CD44-/- Animals Display Decreased Orthodontic Tooth Movement Using a Murine Model

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ABSTRACT

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Future improvements in orthodontic treatment require a comprehensive understanding of the reaction of paradental structures to an applied force, at the cellular and molecular level. Recent advances in molecular biology techniques have established transgenic and knock-out mice as robust tools in studying various physiological and pathological processes. To successfully utilize this tool in orthodontic research, a good model for orthodontic tooth movement (OTM) in mice is needed. The aim of this study was to characterize a model of OTM that can be used in effectively and efficiently in mice. As opposed to prior models, which apply mesializing forces to molars using NiTi coil springs, we fabricated orthodontic springs from 0.010" stainless steel wires designed to move maxillary molars in the palatal direction. The use of radiographs to measure

tooth movement in our method, allows for multiple and frequent measurement to be made from individual animals over the entire experimental period, with good reliability and accuracy. We tested this method in mice lacking the CD44 gene and compared them to control mice. CD44 is a cell surface receptor present on osteoclasts and believed to be important in cell adhesion, migration and function. Our data indicate that CD44-/- animals have reduced overall tooth movement when compared to controls and also have a larger lag phase before tooth movement is observed. These data support existing evidence that CD44 may be important in osteoclast function and bone resorption.

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INTRODUCTION

Biology of orthodontic tooth movement (OTM)

Orthodontists need to understand the physical and biological responses of the periodontal ligament (PDL) to applied force in order to obtain desired treatment outcomes. Our initial knowledge of tissue reactions to orthodontic forces came from landmark histological studies by Sandstedt, which identified areas of bone resorption and bone apposition on the "compression" and "tension" sides of the PDL respectively (1,2). On the compression side, the PDL undergoes necrotic changes, depending on the level of force applied, followed by an inflammatory response to clear the necrotic tissue and finally alveolar bone resorption. On the tension side, the PDL space is enlarged, the principle fibers appear stretched, and new bone formation follows (3-6).

Later, studies began to focus on the role of individual cells in orthodontic tooth movement (OTM). Under tensional stress, the PDL experiences anabolic modeling, which is characterized by addition of sub-periosteal osseous tissue to the existing bone surface. This is mediated by resident osteoblasts and committed pre-osteoblasts as well as newly differentiated cells from the pluripotent stromal cells resident in the adjacent marrow and blood derived multipotent stem cells. Bone formation begins 40 to 48 hours after force application with the rate of formation and the kind of bone produced depending on the levels of applied force (7). Bone resorption on the compression side is carried out by highly specialized, giant multinucleated cells called osteoclasts. These

cells differentiate from blood derived monocytic precursors and are capable of clearing both the organic and inorganic components of bone. Studies have also differentiated between frontal resorption associated with light force application, and undermining resorption involving necrotizing forces causing crushing injury (hyalinization) of PDL tissues (8-10).

Studies designed to identify and characterize the roles of specific molecules involved in OTM have advanced our knowledge of these processes. We now know that various cytokines, growth factors, cell surface receptors as well as structural extracellular matrix (ECM) proteins are involved in differentiation and functioning of osteoblasts and osteoclasts (3,7,11,12). We recognize that osteoblasts, are central players in the resorptive process, secreting many of the key cytokines such as macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor B-ligand (RANK-L) that mediate monocytic recruitment and differentiation into osteoclasts (13). Studies have also detailed intracellular signaling cascades and specific cellular responses to individual molecules thought to be important in OTM.

Current concepts propose that mechanical loading produces strains in the tissues and the subsequent cellular responses are aimed at adaptation to this strain. Hence, both the PDL and bone undergo remodeling processes to adapt to orthodontic forces (3). These responses are not limited to osteoblasts and osteoclasts, but extend to endothelial cells, osteocytes as well as gingival and PDL fibroblasts. Many studies have identified and implicated specific proteins that mediate cellular responses in OTM. Figure 1 shows a

snapshot of just some of the various molecules that have been implicated in tooth movement. One shortcoming of most of these studies is that they simply demonstrate changes in expression profiles either at mRNA level or the protein level. While this information is important, they do not unequivocally ascertain a functional role of these proteins during OTM. The next step in our understanding of OTM would require establishing functional roles for these molecules.

Current models for studying tooth movement

Most of the information that we have about tooth movement has been obtained from the use of animal models. OTM studies in large animals (e.g., dogs, cats and monkeys) answered key questions about mechanical parameters of force that govern tooth movement and root resorption. These parameters include force levels, duration of force, use of continuous v/s intermittent forces, etc. Milestone histomorphological studies that describe the response of paradental tissues to tooth movement were also made in dogs and primates using light and electron microscopy (14-16).

However, the use of rodents, specifically rats, greatly enhanced our understanding of the biology of OTM, especially due to the availability of antibodies and molecular reagents designed specifically for use in rodents and rodent tissues. A systematic review on available literature on experimental tooth movement in animals revealed that out of 320 studies, the majority (roughly 55%) were performed in rats (17). Dogs were the next most frequently used model followed by primates and cats.

Ren *et al.* (2004) reviewed the different methods employed in animal studies to produce tooth movement (17). Around 25% of these studies used the "Waldo's method", in which an elastic band is placed between the first and second maxillary molars to push these teeth apart (18). This method has also been applied to mice (19,20). A criticism of this method is the lack of data on the types of elastics used and the force levels as well as the decay curves obtained. The method also does not lend itself to the accurate measurement of tooth movement.

Most studies, however, use a spring (NiTi coiled spring) that typically extends from the first molars to the incisors. These exert a mesially directed force on the molar, pulling it away from the second molar. Tooth movement is then assessed by measuring the distance from the cemento-enamel junction (CEJ) on the distal surface of the first molar to the CEJ on the mesial surface of the second molar using histological sections made after sacrificing the animals. Studies have also used radiographs, micro-CT, as well as impressions of teeth to measure movement using this method. The advantages of this method lie in the ability to measure the amount of force applied to the teeth using tension gauges before the spring is bonded to the incisors (21). Additionally, NiTi springs are known to deliver fairly uniform forces over a considerable range (22-24). Despite these advantages, there are some drawbacks to this method of measuring tooth movement. Firstly, rodent molars are known to exhibit physiologic distal migration, which may result in the overestimation of OTM. On the other hand, mesial movement of the first molar may cause the second molar to move mesially as well, due to the action of trans-septal

fibers. This may result in underestimation of OTM. Additionally, many studies use animals that are between 5-8 weeks old and these animals may experience physiologic growth of the snout. Also, mouse incisors are continuously erupting, at a rate of ~160 μm/ day (25,26), which can dramatically alter the force levels and vectors experienced by the molars. All these factors can confound the assessment of tooth movement and are difficult to measure and control. Also, since the springs are typically bonded to the occlusal surface of the maxillary first molar, many researchers extract the opposing (mandibular) first molar to prevent occlusal contacts from debonding the appliance. This is not only stressful for the animals but also allows for an occlusal vector of tooth movement. Since the contralateral side often serves as a control, the maxillary molars on this side may erupt more than the experimental side and make interpretation of results difficult. Instead for CEJ measurements as described above, King *et al.* (1991) presented a modified method of placing subperiosteal implants (broach/amalgam) to serve as stable landmarks and used radiographs for assessing tooth movement (27). They were hence able to measure tooth movement on individual animals every 48 hours without necessitating sacrifice and also accounted for distal drift of molars in their study.

Another OTM model uses a unilateral cantilevered 0.012" NiTi wire that is looped around the maxillary incisors and has an arm extending distally to the first molar. This arm lays palatal to the right first molar when passive. Once bonded, the wire is activated by flipping the arm to the buccal surface of the molar, applying a palatally directed force on the first molar. Hence, the tooth movement produced would reduce the intermolar distance, which can be measured using calipers, radiographs or histological

sections at desired time points. The mandibular incisors are usually trimmed to avoid loss of appliance. Since this method does not rely on mesial movement, it eliminates some of the confounding factors described above and does not necessitate the extraction of mandibular molars. However, growth at the midpalatal suture would tend to confound intermolar OTM measurements.

Need for a mouse model of tooth movement

Mice have been used in biomedical research for over three centuries. There are several advantages to using mice over other animals for research. Mice are probably the easiest animals to maintain in a laboratory setting. They have a high reproductive rate in a short period of time and an accelerated lifespan keeping the costs, space, and time required to perform research manageable. Hence, they serve as a cost-effective and efficient research tool. Since they have been so utilized, there are usually precedents to follow in their use and technicians are generally skilled in taking care of them.

