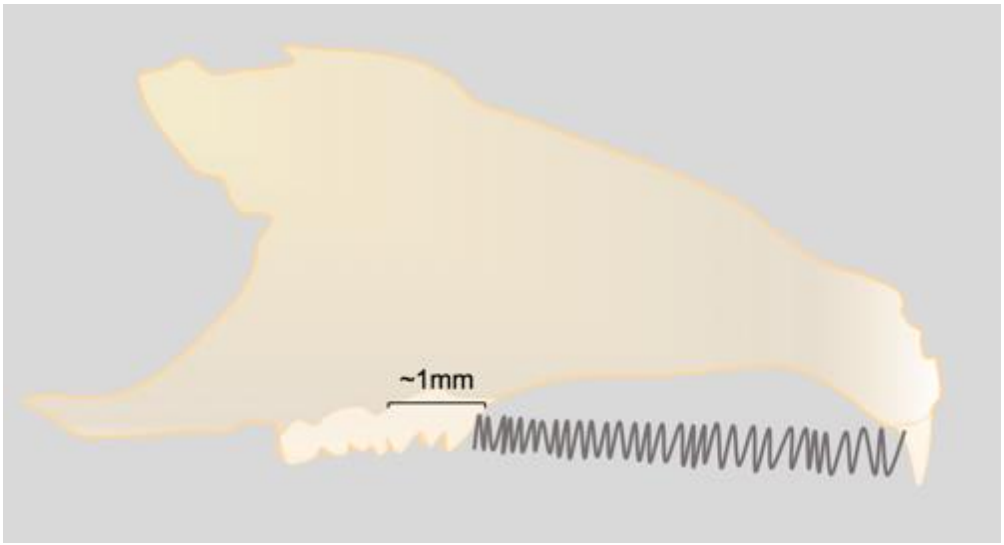

Figure 3. Orthodontic spring design. Springs are bonded to the maxillary first molar and the maxillary inscisors.

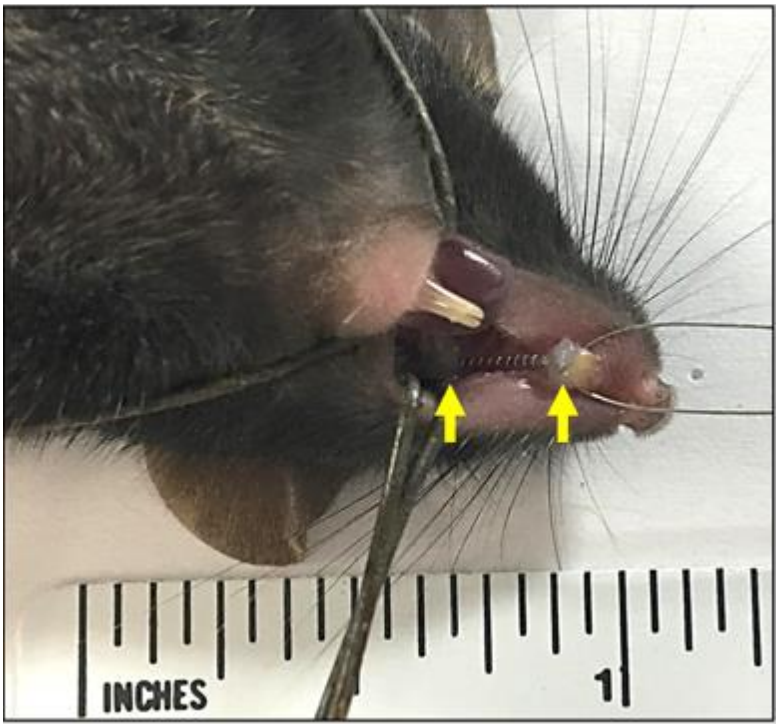

Figure 5. Bonded orthodontic springs. Yellow arrows indicate points of the spring, bonded with orthodontic resin.

