THE ROLE OF AUTOPHAGY DURING ORTHODONTIC TOOTH MOVEMENT

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ABSTRACT

Yina Li: The Role of Autophagy During Orthodontic Tooth Movement
(Under the direction of Ching-Chang Ko)

Orthodontic tooth movement (OTM) depends on efficient remodeling of surrounding alveolar bone. While a well-controlled inflammatory response is essential during such biological processes, the precise mechanism by which how inflammation is regulated hasn't been fully understood. Autophagy, a conserved catabolic pathway, has been shown to protect cells from excessive long lasting inflammation in nervous systems and other disease conditions. We hypothesize that autophagy plays a role in regulating inflammation during OTM. By using a split mouth design in adult male mice at different time points (days 0, 1, 3, 5, 7, 10 and 14) after 30 gram of force loading, we found that autophagy activity increased shortly after loading (as early as day 1) and was closely associated with inflammatory cytokine expression as well as osteoclast activation (by TRAP staining). Autophagy activation appeared to be at the protein, not mRNA, level. Daily administration of rapamycin, autophagy activator, in adult male mice led to reduced tooth movement amount as well as inflammatory signal after loading, suggesting a negative effect of autophagy on inflammatory response during OTM. To our knowledge, this is the first time that research showed autophagy plays a role during orthodontic tooth movement, likely via negative regulation of inflammatory response. More molecular and cellular analyses are needed to elucidate the underlying mechanism that governs the regulation of inflammation by autophagy pathway.

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LIST OF ABBREVIATIONS

BMP	Bone Morphogenic Protein
ClCN7	Chloride channel 7
CSF	Colony-Stimulating Factors
COX	Cycloxygenase
ECM	Extracellular Matrix
eNOS	endothelial Nitric Oxide Synthase
FAK	Focal Adhesion Kinase
HIF	Hypoxia Inducible Factor
iNOS	inducible Nitric Oxide Synthase
IL	Interleukin
MAP1LC3	Microtubule-associated protein 1 light chain 3
MMP	Matrix metalloprotease
mTOR	mammalian/mechanistic Target of Rapamycin
mTORC1	mammalian/mechanistic Target of Rapamycin Complex 1
NiTi	Nickel Titanium
NLRs	NOD-like receptors
NO	Nitric Oxide
OIIRR	Orthodontic-induced inflammatory root resorption
OPG	Osteoprotegerin
OTM	Orthodontic Tooth Movement
PDL	Periodontal Ligament
PE	Phosphatidylethanolamine

PG	Prostaglandin
RANK	Receptor Activator of Nuclear factor Kappa B
RANKL	RANK Ligand
STAT	Signal Transducers and Activators of Transcription
TGF-β	Tissue Growth Factor β
TIMP	Tissue Inhibitors of Metalloproteases
TLRs	Toll-like receptors
TNF-α	Tissue Necrosis Factor-α
TRAP	Tartrate Resistant Acid Phosphatase
ULK1/2	unc-51 like autophagy activating kinase 1/2
VEGF	Vascular Endothelial Growth Factor

LIST OF SYMBOLS

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®	Registered Trademark

REVIEW OF THE LITERATURE

Orthodontics is a special discipline dedicated to the investigation and practice of moving teeth through the bone. Moving teeth through the dentoalveolar complex is a synergistic sequence of physical phenomenon and biological tissue remodeling. The physical behavior of tooth movement due to orthodontic force relies on Newton's Laws. The tooth biological system reacts to variation in force magnitude, time of application and directionality through receptor cells and signaling cascades that ultimately produce bone remodeling and orthodontic tooth movement (OTM).

Periodontium: the Tooth Supporting Complex

Periodontium is the investing and supporting attachment of the teeth to alveolar bone. It includes both the soft tissues of periodontal ligament (PDL) and gingiva as well as the hard tissues of cementum and alveolar bone (Figure 1.1).

The ability of teeth to move through the bone relies on the PDL, which attaches the tooth to the adjacent bone. The PDL is a dense fibrous connective tissue structure that consists of collagenous fiber bundles, cells, neural and vascular components and tissue fluids. Its primary function is to support the teeth in their sockets while allowing teeth to withstand considerable chewing forces. On average, the PDL occupies a space about 0.2mm wide. Depending on its location along the root, PDL width can range from 0.15-0.38mm, with its thinnest part located in