Moreover, the main reason that mice are the most widely used animals in biomedical research (29) is due to our ability to experimentally manipulate their genome. Genes can be injected directly into the fertilized egg generating a transgenic animal, enabling the manipulation of specific genes believed to be important in pathophysiology. Subsequently, scientists developed techniques that permitted the removal of genes of interest, generating “knock-out” mice. Moreover, genes can be replaced by other mutant genes using homologous recombination or targeting, thereby

allowing the study of specific mutations in disease models. New technologies allow scientists to not only knock out genes of interest, but to do so in a specific tissue at a specific time during development, refining their ability to address fundamental biological questions.

Since over 95% of the mouse genome is similar to humans, mouse genetic research is particularly applicable to studying human pathophysiology . Moreover, naturally occurring, spontaneous mutations that cause genetic diseases in humans also often cause similar afflictions in mice. Mice also serve as a good tool to study complex diseases/traits, which involve the interaction of multiple genes. This is because highly inbred strains are available, that control for the effects of genetic background which can confound interpretation of data while studying these traits. Hence, their genetic standardization helps assure experimental reproducibility.

While genetic manipulation is possible in other rodents such as rats, the use of mice over the past 50 years has generated an extensive body of data along with thousands of distinctive strains. The Knockout Mouse Project (KOMP) and the International Knockout Mouse Consortium (IKMC) have set an ambitious goal of knocking out all of the genes in the mouse genome and are making progress in achieving this goal. Currently, over 6000 unique mouse models are available for study, with approximately 600 new strains being added each year. Hence, mice can serve as an invaluable tool we have at our disposal, to test the functional relevance of specific genes/proteins in OTM.

CD44

CD44 is a transmembrane glycoprotein that is part of a large family of cell adhesion molecules. Together with selectins, integrins and cadherin, the CD44 family of protiens mediate contact between cells and the extracellular matrix. Members of this family are encoded by one single, highly conserved gene present on chromosome 11 in humans and chromosome 2 in mice and are expressed in a wide variety of cells (30). All alternatively spliced CD44 isoforms contain a large constant ectodomain, a variable transmembrane domain and a cytoplasmic domain (31). The ectodomain, excoded by the first five (invariable) exons of the CD44 gene, contains motifs that function as docking sites for several extracellular matrix (ECM) molecules. Interactions of CD44 with these ECM molecules such as hyaluronan, collagen, laminin and fibronectin seem to promote matrix-dependent migration.

Studies using specific antibodies directed against CD44 molecules that interfere with their binding capacity have provided evidence of functional roles of CD44 in organ development, neuronal axon guidance, numerous immune functions and hematopoiesis. Recent studies have used mice with germline deletions of different portions of the CD44 gene and implicate CD44 in various physiological and pathological processes including tumor progression and metastasis (32). However, CD44-null mice are viable and have relatively mild phenotypes with abnormalities in myeloid-progenitor migration, bone-marrow colonization and in the homing (or migration) of lymphocytes (33).

Studies have demonstrated the expression of the standard or hematopoietic CD44 isoform (sCD44) in human (34) and rat (35) osteoclasts. Immunostaining of mouse distal femur showed that CD44 protein is expressed in bone marrow cells, osteoblasts, osteoclasts, and osteocytes (36). Although hyluronan is the principal ligand, CD44 was also found to interact with the bone specific ECM molecule osteopontin (OPN) (37). Weber *et al.* demonstrated that OPC CD44 interaction mediates chemotaxis and attachment of monocytic cells (38). Once mature, osteoclasts undergo alternative cycles of bone resorption and motility, requiring rapid attachment and release from the extracellular matrix. This is facilitated by cellular structures called podosomes that are essential for various functions including adhesion, invasion, and migration (39). Moreover, actively resorbing osteoclasts exhibit a sealing zone that defines the area of enzyme secretion and matrix degradation which also consist of a dense network of podosomes interconnected with radial bundles of actin (40). CD44 was found in osteoclast podosomes and identified as a non-integrin receptor ($\alpha v\beta 3$) involved in osteoclast adhesion and migration (41,42). Cao *et al.* reported that CD44 was also important in RANKL expression in bone marrow stromal cells (36).

Hence, evidence exists that CD44 plays a role in various stages of osteoclastogenesis, including the recruitment and migration of monocytic cells, their differentiation into osteoclasts and in the function of active osteoclasts. Given that osteoclastic bone resorption is a vital step in OTM, we hypothesized that CD44-/- animals may display deficiencies in this process.

RATIONALE AND GOAL OF STUDY

Improvements in molecular biology techniques have allowed for manipulation and targeting proteins and genes to study their function and regulation in cultured bone cells. However, study of gene regulation and function in vitro, requires validation in vivo. With recent advances in genetically manipulated animal models, study of individual gene function is becoming possible. Such models have been widely utilized in bone research. However, very few studies have used such models to analyze orthodontic tooth movement. Notably, only 5%, of the 320 studies reviewed by Ren *et al.* (2004) used mice (17). Most use appliances that are poorly characterized, differing in the kind of appliances used, direction of tooth movement, amount of force used and methods for measuring tooth movement. Moreover, these models undoubtedly require a high level of surgical skill and experience , but none report failure rates or placement times, making it difficult to assess their efficiency.

The goal of this study was to develop and characterize a model for tooth movement in mice with the following criteria:

- Ease of appliance design, placement and activation
- Ability to measure tooth movement accurately and reliably
- High efficiency, with the ability to perform multiple measurements from an individual animal over the duration of study

To achieve this, we modified an existing appliance design and used it to study tooth movement in CD44-/- and control C56BL/6J mice.

MATERIALS AND METHODS

Animals

CD44-/- animals were obtained from Dr. Tak Mak, University of Toronto as a kind gift (32). Eight CD44-/- and wild type C56BL/6J (WT) mice (4 males and 4 females in each group) were used for the tooth movement studies. Animals were acclimatized for at least 2 days under experimental conditions and received a diet of ground laboratory chow and distilled water ad libitum unless otherwise noted. Animals were housed on a standard 12-hour light/dark cycle. All animals were 8 weeks old and weighed approximately 20 grams at the time of appliance placement.

Appliance Placement

An austenitic stainless steel wire of diameter 0.010" with a modulus of elasticity of 28.5×10^6 psi (Rocky Mountain Orthodontics #E00003, Denver CO) was used to make the each appliance. The design adapted from appliances used by King *et al* in pilot studies. As shown in figure 2, the appliance consisted of a 3 mm loop to encircle the incisors with two arms extending distally, approximately 8.8 mm in length. A bevel was placed distal to the loops so as to position the arms parallel to the plane of the loop but 1.5 mm away in a sagittal view. This bevel accommodates for the difference in the level between the cervical margin on the incisors and the first molar.

Appliances were placed at day 0. Animals were anesthetized using a cocktail of 65 mg/kg ketamine (Ketaset®III, Fort Dodge, IO) and 4.4 mg/kg xylazine (AnaSed®, Shenandoah, IO) in 0.9% saline (Hospira Inc, Lake Forest, IL), diluted to a final dose of 0.01 ml/gm and injected intra-peritoneal using a 25 gauge needle. Once anesthetized, animals were placed on their backs and restrained on a heated acrylic platform. Animals were monitored constantly for signs of respiratory distress. The lower jaw and tongue were gently retracted using dental floss and the cheeks were retracted using a custom-made cheek retractor. The appliance was then placed on the incisors with the ends of the arms lying palatal to the first molars. The loop was bonded to the incisors using Transbond™XT (3M Unitek, Monrovia, CA) and light cured ensuring that the distal arms stay firmly approximated to the palate (Figure 2b). The appliance was activated by flipping the arm to the buccal surface on the right side of each animal, so that the distal end of the wire rested in the buccal cervical undercut. The mandibular incisors were trimmed to prevent de-bonding of the appliance. Radiographs were taken as described below and animals were returned to a heated cage and monitored for recovery from anesthesia. Upon recovery, animals were injected sub-cutaneously with 0.05 mg/kg bueprenorphine (Buprenex®, Reckill Benkiser Ph, Richmond VA) for pain control. Animals were returned to the housing facility and administered bueprenorphine every 12 hours for a total of 36 hours after appliance placement. Animals were fed a water-softened chow for 48 hours and their weights were recorded throughout the experimental period, with weight loss and clinical condition used as an indicator of excessive pain or distress.