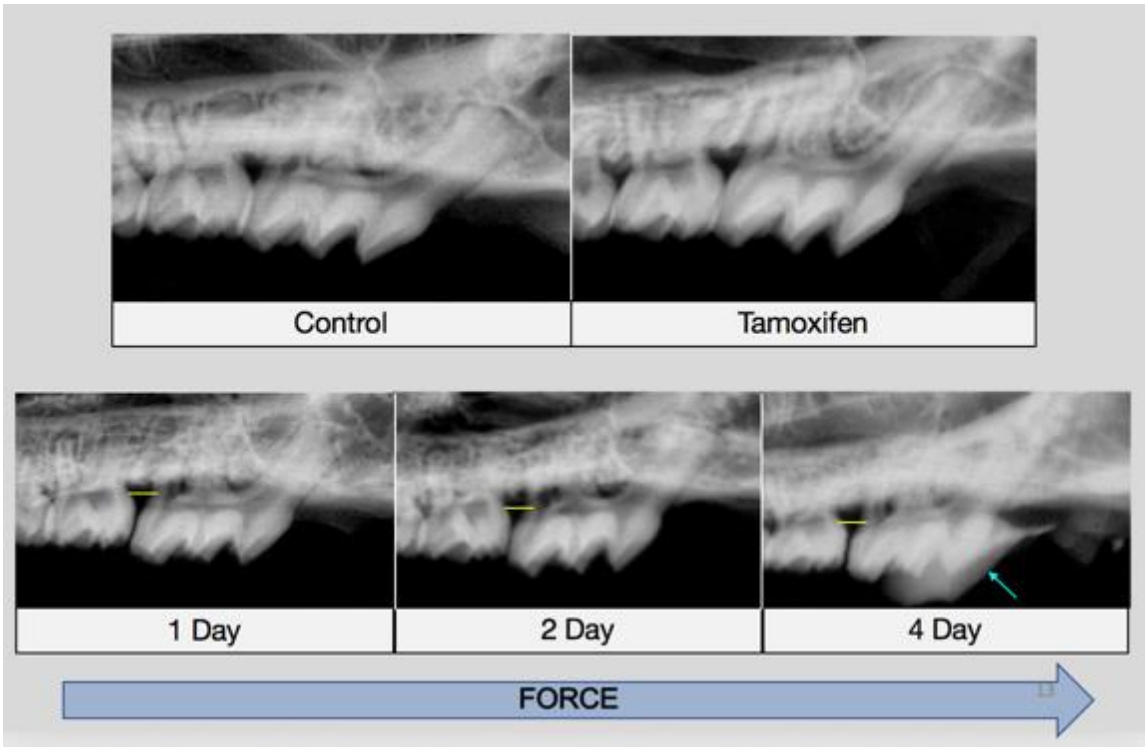

Figure 6. Radiological results. Yellow lines indicate space generated by orthodontic treatment. Blue arrow pointing to artifact of remaining orthodontic resin residue.

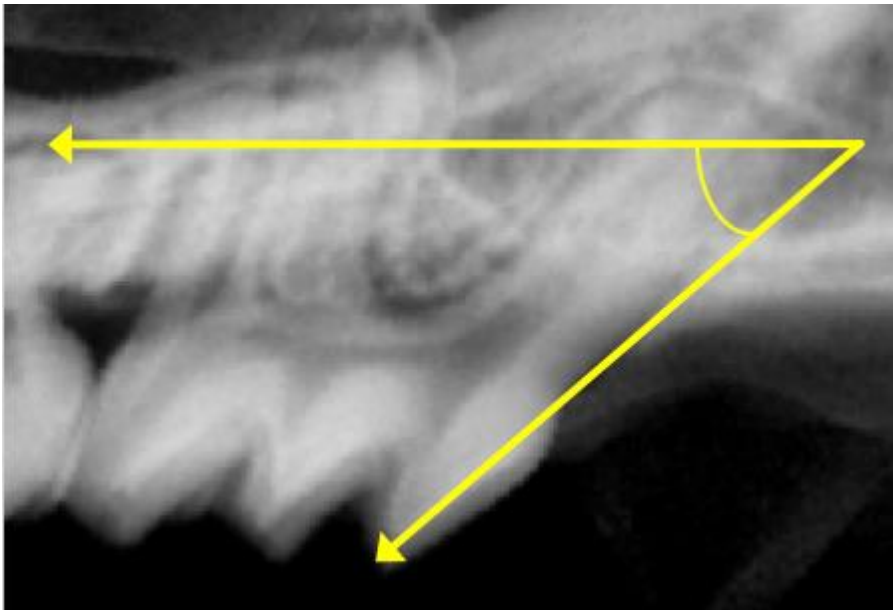

Figure 7. Angle of first molar.

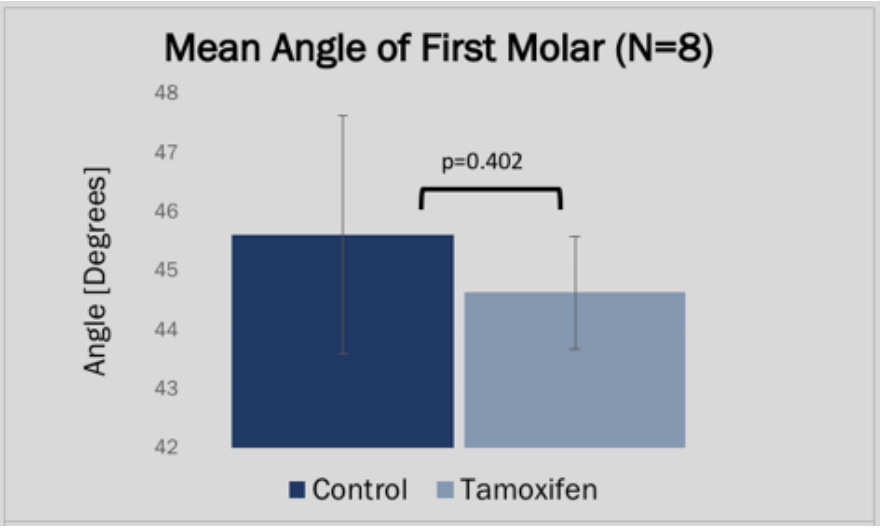

Figure 8. Difference of means of control and tamoxifen angles of first molar.