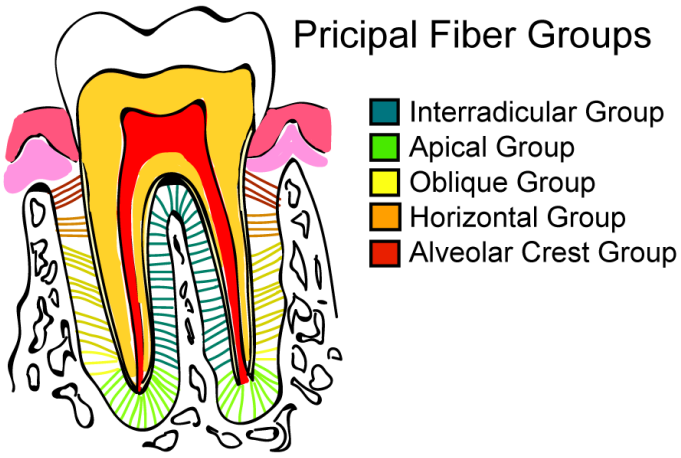


Figure 1.1. Components of the periodontium. Different types of principal fiber groups are indicated with different colors. White crown: animal. Yellow: dentin. Red: dental pulp. Pink: Gingiva. Black outline: alveolar bone.

the middle third of the root. PDL space also decreases progressively with age[1]. Most PDL space is taken up by bundles of collagen fibers (mainly Type I) that are embedded in the intercellular substance. The terminal portion of the fibers that insert in the cementum and alveolar bone is termed Sharpey's fibers. These fibers can be divided into the principal fibers, the accessory fibers and the oxytalan (elastic) fibers. According to their orientation and location along the tooth, the principal fibers can be further categorized into the transseptal fiber (or interdental ligament) and alveolodental ligament (Figure 1.1). Transseptal fibers extend interproximally connecting the cementum of adjacent teeth to maintain tooth alignment, and the alveolodental ligament group of fibers helps teeth withstand compression forces during mastication. In addition to principal fibers, accessory fibers run from alveolar bone to cementum in different planes, more tangentially to prevent rotation of the tooth. Besides PDL fibers, paradental cells of different functions reside in the PDL space, including: 1) synthetic cells like fibroblasts which make up 50-60% of total PDL cellularity, osteoblasts, and cementoblasts; 2) resorptive cells such as

osteoclasts, fibroblasts, cementoclasts; 3) progenitor cells including undifferentiated mesenchymal cells; 4) defense cells such as macrophages, mast cells and lymphocytes; and 5) epithelial cells, i.e. remnants of the epithelial root sheath of Hertwig[1]. Together, these various cells participate in the homeostasis of the periodontium. Finally, the PDL space is filled with tissue fluid known as interstitial fluid, which is ultimately derived from the vascular system. This fluid-filled chamber allows the PDL space to evenly distribute forces loaded onto teeth, serving as a shock absorber.

The alveolar bone is a mineralized connective tissue that consists of, by weight, mineralized tissue (60%), organic matrix (25%) and water (15%)[2]. While the majority of alveolar bone is trabecular, a plate of compact bone called the lamina dura lies adjacent to the PDL space. PDL fibers anchor to the alveolar bone by piercing through the lamina dura, while the other ends connect to the cementum (Figure 1.1). Multiple cell types, namely osteoblasts, osteoclasts and osteocytes, play critical roles in the homeostasis and function of the alveolar bone. In addition, macrophages, endothelial cells and adipocytes can also be found within the alveolar bone. Osteoblasts are mononucleated and specialized "bone forming" cells. Both osteoblasts and fibroblasts can synthesize Type I collagen matrix. Osteoblasts differ from fibroblasts because they can express Runx2 (aka. Cbfa1), a master switch for osteoblast differentiation from mesenchymal progenitor cells[3]. The number of osteoblasts decreases with age, leading to an imbalance of bone deposition and resorption[4]. Osteocytes are derived from osteoblasts that are embedded in mineralized bone during bone apposition. During this process, minerals such as hydroxyapatite, calcium carbonate and calcium phosphate get deposited around the osteocyte, forming lacuna, the space that an osteocyte occupies during its entire lifespan (Figure 1.3). Lacunae are connected via narrow channels known as cannaliculi, where dendrites of osteocytes

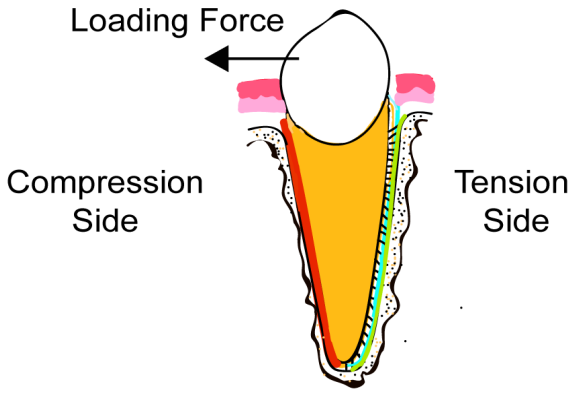
contact and communicate via gap junctions. Unlike the "bone forming" osteoblasts and osteocytes that arise from the mesenchymal cell lineage, the "bone resorbing" osteoclasts originate from a different progenitor population, the hematopoietic/monocyte lineage, and are formed by the fusion of multiple monocytes becoming "multinucleated". Osteoclasts are characterized by their high expression of Tartrate Resistant Acid Phosphatase (TRAP), Cathepsin K, Chloride channel 7 (ClCN7), and Osteoprotegerin (OPG). Cathepsin K is a protease capable of catabolizing bone matrix proteins such as elastin, collagen and gelatin. ClCN7 shuffles chloride ions through the cell membrane, thereby maintaining osteoclast neutrality. OPG (aka osteoclastogenesis inhibitory factor or tumor necrosis factor receptor superfamily member 11B) is an osteoblast expressed decoy receptor for the receptor activator of nuclear factor kappa B ligand (RANKL), thus inhibiting osteoclast differentiation by blocking RANK and RANKL docking. RANKL is expressed on osteoblasts, and it promotes osteoclast differentiation by binding to RANK on osteoclast precursors [5, 6].