Tooth movement measurement

Tooth movement was measured using radiographs. For measurements on day 0 animals were radiographed while under anesthesia. For the subsequent time points, mice were placed in a gas box (Summit Medical, NJ) filled with 2-4% isoflurane (Forane®, Baxter, IL) for short-term anesthesia. Once anesthetized, animals were checked to ensure that the appliances were intact and active. Mice were then repositioned in the chamber, facing down and centered over a Kodak Ultra speed (DF-50, Eastman Kodak, NY) size 0 film (Figure 3). An x-ray source was positioned 450 mm from the film applying a 50 kVp, 7.5 mA exposure for 0.5 seconds. Animals were repositioned and re-exposed for a total of 3 radiographs at each time-point. Radiographs were then developed and scanned (Epson Expressions 1680, CA) at 3200 dpi and analyzed using a standard imaging software (Adobe Photoshop CS5 version 4.0). Five measurements of the diameter of the wire as shown in figure 4, were averaged to calibrate each film. The distance between the inner corners of the ends of the two arms was then measured in millimeters. The difference in the distance between a time-point and the preceding time-point was calculated as the amount of tooth movement obtained between those two time-points.

Calculation of force/deflection curve

To analyze the amount of force applied by the wire on the molars and to estimate force decay, we determined a force deflection curve for our appliance. An appliance was fabricated mounted on a rigid support with the loop cemented and the arms extending

out, parallel to the base (Figure 6a). A surgical silk suture was tied to the end of one arm and extended over a pulley and attached to plastic cup that can hold weights. X-rays were placed below the wire and exposed without the cup, with the cup only and with known weights loaded into the cup. After using the largest weight, the wire was radiographed once more to ensure that no permanent deflection occurred. The procedure was repeated two more times using the same weights. Radiographs were then processed and analyzed as described above, to measure the deflection obtained for each weight.

Statistics

The means and standard error for inter-molar distance was calculated for each animal at each time point as described above. Analysis of variance (ANOVA) was performed to examine differences between CD44-/- and WT groups at each time point and across time points for each group. Pairwise comparisons were performed using Student's t test where indicated ($p<0.05$). Repeated measures ANOVA was performed to adjust for day 1 movement to analyze significance of subsequent data. In all figures (*) indicates significant differences between CD44-/- and WT animals while (#) indicates significant difference in tooth movement compared to day 1 ($p <0.05$). To measure reliability of using radiographic measurements, mean standard error was calculated for measurements made using calipers and radiographs. Average mean difference in measurements obtained using radiographs and calipers at each position for each skull was also calculated with confidence limits determined at the 95% level.

RESULTS

Appliance design and tolerance

Of the 16 total animals used in this study, 3 animals did not survive the procedure (1 CD44-/- and 2 WT mice) due to failure of recovery from anesthesia (Table 1). The average time for placing and activating the appliance per animal was ~10 minutes (range 7-16 minutes). The average time required for animals to recover from anesthesia was ~ 27.5 minutes. Surviving animals were weighed at each time point as a measure of discomfort. The animals tolerated the appliances very well and assumed a normal feeding pattern within 24-48 hours. While all animals were ~8 weeks old at time of appliance placement, the CD44-/- animals were significantly smaller compared to WT animals at start of experiment (Table 2). Figure 8 shows that the animals experience a minor loss in weight initially, which is quickly recovered over the experimental period.

We had first attempted to use a unilateral cantilevered appliance using a 0.012" NiTi wire, used in pilot studies by King *et al*. However, we encountered high appliance failure rates (58%). Failure rate was calculated as a percentage of animals surviving anesthesia that either lost the appliance, or lost activation of the appliance, on or before day 5. Hence, we modified the design to use a 0.010" stainless steel wire, which enabled more accurate loop fabrication and adaptation to the incisors. Our use of a bilateral design, which incorporated a second loop around the incisors, also increased surface area

for adhesion. The failure rate we obtained using this modified design was ~38 % (Table 1).

Reliability of method

We tested the accuracy and precision of measurements obtained using the radiographic method described here (Materials and Methods). To achieve this, we cemented appliances onto a dry skull of a mouse as shown in figure 5a. Two different configurations of the wire were tested: position 1 where the distal ends of the wire lay buccal to the first molars and position 2 where the distal ends of the wire lay palatal to the first molar. To record the true value, measurements in millimeters were made using a digital caliper (Carerra Precision ™). The skulls were then placed on an x-ray film and exposed. This procedure was repeated 3 times in each configuration using 3 different skulls. The radiographs were developed and analyzed using the diameter of the wire to calibrate the measurements and account for magnification (Materials and Methods). Mean measurements obtained for each position using the digital calipers (grey bars) and the radiographic method (white bars) do not show statistically significant difference using the Student's *t* test (Figure 5b, $p<0.05$). The average absolute error using the radiographs was 79 μm and the mean difference in measurements obtained using the radiographs and the calipers was 12.5 μm with a 95% confidence interval of (-) 4.6 μm to (+)29.6 μm. We also calculated a retest correlation at 0.99 suggesting that the method has high reliability (Figure 5c).

Tooth movement in CD44-/- mice and WT mice

We next measured tooth movement in CD44-/- mice and WT C56BL/6J mice. As described in Materials and Methods, appliances were cemented onto CD44-/- and wild type (WT) mice on day 0, followed by taking radiographs to measure initial inter-molar width. Animals were then returned to their cages and monitored for signs of pain and activity. Tooth movement measurements were made 24 hours later (Day 1) and every 48 hours thereafter. Animals were also checked to see if the appliances were still present and active at each time point by anesthetizing them with isoflurane and gently retracting their cheeks to view the distal end of the active arm. Of the seven surviving CD44-/- mice, 1 animal lost activity of the wire on day 3 of tooth movement and 2 animals lost their appliances on or before day 5. Of the six WT mice, 2 animals lost their appliances on or before day 5 (Table 1). The remainder of the animals (4 CD44-/- and 4 WT) had their appliances present and active at least until day 11 of tooth movement. Following this time point, animals lost their appliances sequentially in each group.

Figure 7a shows tooth movement averaged from all animals in each group +/- SE (blue = CD44-/- and red = WT) over the experimental period. Both groups displayed a large initial movement (Day 1), followed by a lag period (where no significant movement is seen), after which varying tooth movement was observed. As seen in Figure 7a the two groups show significant difference in tooth movement at all time points (Pairwise comparisons by Students *t* test *, $p<0.05$). Moreover, for the WT group, significant tooth movement was first observed at day 7 after the lag phase; while in the CD44-/- animals,

significant tooth movement was first observed at day 9 (Figure 7a, # $p<0.05$) after the lag phase.

Tooth movement at day 1 in the WT group averaged at ~ 0.8 mm, while in CD44-/- animals, tooth movement observed at day 1 was significantly less (average ~ 0.6 mm). However, this does not reflect orthodontic movement since bone remodeling and osteoclastic activity are not contributing to this phenomenon. To ensure that this initial difference does not influence subsequent observations, we reanalyzed the data by normalizing tooth movement to day 1. Although there is greater individual variation seen within the groups when analyzed in this manner, the CD44-/- animals displayed significantly less tooth movement when compared to WT controls at and after day 5 (Figure 7b, repeated measures ANOVA, * $p<0.05$).

As can be seen in Table 2, CD44-/- animals weighed significantly less than WT controls of the same age at the start of experiments. We hence analyzed our data, normalizing to initial weight of animals (Fig 7c) and found that there is no statistically significant difference between the two groups initially (days 1-5), beyond which the difference between groups is significant (Fig 7c, * $p<0.05$). Again, WT animals begin to show tooth movement by day 7, while the CD44-/- animals display a small, although significant movement only after day 9 (Fig 7c, # $p<0.05$). Taken together, these data indicate that CD44-/- animals display significantly less tooth movement compared to WT controls and a larger lag phase before tooth movement is observed.

Force level and decay estimates

To estimate the amount of force applied by the wire and force decay over the experimental period, we determined the force/deflection curve. Figure 6a shows a schematic of the set-up used to apply known weights to one end of an appliance cemented at the loop to a rigid base. Radiographs were used to calculate the deflection obtained of each weight and values were plotted to obtain a standard curve (Figure 6b). The initial wire deflection measured from radiographs taken before and after activation of the appliance averaged at 1.2mm in both groups. Hence the estimated force applied to the molar as calculated from the standard curve is ~ 45 cN. Table 3 shows the estimated force at each time point subsequent to activation calculated based on the deflection of wire at each point. After the initial movement at day 1, the mean force level in WT animals is ~ 16.8cN while that in the CD44-/- animals is 23.4cN.

DISCUSSION

Mouse model for orthodontic tooth movement

Very few studies on OTM have used mice as a model for tooth movement. Notably, of 320 animal studies on OTM reviewed by Ren *et al.*(43) only 5%, had used mice. Certainly, the most challenging aspect of using mice is the small scale and difficulty in accessing the working field. Moreover, since the amount of tooth movement expected is so small, an accurate and sensitive method for measuring it is important. As with most studies in rats, tooth movement in mice has been achieved using NiTi springs which apply a mesializing force to the first molar (44-47). Few studies have employed elastic bands, but force levels obtained are large, with high decay rates (48).