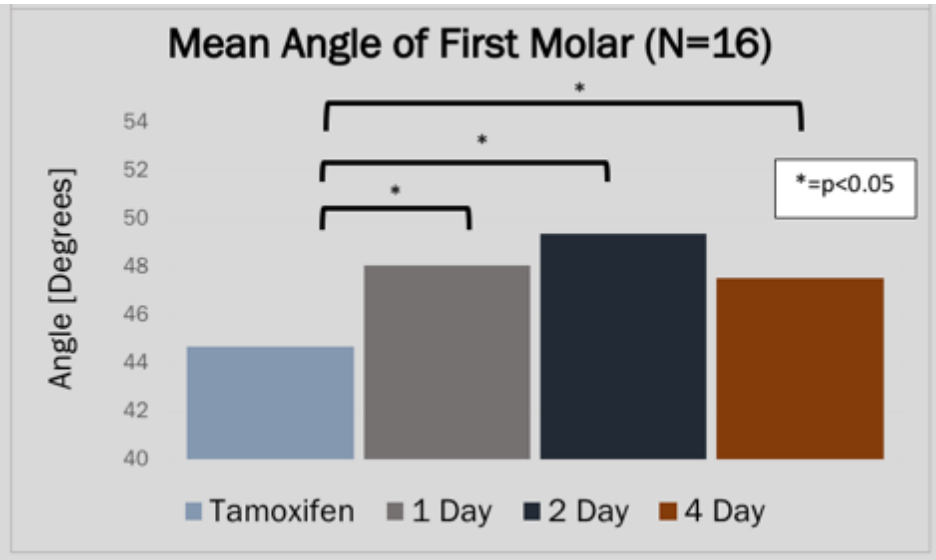

Figure 9. Difference of means comparing each orthodontic time point to tamoxifen (untreated) sample to determine if movement occurred in the orthodontic samples.

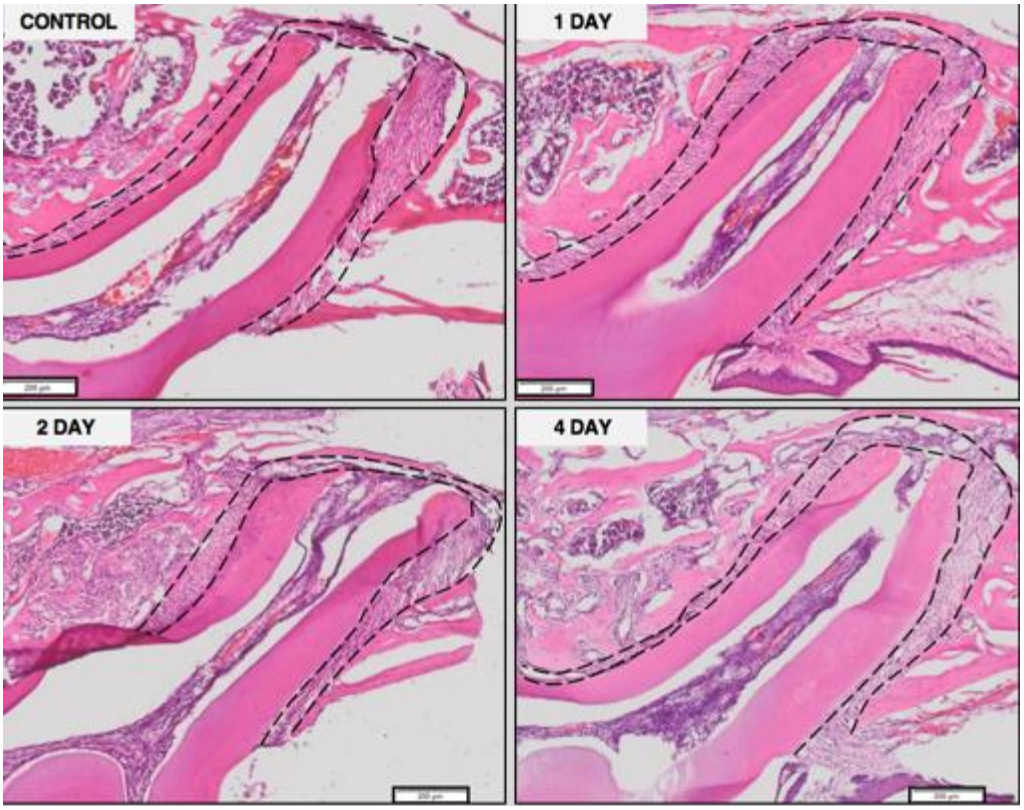

Figure 10. Histological results. HE staining clearly distinguishes between the dental tissues and the tissues of the periodontium. The periodontal ligament space is outlined.