We initially used 0.012" Niti wires with a unilateral appliance design. However, due to the small scale of mouse incisors, precise loop fabrication was difficult using NiTi wires. We hence switched to a 0.010" stainless steel wire, which enabled better loop fabrication. As is apparent from figure 4, it is difficult to distinguish the molars in mice using radiographs. Incorporation of the bilateral appliance design provided a radiographic landmark from which tooth movement measurements can be made, especially because placement of subperiosteal implants in mice proved challenging.

In reviewing available literature, it is difficult to find previous reports on failure rates, which are undoubtedly high. King *et al.* (1991) carried out a large study using 300 rats and placed NiTi springs to mesialize molars with different force levels. They reported a ~ 21 % failure rate in the animals that survived the procedure (27). In a study in mice using NiTi coil springs, Pavlin *et. al* (44) had a failure rate of ~16%. In our study, failure rates were ~ 38% as calculated by loss of appliance or loss of activity on or before day 5. In order to minimize the number of animals needed and improve the power of future studies, it would be imperative to further reduce failure rates. Some modifications that we propose to try in future studies are to use a self-etching primer on the incisors before cementation (without the use of a rinsing step), gentle filling between the incisors to improve the mechanical bond to the cement and gentle micro-abrasion of the stainless steel wire around the loop. Regular trimming of lower incisors during the experimental period may help in maintaining the wire for a longer period, if prolonged experimental time points are desired.

Most of the previous studies, analyze tooth movement at few time points, which are further apart (e.g. weekly measurements). As a result, it is difficult to pinpoint the phases of the tooth movement cycle. Moreover, the methods used to measure tooth movement necessitates sacrificing animals at each time point and hence a large sample size. A major advantage of using radiographs to measure OTM, as presented in this study, is the ability to make longitudinal measurements. This helps to reduce the number of animals needed, enables more frequent measurements (for e.g. every 48 hours as described here) and is more robust statistically compared to cross-sectional data. Our

study enabled us to perceive a prolonged lag phase in CD44-/- animals, which would have been difficult to resolve if we only analyzed weekly measurements (Figure 7). A detailed description of tooth movement kinetics would also allow for appropriately designed experiments that focus on histologic analyses and cellular activity in response to OTM. Additionally, few studies report failure rates of appliance and none have mentioned the amount of time required to place appliances, which are likely high. We were able to cement and activate appliances relatively quickly (average ~10 mins) and since extraction of opposing teeth was not required, the level of operator proficiency demanded by this method is not very high. Together, the advantages afforded by this method may outweigh the disadvantage of the high failure rate observed in our study and can potentially improve the cost-effectiveness ratio of these experiments.

We estimate the amount of force applied to the molars upon appliance activation to be 45cN. This value is significantly higher than the force suggested by Pavlin *et al* (44) who used NiTi coiled springs activated at 20 cN. In fact most rat studies use forces in the range of 10-40 cN (49). Given that mice are 2-3 times smaller than rats, it would be prudent to reduce the initial forces delivered by the appliance for studies focusing on OTM. Future studies using a smaller diameter wire (e.g. 0.08") will reduce the stiffness of the wire while improving the range and springiness of the wire.

It is clear that a larger initial tooth movement would also result in a larger decay in force levels. In our WT animals, the force level decays to 37% of original (~16.8cN), by day 1 (Table 3). While this is a relatively large amount of force decay, levels are still

in the physiologic range expected to maintain tooth movement. In their study Pavlin *et al*. found that appliance activation decreased to ~33% and 38% of original levels by 6 hours and 1 day respectively. To overcome this decay, they were able to reactivate the NiTi coiled springs at day 5, which would not be possible using our method. However, any attempt to reactivate appliances (using either the method described here or using NiTi springs) would require re-anesthetizing of animals and administration of pain medication. A potential argument against these additional steps is its effect on animal health and loss due to anesthesia. Moreover, re-activation may be considered inadvisable considering the high initial activation.

Tooth movement in CD44-/- and WT mice

Figure 7a shows that there is a significant difference is tooth movement between the CD44-/- group and the WT group at Day 1 of force application. This initial phase does not reflect classical orthodontic tooth movement involving tissue adaptations but is more of an instantaneous tooth displacement within the socket accompanied by the bending of alveolar bone and is a reflection of the material properties of the periodontal tissues (50-52). Initial histological studies by Dr. Popowics (personal communication) suggest that the width of the PDL is essentially similar in CD44-/- and WT animals. Hence, the instantaneous tooth displacement that would occur after force application should be similar in both groups. However, Cao *et al.* (2005) used μCT and quantitative histomorphometry analysis of tibial and femur bones from CD44-/- mice and WT mice and reported subtle but significant differences (36). While total bone area was the same in

CD44-/- and WT animals, the tibias in CD44-/- mice were shorter with increased bone mass. Cortical thickness was increased and medullary area decreased, changes consistent with reduced endocortical resorption. It is possible that this increased cortical thickness could significantly reduce the initial bone bending and hence the amount of initial movement observed in the CD44-/- animals.

When analyzing data by normalizing to day 1 measurements, we observed that CD44-/- animals display significantly less overall tooth movement compared to WT controls and have a longer lag phase (Figure 7a,b). Since bone resorption is necessary for tooth movement, our data suggest that CD44-/- animals may display deficiencies in osteoclastic activity. These deficiencies may arise from reduced osteoclast numbers (either due to reduced monocytic migration to resorptive sites or impaired differentiation to osteoclasts), or due to impaired osteoclast function. In vitro data from previous studies suggest that CD44 may be important in chemotaxis and attachment of monocytic cells (38) and may also be involved in osteoclast adhesion and migration (41,42). CD44 was also found to be important in RANKL expression in bone marrow stromal cells, a necessary step for osteoclast maturation (36). Future studies, focusing on histologic analysis of teeth and surrounding bone in CD44-/- animals subjected to OTM, would help in analyzing its function in osteoclasts.

Although we used all animals ~ age 8 weeks old, Table 2 shows that the mean weight of the two groups was significantly different. Earlier studies characterizing the phenotype of CD44-/- animals do not report differences in size or weights compared to

controls (33). Given our small sample size, the uneven distribution of males and females in the two groups can explain the difference in the weights of the animals (53,54). To analyze if sex of the animals influences tooth movement data, we performed a repeated measures ANOVA adjusting for the sex of each animal and found no significant gender effect on tooth movement. Normalization of tooth movement to initial body weight (Figure 7c) results in an elimination of this initial difference seen at day 1.

To ensure that application of orthodontic force is indeed resulting in tooth movement via bone resorption, we sectioned and stained the maxilla of a CD44-/- animal at day 11 of OTM. Hematoxylin/eosin staining revealed areas of bone resorption at the palatal alveolar crest on the experimental side with some stretching of PDL fibers apical to it, while no resorption was noted on the control side (Supplemental figure).

In our WT animals, the force level decays to 37% of original (~16.8cN), while the CD44-/- decays to 52% of original (~23.4cN). We know that higher force can slow down tooth movement, potentially due the need for undermining resorption (6,9,55). While the amount of force required to initiate undermining resorption in mice is not well defined, we cannot rule out the possibility that the CD44-/- animals experience reduced tooth movement due higher forces present at day 1 as compared to the WT animals (Table 3). However, given that both the groups experience a heavy initial force (45cN) we suspect that both groups have hylanized PDLs and will require undermining resorption for tooth movement to occur. A detailed histological analysis of the paradental structures in these animals would address this issue.

Growth at the mid-palatal suture should also be considered when using this method to measure tooth movement. Griffiths *et al.* analyzed the growth and maturation of the palatal complex in WSW strain mice commencing at birth and at successive intervals of 5, 10, 15, 20, 35, and 90 days using histological sections. At age 35 days (~5 weeks), they did not observe an increase in the inter-molar width when compared to the 20 day animals (56). By 90 days they observed hyaline cartilage in a narrow band along the entire height of the suture, while the bone of the palate was organized from its earlier cancellous form into a mature compact structure. In our study we used mice what were 8 weeks old, but the background of these mice is different (C57BL/6) from that used by Griffiths *et al*. C57BL/6 mice are known to reach skeletal maturity by age 4-6 months depending of the sex of the animal (53). Hence it is possible that our mice may have some growth remaining at the mid-palatal suture. If there is a difference between CD44-/- animals and WT animals with regards to growth at the suture, it could influence the data obtained. A detailed analysis of palatal growth temporally in each strain would be required to rule out its effects on measurements of inter-molar width and OTM.

Application of mouse model of OTM

The most evident application for a mouse model of OTM is in furthering our understanding of this process at the cellular, molecular and biochemical levels. To illustrate. Taddei *et al.* (57) carried out OTM in CCL3-/- and CCR1-/- mice and demonstrated the importance of this chemokine and its receptor in bone remodeling.

Recent publications have also elucidated the important role played by osteocytes in bone homeostasis and also in OTM. Nakashima *et al.* demonstrated that osteocyte synthesis of receptor activator of NF-κB ligand (RANKL) plays a crucial role in osteoclastogenesis (58). Using the 9.6-kb *Dmp1* promoter, Tatsumi *et al*. established mice that expressed diphtheria toxin receptor (DTR) on osteocytes, so as to selectively ablate them by timely injection of diphtheria toxin. They found that osteocyte-ablated mice had markedly increased empty lacunae in femoral cortical bones and were resistant to unloading-induced bone loss, indicating that osteocytes play a crucial role in bone remodeling induced by mechanical loading (59). Importantly, Matsumoto *et al*. utilized these DTR-Tg mice and carried out OTM studies on them, outlining the crucial role played by osteocytes in this process (60). Establishment of a standardized model for OTM will allow for designing future studies in genetically engineered mice, focusing on the roles of individual genes and proteins involved in OTM as well as root resorption and repair.

Recent studies have also focused on the use of bioactive agents in modulating tooth movement, especially agents that can reduce osteoclastic activity. For e.g. Salla *et al.* used IL-1Ra injection in mice and showed that it reduced both orthodontic tooth movement as well as osteoclast numbers as evaluated by TRAP staining (61). Similarly, Karras *et al*. administered alendronate, a known inhibitor of osteoclastic activity, and found reduced tooth movement in rats that received the drug when compared to controls (62). These studies suggested that agents that inhibit osteoclastic activity may find application in reducing orthodontic relapse. If local delivery of these agent can be achieved, they may also find application in cases where absolute anchorage is required,

for e.g. retraction of anterior teeth into extraction spaces without reciprocal mesial movement of molars. Indeed, Kohara *et al.* have utilized IL-12 injection adjacent to the first molar of mice subjected to OTM display reduced tooth movement as well as reduced root resorption (63). Our study shows that CD44-/- animals display reduced tooth movement compared to WT controls (Figure 7). Given the potential function of CD44 in osteoclast differentiation and activity, it may serve as a target for future drug therapies for controlling relapse. Monoclonal antibodies against CD44 (RG7356) are available and have been shown to be cytotoxic for leukemia B cells, both in vitro and in vivo, without affecting normal B cells (64). If local application of this antibody proves successful in inhibiting osteoclast activity and OTM, it could potentially find an application in anchorage control and relapse control.

Another application of the tooth movement model is in studying the effects of mechanical loading on bone. Previous experimental models to study this have included animals subjected to some form of exercise ranging from jumping, treadmill running, and swimming (65-69). While OTM model is not the most ideal for studying physiologic mechanical loading, researchers argue that certain data from OTM studies can be extrapolated to bone remodeling in other areas of the skeleton.

CONCLUSION

We have characterized a model of analyzing OTM in mice, which is relatively easy to carry out with moderate failure rates and considerable accuracy. While the amount of tooth movement obtained is relatively small, the method is sensitive enough to detect differences between phenotypically different strains with extremely small sample sizes. The method also allows for analyses of tooth movement curves (if desired) with a relatively small test group, by permitting longitudinal measurements at multiple time points. Using this method, we present pilot data demonstrating reduced tooth movement in mice lacking the CD44-/- gene, characterized by less initial deflection, longer lag phase and a reduced post-lag phase. In line with existing evidence, these findings support the notion that CD44-/- is important in osteoclast recruitment and function. Follow up studies are needed to demonstrate histological and cellular responses of paradental tissues in response to orthodontic forces.

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Table 1. Failure rate calculated as a percentage of animals surviving anesthesia that either lost the appliance, or lost activation of the appliance, on or before day 5

Group	Initial (N)	Animals surviving Anesthesia (N')	Loss of appliance/activity on/before day 5 (F)	Final (n)	Failure rate (F/N' %)
WT	8	6	2	4	
CD44-/-	8	7	3	4	
Total	16	13	5	8	38.46

Table 2. Gender distribution and mean weight of animals in each group that were included in the final analysis (* indicates significant difference between groups using Students *t* test, p>0.05)

Group	Males n	Females n	Initial mean weight (grams)	S.D.
WT	3	1	21.25	1.92
CD44-/-	2	2	17*	1

Figure 1. Abridged list of genes, intracellular signaling molecules, cytokines and growth factors thought to be important in various cellular processes involved in tooth movement after application of an orthodontic force.

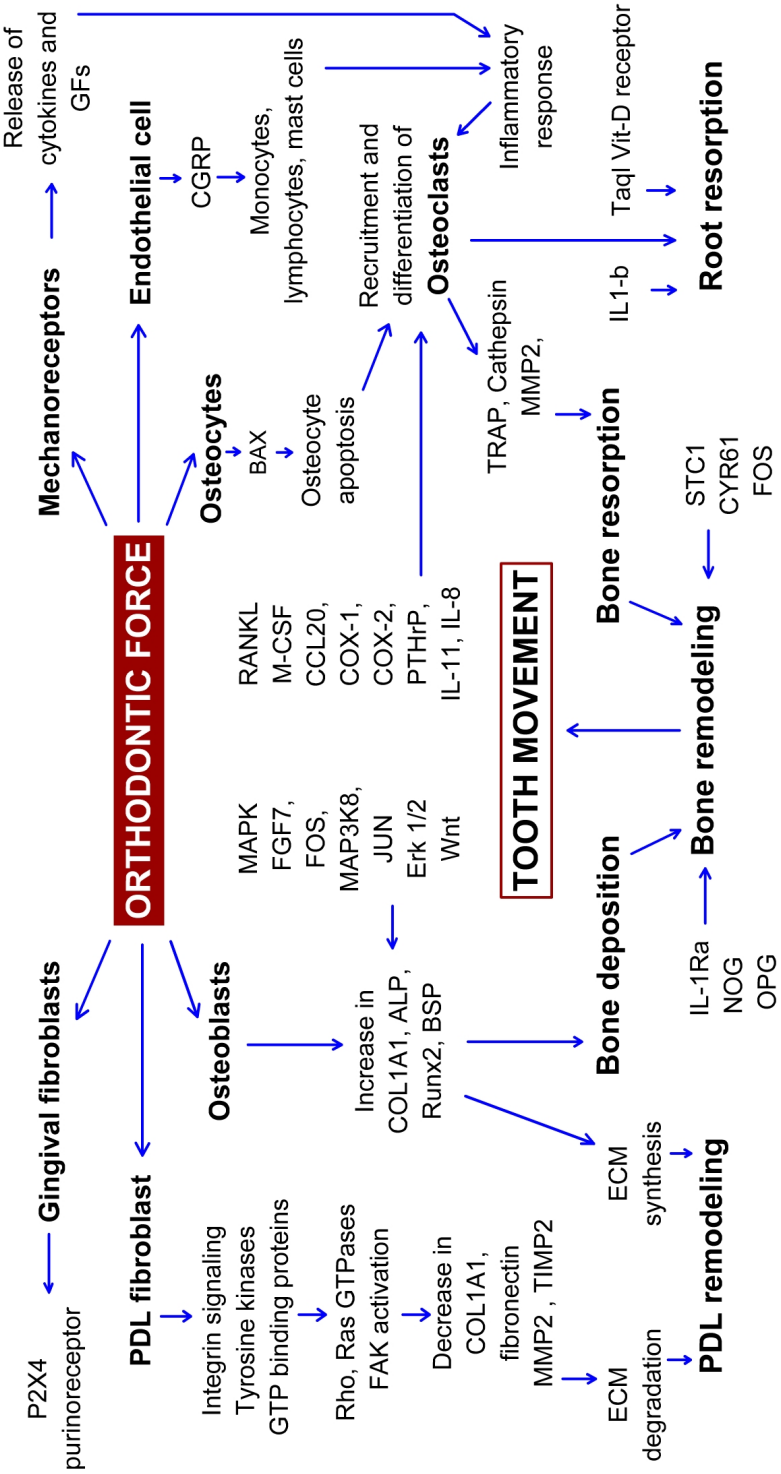
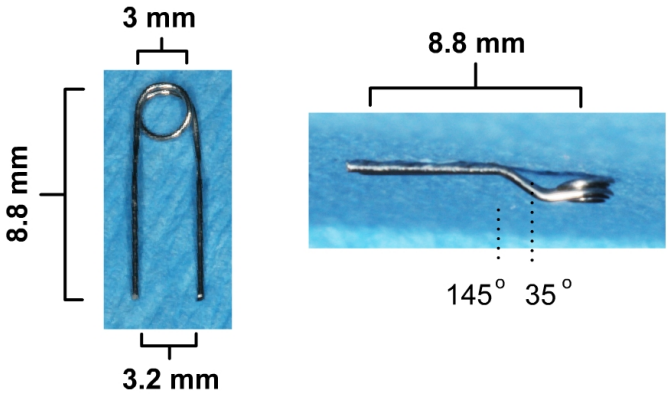


Figure 2. A. Representative appliance fabricated from a 0.010" stainless steel wire with a 3 mm loop and two arms extending distally ~8.8 mm long beveled from the plane of loop. **B.** Mouse with appliance in position with arms lying palatal to maxillary first molars.