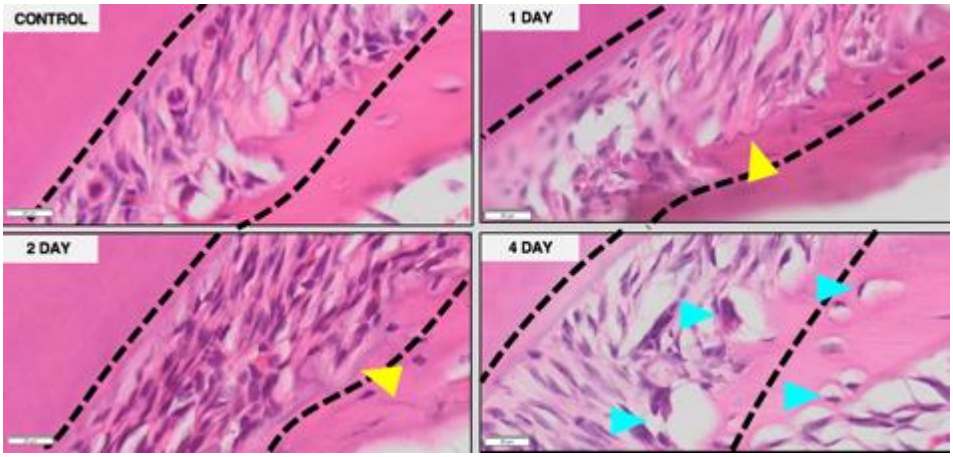

Figure 11. Histological results focusing on the mesial crown area of the alveolar crest, expected to undergo pressure. Yellow arrows indicate areas where increased acidic matrix is present when compared to the control. Blue arrows indicate osteoclast cells with resorption bays.

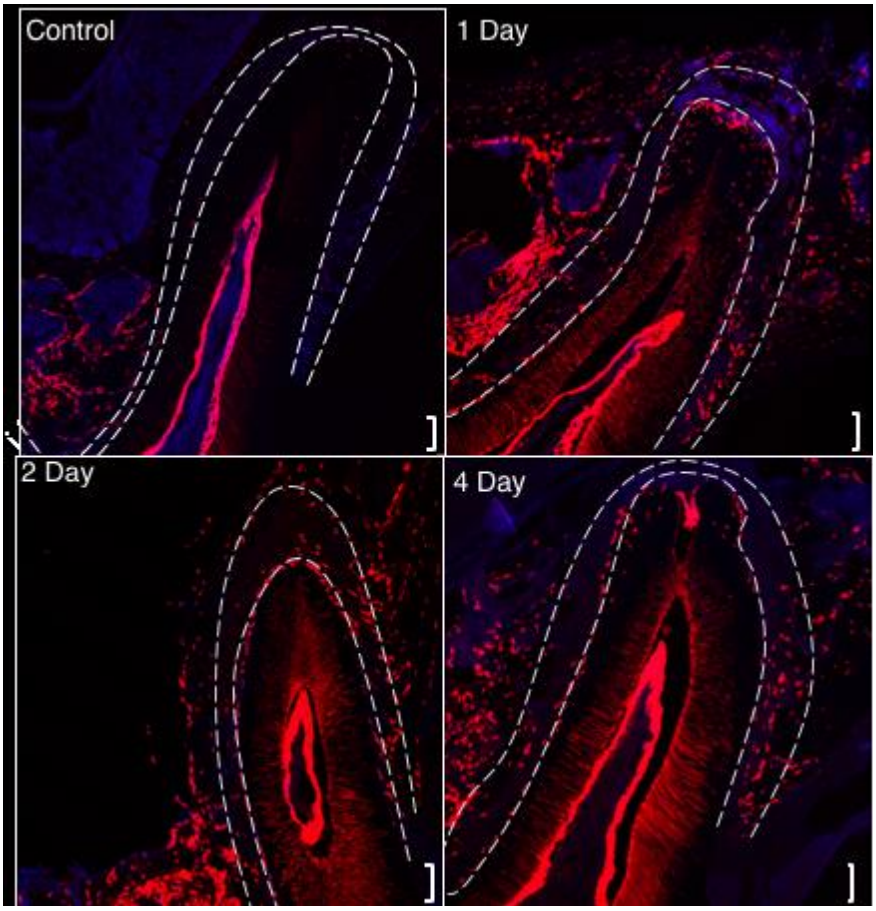

Figure 13. Characterization of the 3.2Col1a1 signal. Periodontal ligament outlined. Red- 3.2Col1a1. Blue- DAPI. Scale- 100um.

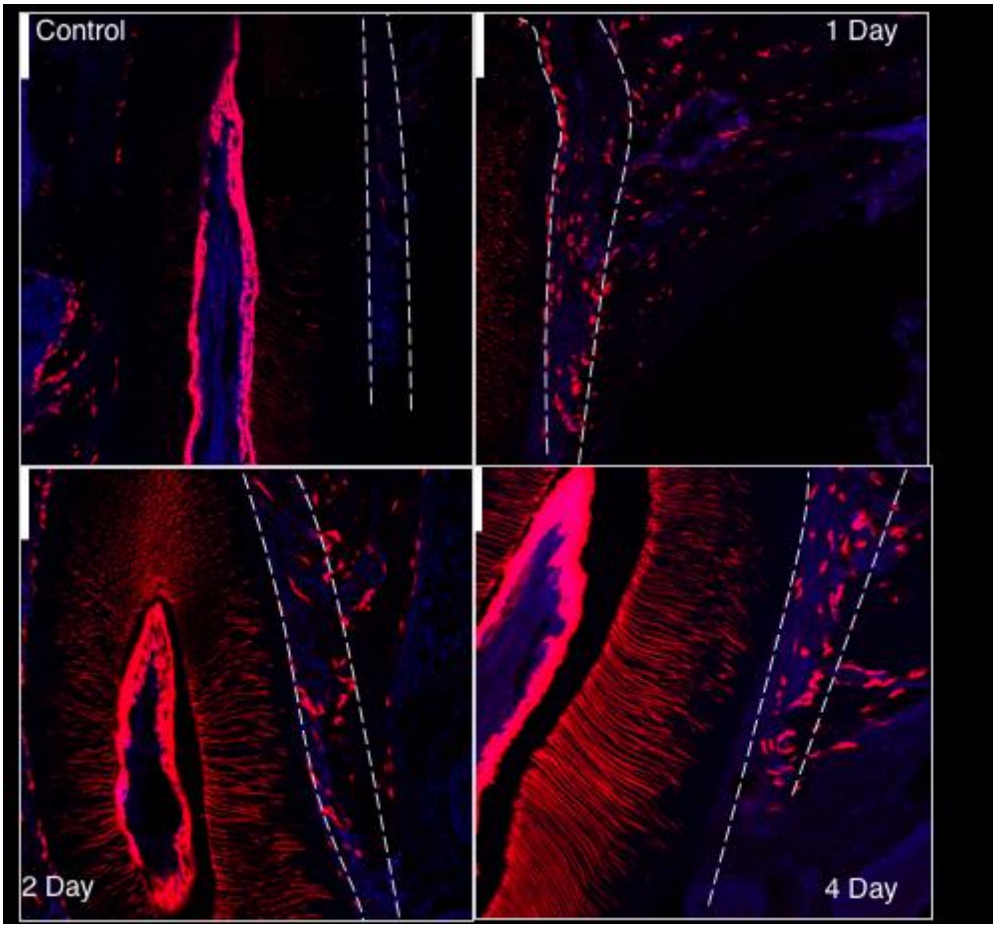

Figure 14. Mesial crown area near the alveolar crest, expected to experience pressure. Periodontal ligament outlined. Red- 3.2Col1a1. Blue- DAPI. Scale- 100um.

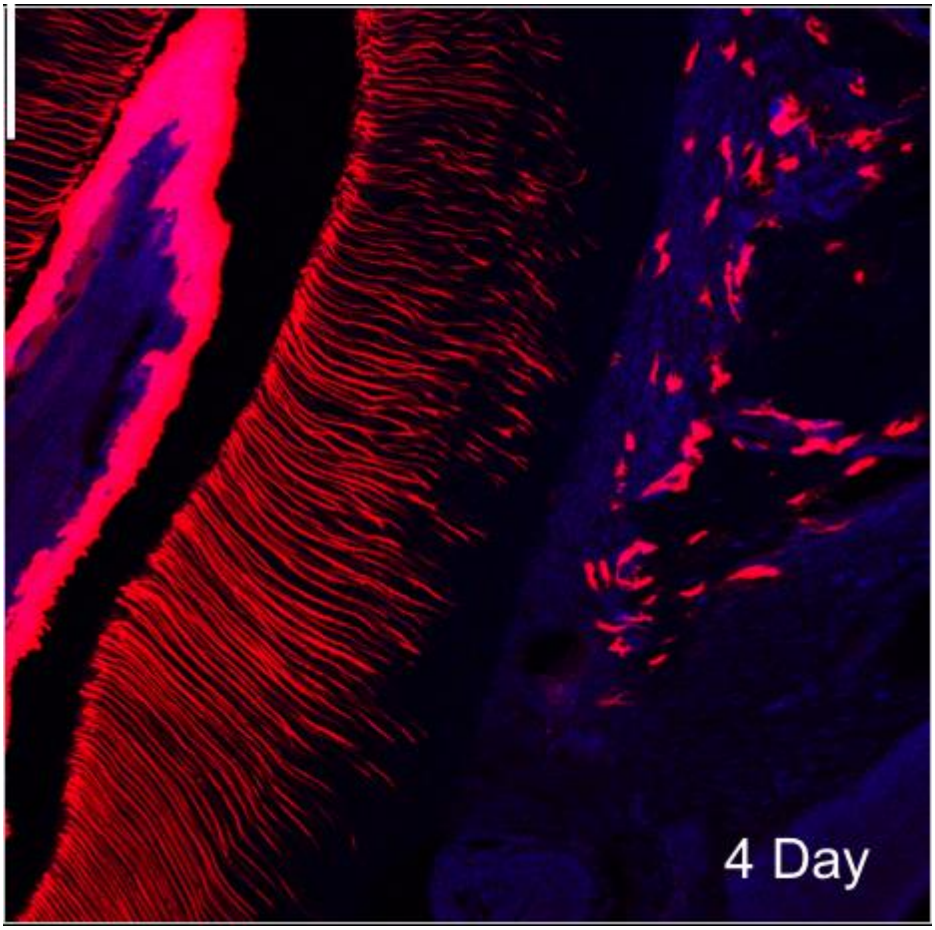

Figure 15. Odontoblast labeling in response to orthodontic tooth movement. Red- 3.2Col1a1. Blue- DAPI. Scale- 100um.