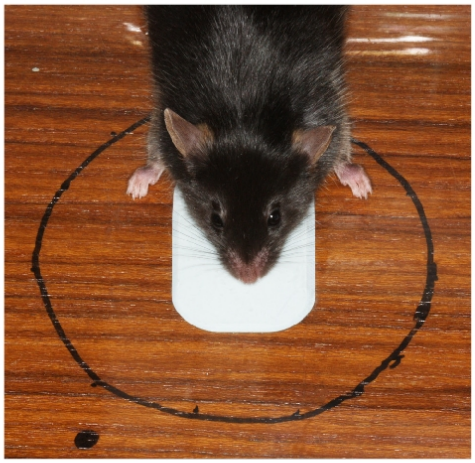
A



B

Figure 3 A. Mouse positioned over an x-ray film with inter-pupillary line parallel to floor, in gas box filled with isoflurane. **B**. X-ray source positioned 450 mm from film.

A



B

Figure 4. Representative radiograph of a mouse skull with appliance demonstrating areas where measurements are made to calibrate and quantify inter-molar width

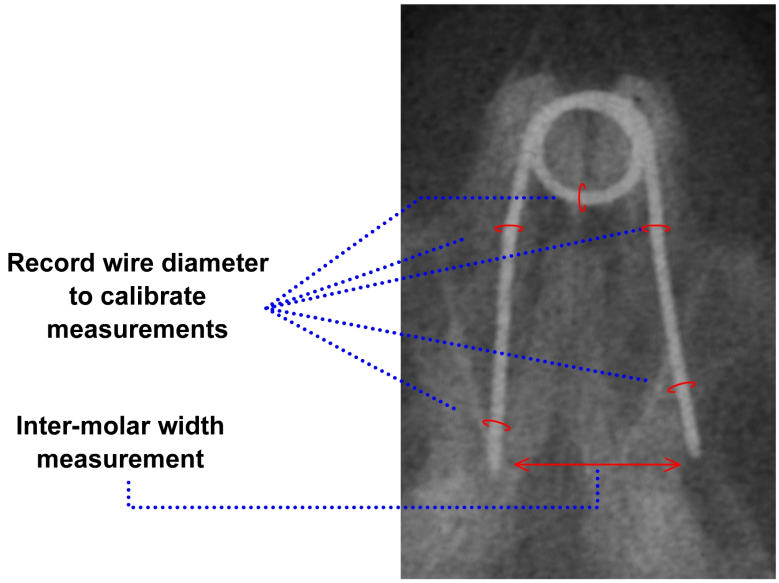
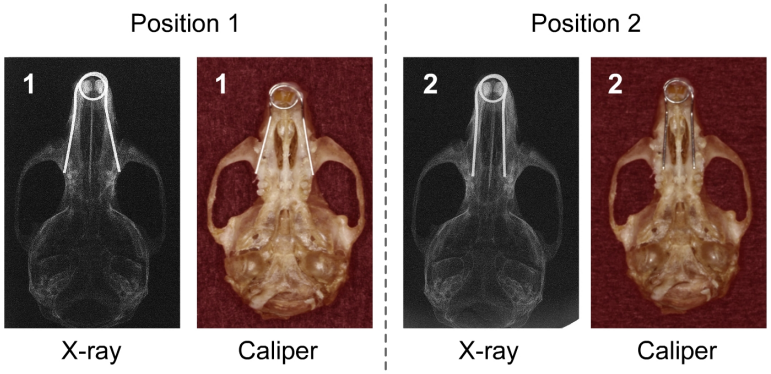
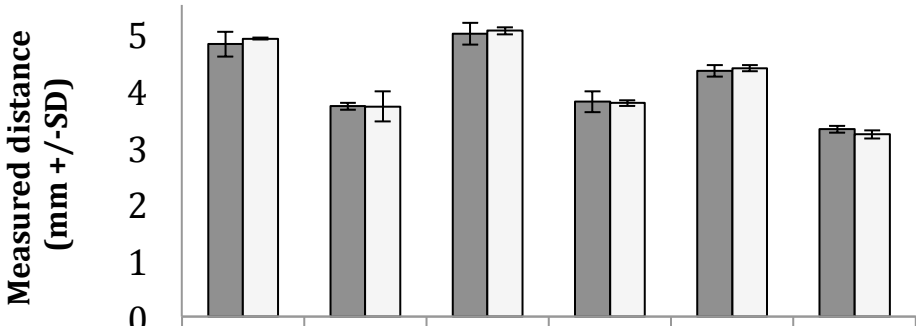


Figure 5. A. Representative mouse skull with appliance cemented and configured in two positions: 1, with arms lying buccal to molars (activated) and 2, with arms lying palatal to molars (unactivated). **B.** Mean distance measured using the x-ray method (grey bars) and the calipers (white bars) in mm (+/-SD) for three different skulls in each position (values shown in table below each bar). **C.** Correlation between measurements obtained for each position using the two methods.

A



B



	1	2	3	4	5	6
X-ray measurements	4.806	3.712	4.988	3.793	4.337	3.306
Caliper Measurements	4.903	3.707	5.043	3.767	4.383	3.213

C

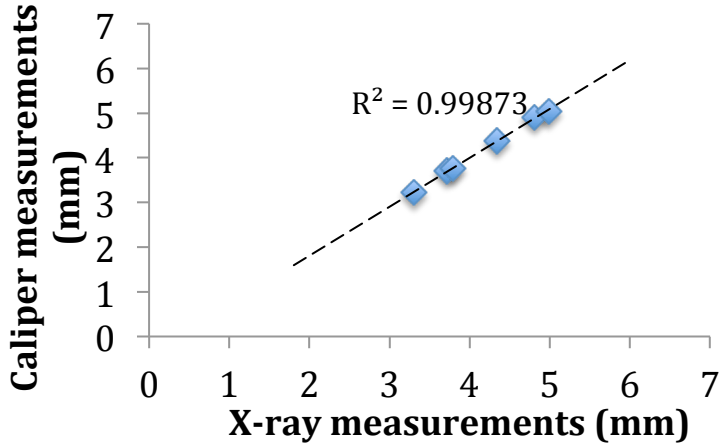
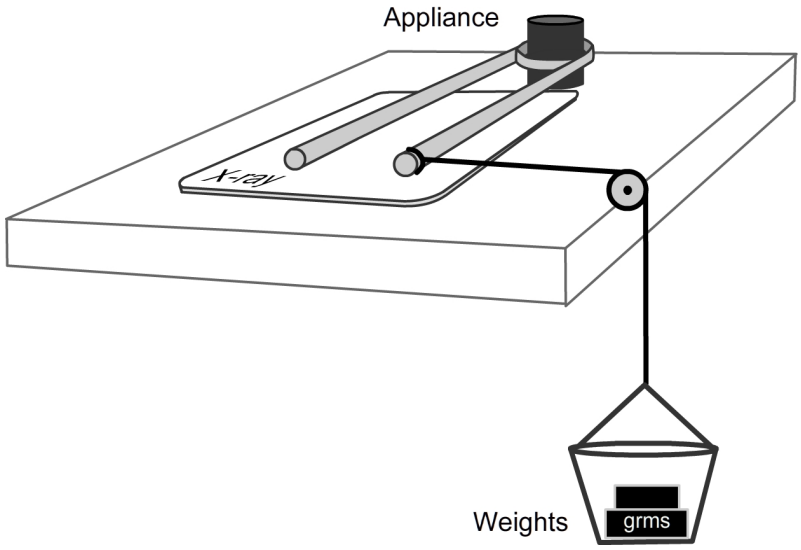


Figure 6. A. A schematic drawing of method used to obtain a force/deflection curve for the appliance. **B**. Standard curve for deflection of wire (mm) obtained under application of know amount of force (cN) with equation used to estimate force level for a given deflection of wire.