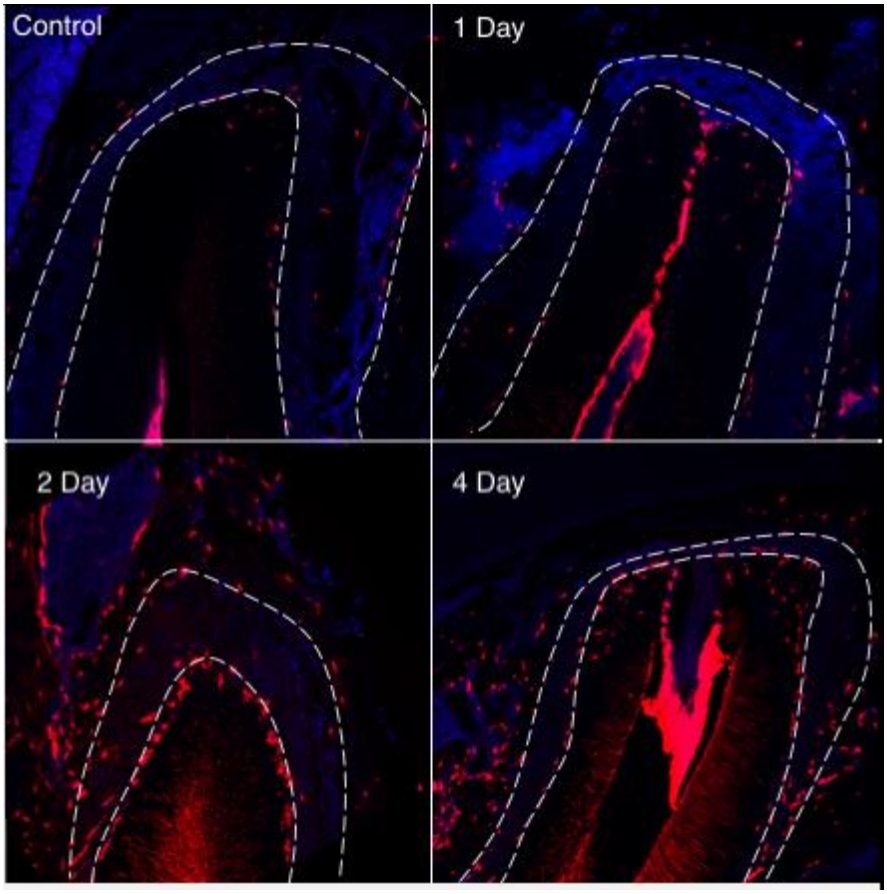

Figure 16. Root apex. Periodontal ligament outlined. Right side of root expected to undergo tension while left side is expected to undergo pressure. Red- 3.2Col1a1. Blue- DAPI.

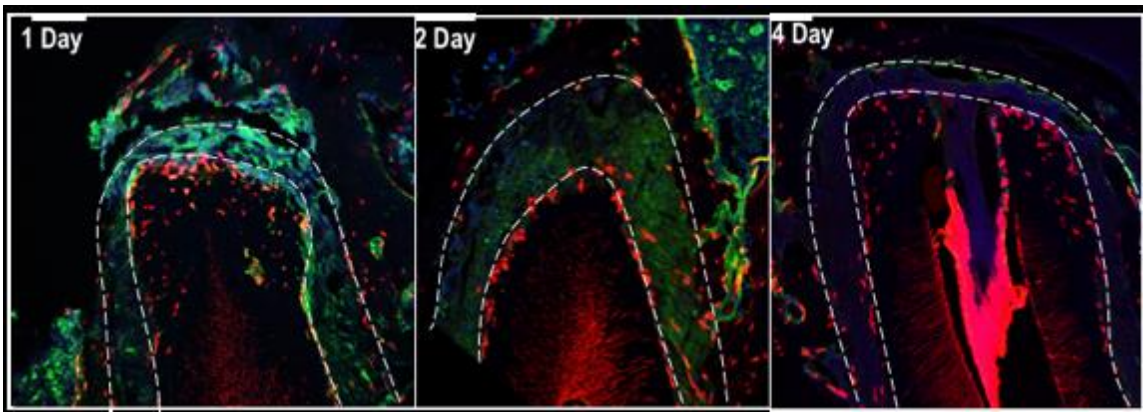

Figure 17. Ki67 immunostaining. Root apex. Right side is expected to undergo tension while left side is expected to undergo pressure. Periodontal ligament outlined. Green- Ki67. Red- 3.2Col1a1. Blue- DAPI. Scale- 100um