A



B

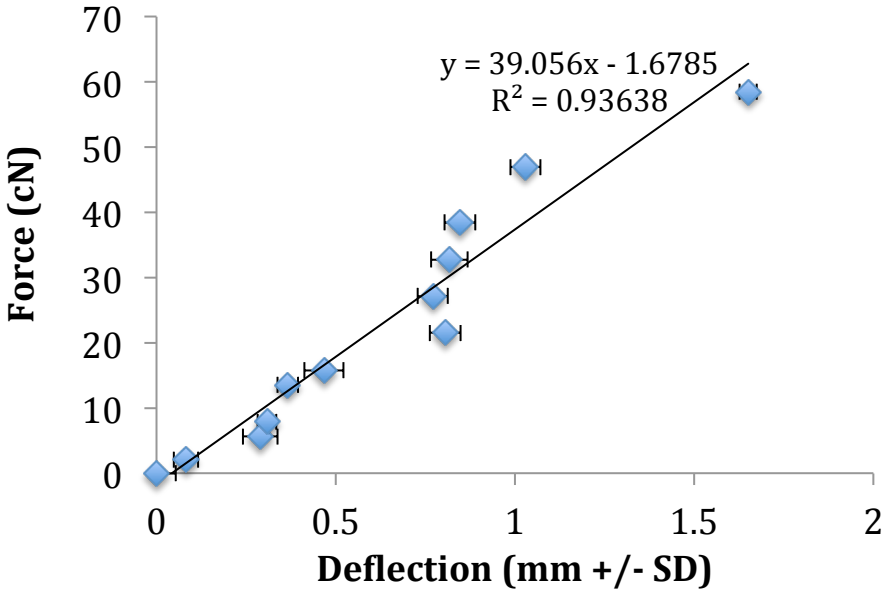
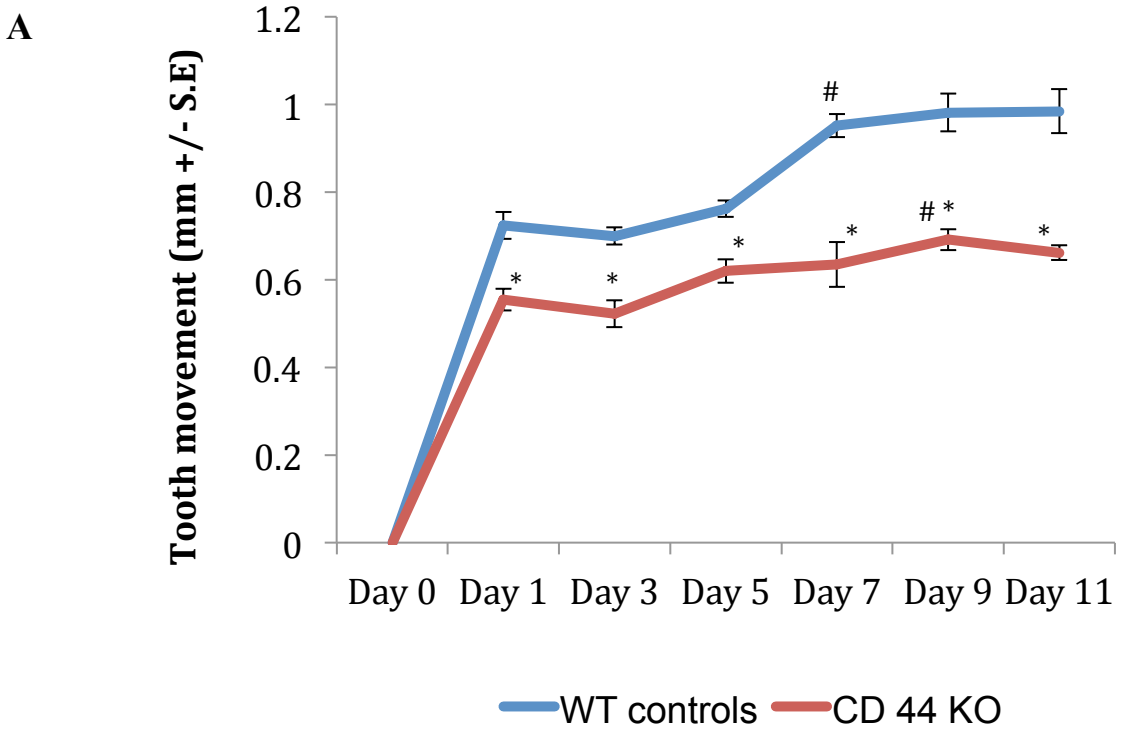
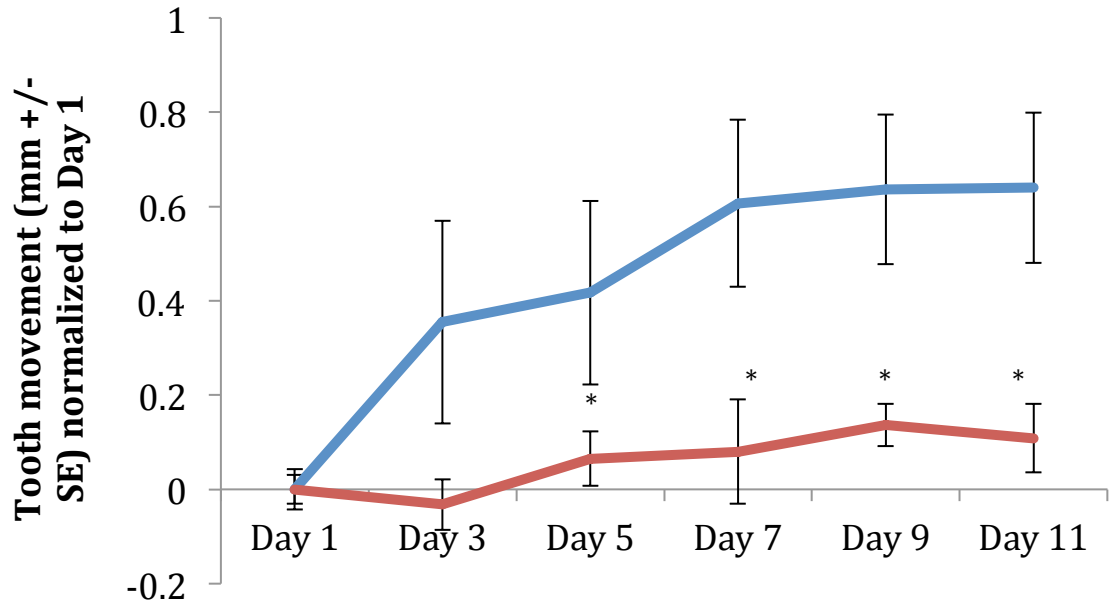


Figure 7 A. Tooth movement curve for WT (blue) and CD44-/- (red) animals measured in mm (+/- SE, # indicates significant tooth movement at a time point within each group compared to day 1 and * indicates significant difference between two groups at each time point, $p<0.05$). **B.** Tooth movement curves for each group normalized to day 1 movement. **C.** Tooth movement curves for each group normalized to weight of animals (grms) at day 1.



B


Tooth movement (mm +/- SE) normalized to Day 1
1
0.8
0.6
0.4
0.2
0
-0.2
Day 1
Day 3
Day 5
Day 7
Day 9
Day 11
*
*
*
*