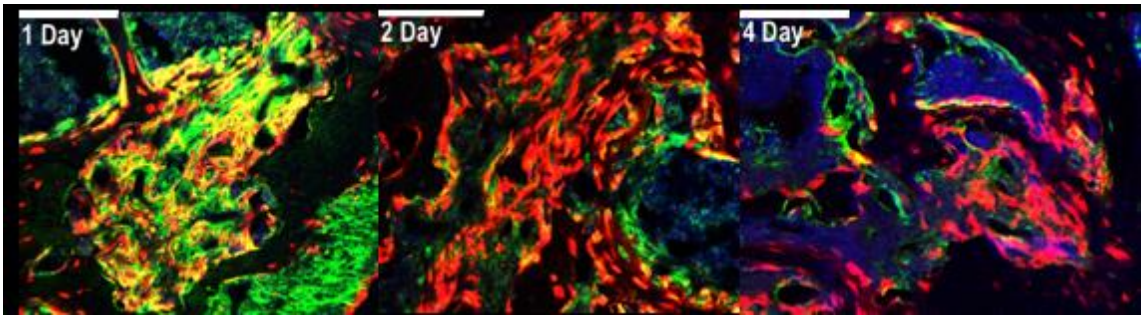

Figure 18. Ki67 immunostaining. Bone marrow. Green- Ki67. Red- 3.2Col1a1. Blue- DAPI. Scale-100um

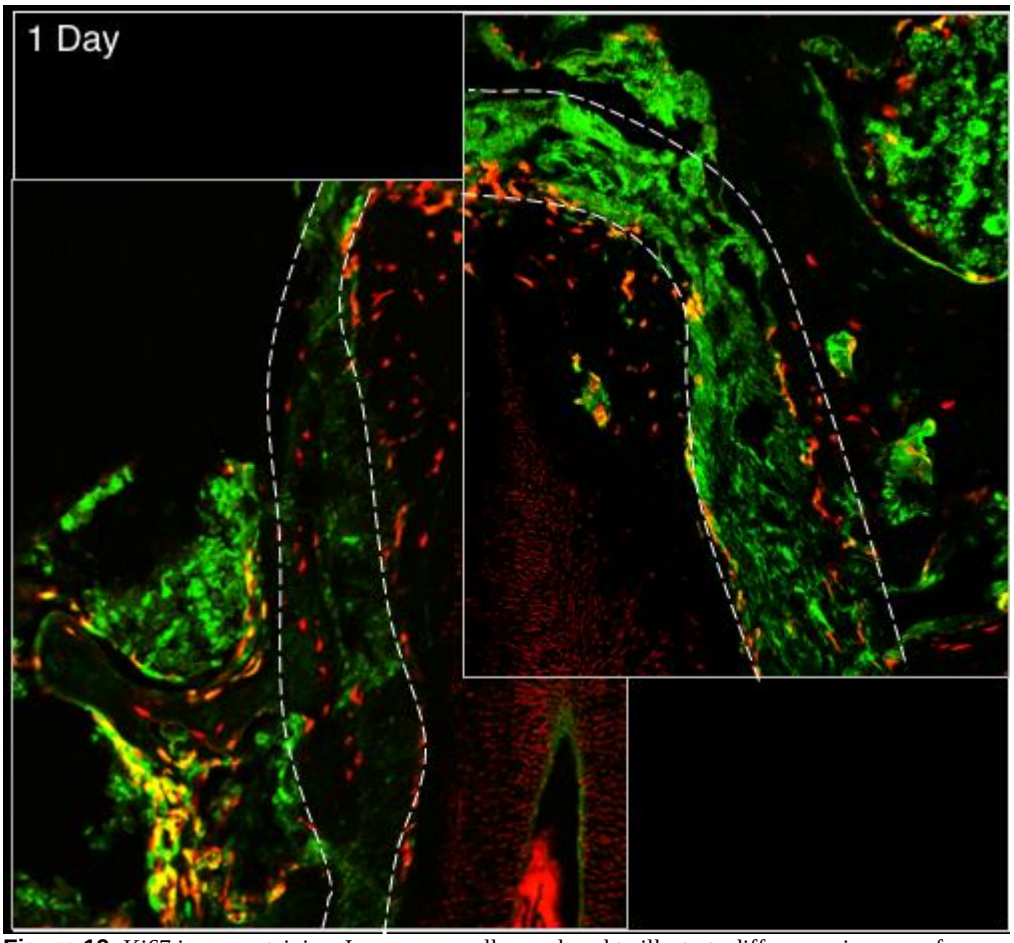

Figure 19. Ki67 immunostaining. Images manually overlayed to illustrate differences in areas of expected pressure (left) and tension (right) in terms of Ki67 expression. Note bone marrow Ki67 intensity adjacent to periodontal ligament on left panel. Green-Ki67. Red-3.2Col1a1.