C

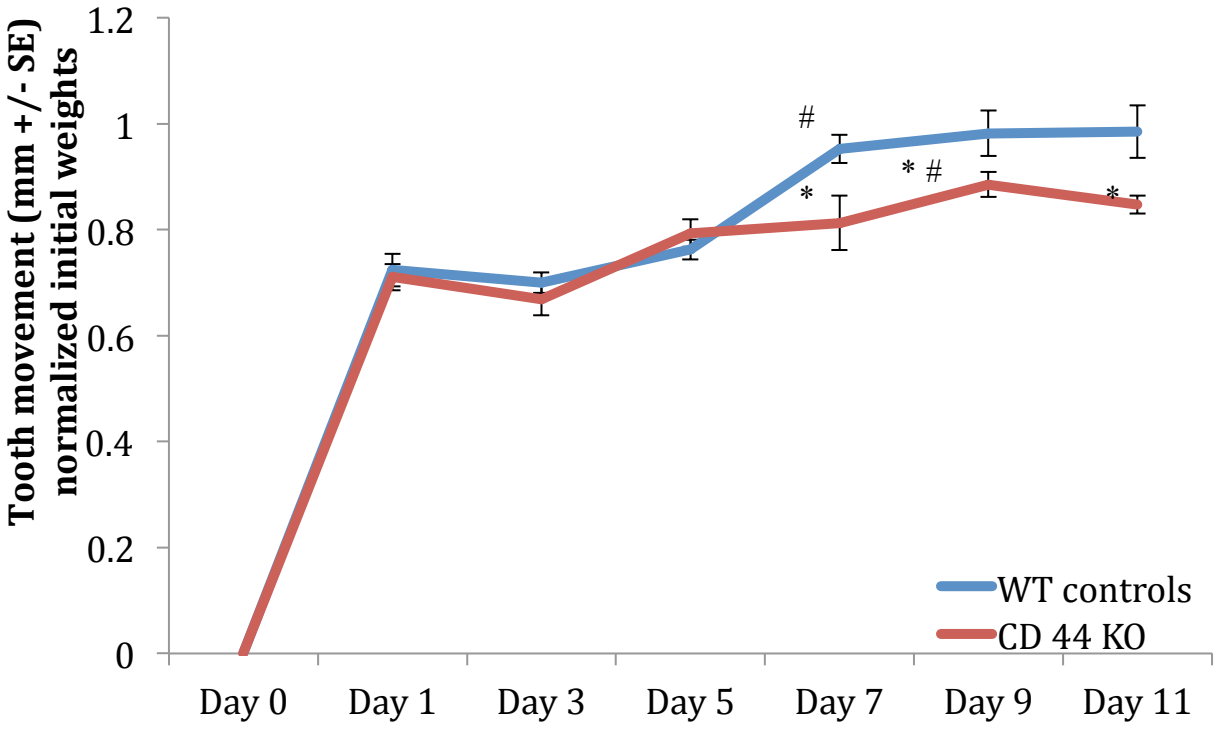
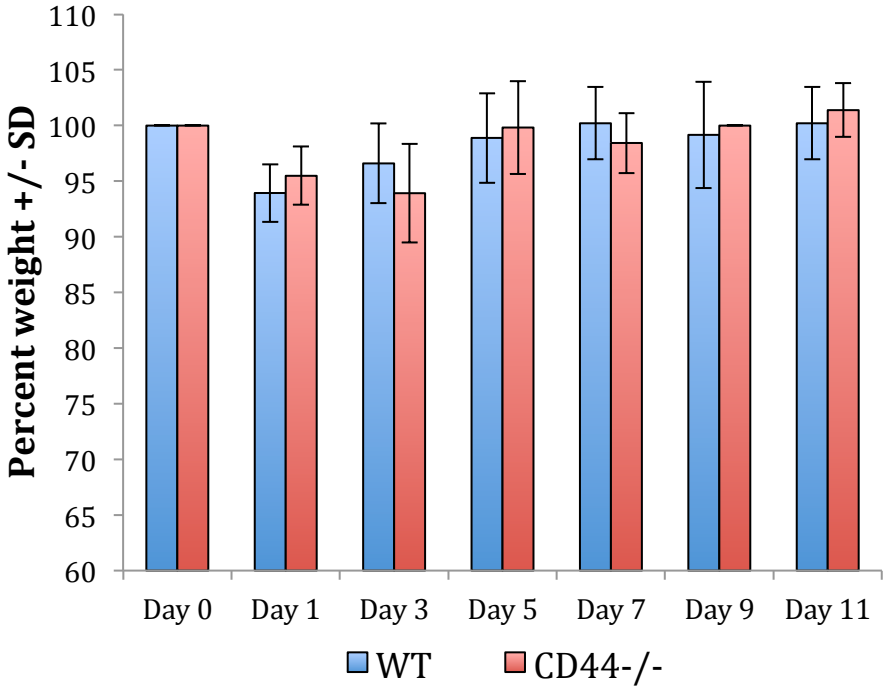

Tooth movement (mm +/- SE) normalized initial weights
1.2
1
0.8
0.6
0.4
0.2
0
Day 0
Day 1
Day 3
Day 5
Day 7
Day 9
Day 11
#
* #
*
*
WT controls
CD 44 KO

Table 3. Estimation of force levels exerted by appliance on molars at experimental time points, based on the standard F/ ∂ curve.

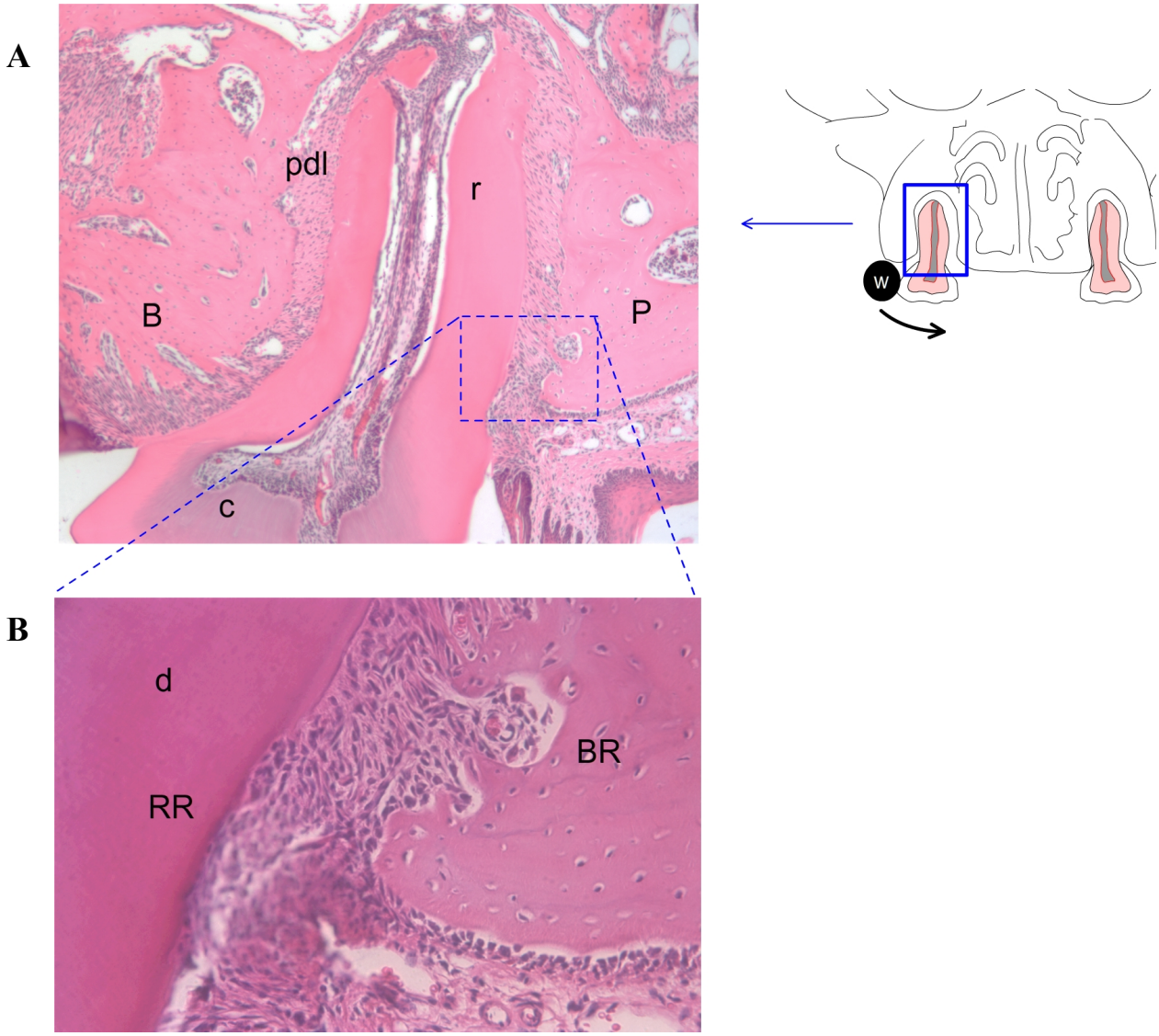
	Estimated force level (cN)	
	WT	**CD44-/-**
Day 0	45.0	45.0
Day 1	16.8	23.4
Day 3	17.8	24.7
Day 5	15.3	20.9
Day 7	8.0	20.3
Day 9	6.8	18.1
Day 11	6.7	19.2

Figure 8. Mean weight of WT and CD44-/- animals expressed as a percentage of initial weight +/- SD



Supplemental Figure

Hematoxylin/eosin stained sections of periodontal sites of a CD44-/- mouse maxilla at day 11 of OTM. **A.** Experimental side at 10x magnification. Note areas of resorption at the crest of palatal alveolar bone (blue box). **B.** 40x magnification of area depicted in blue box (A). Note resorption lacunae seen at the crest of the alveolar bone on the palatal side. **C.** Control side at 10x magnification. Note uniform PDL space and lack of resorption at crest of palatal alveolar bone. (c: crown, r: root, pdl: periodontal ligament, B: buccal bone, P: palatal bone, d: dentin, RR: root resorption, BR: bone resorption))



C