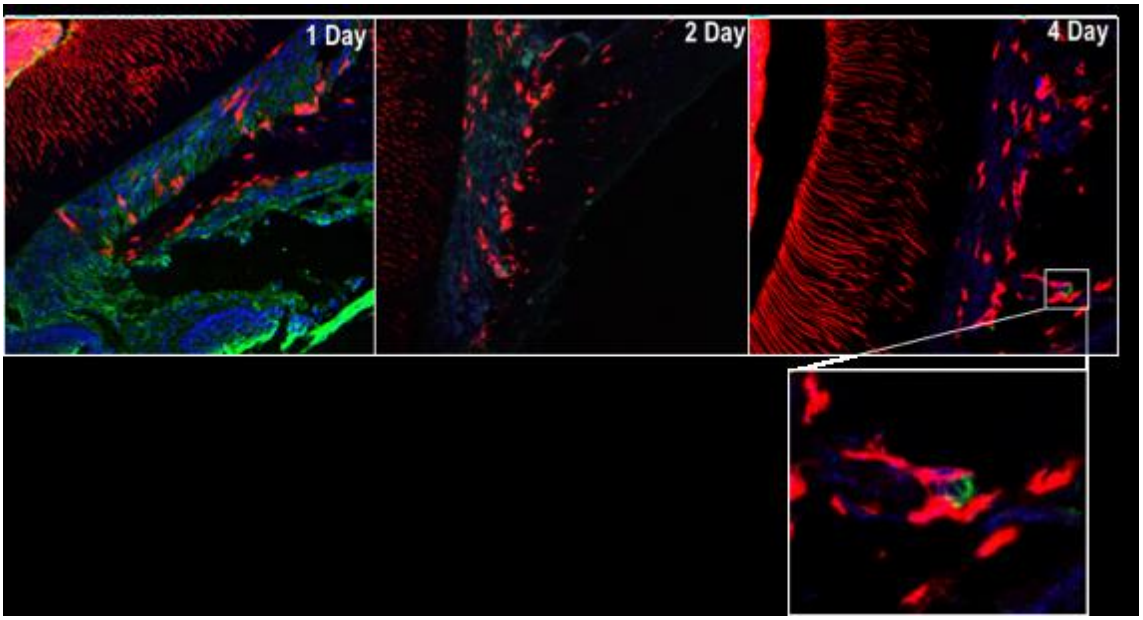

Figure 20. CathepsinK immunostaining. Mesial crown area expected to undergo pressure, near alveolar crest.Note expression in dental pulp, periosteum, and periodontal ligament. Green- CathepsinK. Red- 3.2Col1a1. Blue- DAPI.

APPENDIX B
TABLES

	Day						
Treatment	1	2	3	4	5	6	7
None							
Tamoxifen							
Orthodontics 4 Day			Ortho				
Orthodontics 2 Day					Ortho		
Orthodontics 1 Day						Ortho	

Table 1. Timeline design. Red- tamoxifen induction. Grey- 4-day treatment group. Navy- 2-day treatment group. Orange- 1-day treatment group. Black- sacrifice.

Response to the following questions:

1. Were the original, specific aims of the proposal realized?

   The specific aims of this project were as follows:

   Specific Aim 1

   Mice with the tamoxifen inducible 3.2col1a1-Cre construct will be crossed with mice containing Rosa26dtTomato. The resulting 3.2col1a1Cre/Rosa26tdTomato transgenic mice will then be subjected to orthodontic tooth movement. When treated with tamoxifen, the Cre will excise the Stop cassette in the Rosa26tdTomato construct, and allow expression of the dtTomato fluorescent protein.

   Specific Aim 2

   4-6 week old mice will be divided into 2 groups and will have nickel/titanium springs that generate either .03N or .3N of force attached to their left maxillary incisor and maxillary molar with a light cured composite. The right side will serve as the control.

   Specific aim #1 was realized. We were able to generate and utilize 3.2col1a1Cre/ Rosa26tdTomato transgenic mice and subject them to orthodontic tooth movement.

   Specific aim #2 was partially realized. We were able to test the .03N nickel/titanium springs; however, time constraints did not allow us to test the animals' reaction to orthodontic tooth movement with the 0.3N nickel/titanium springs.

2. Were the results published? **The results have not yet been published.**

   a. If so, cite reference/s for publication/s including titles, dates, author or co-authors, journal, issue and page numbers. **N/A**

   b. Was AAOF support acknowledged? **N/A**

   c. If not, are there plans to publish? **There are plans to publish the results.**

3. Have the results of this proposal been presented? **No.**

   a. If so, list titles, author or co-authors of these presentation/s, year and locations. **N/A**

   b. Was AAOF support acknowledged? **N/A**

   c. If not, are there plans to do so? **The results will be presented orally at the 2019 AAO annual meeting in Washington, D.C.**

4. To what extent have you used, or how do you intend to use, AAOF funding to further your career?

The funds from this Center Award allowed me to "jump-start" a line of research that was falling dormant. It also allowed me to use an investigative tool, transgenic mice, which would not have been available to me. Awards such as this allow investigators to leverage the awarded funds to enter into collaborations with well-established labs.

Because of the limits on my research time and facilities, I will be able to use funding from a source such as the AAOF to develop fruitful collaborations. These collaborations will allow me to increase and improve my scholarly activities, which his essential for career advancement.