Orthodontic Tooth Movement: the Biological Response to Sustained Force

Orthodontic tooth movement is a process that combines physiologic alveolar bone adaptation to mechanical strains with minor reversible injury to the periodontium[7]. Under normal/healthy conditions, such movement is carried out by highly coordinated and efficient bone remodeling, which requires coupling of bone formation following bone resorption. The classic pressure-tension theory proposes chemical, rather than electric, signals as the stimulus for cellular differentiation and ultimately tooth movement. This theory proposes that, within a few seconds upon force loading, the tooth shifts its position within the PDL space, resulting in PDL

compression in some areas and PDL stretch or tension in others (Figure 1.2). While blood flow is decreased on the compression side, it is maintained or increased on the tension side. If the loading force is sustained, the alteration in blood flow quickly (in minutes) changes the oxygen tension (O_2:CO_2 level) and the chemical environment by releasing biologically active agents such as prostaglandins (PGs) and cytokines (e.g. Interleukin (IL)-1β). These chemical mediators differentially affect cellular activities in the compression vs. tension areas within the PDL, promoting a net outcome of bone resorption at the compression side and bone formation at the tension side. Force magnitude is associated with varied cellular responses on the compression side. Heavy force cuts off blood blow, resulting in cell death under compression (hyalinization). As a result, no osteoclast differentiation occurs within the compressed PDL space; instead, a delayed recruitment/differentiation of osteoclasts from adjacent bone marrow space is responsible for the "undermining resorption" that removes the lamina dura next to the compressed PDL. Tooth movement follows completion of these processes on the compression side, but not before. Therefore, it usually takes 7-14 days for tooth movement to occur when heavy force is applied. By contrast, light force only reduces blood flow, allowing quick recruitment of osteoclasts either locally (the first wave) within the PDL or via blood flow (the larger second wave). These osteoclasts remove the lamina dura in the process of "frontal resorption." Tooth movement begins soon thereafter, usually within 2 days after light force application. Clinically, it is almost impossible to avoid blood vessel occlusion completely, thus hyalinization always occurs to a certain degree and tooth movement is a result of combined undermining and frontal resorption[8]. During the time of resorption and tooth movement happening at the compression side, the PDL space at the tension side, somewhat lagging behind,

become enlarged, and the osteoblasts are recruited locally in the PDL (from progenitor cells) and begin remodeling on the tension die by forming bone there[9] (Figure 1.2).



	Compression	Tension
Increase	Cox2 → PGE_2 → RANKL, M-CSF TNFα MMPs eNOS → NO IL-1β	IL-10 → OPG TGFβ TIMPs iNOS → NO
Decrease	OPG	RANKL
Outcome	↑ Osteoclasts ↑ Resorption ↓ Apposition	↓ Osteoclasts ↓ Resorption ↑ Apposition

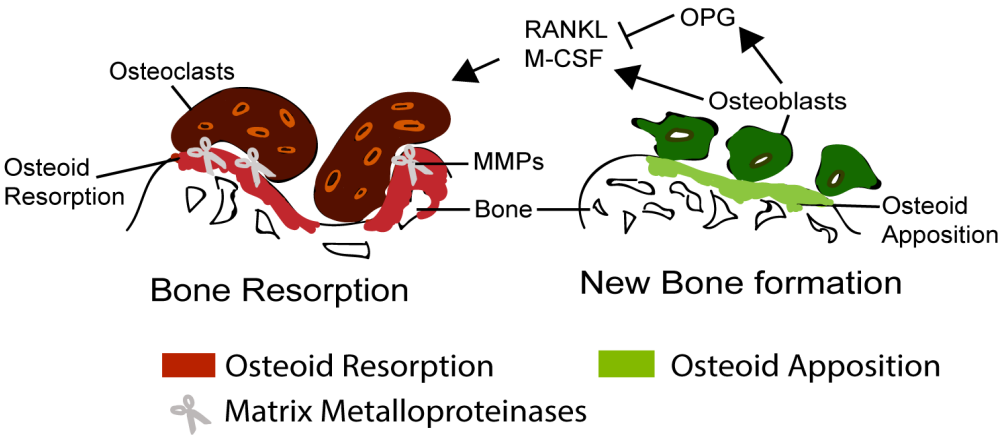


Figure 1.2. Signaling pathways associated with compression and tension due to orthodontic loading. Distinct signaling factors are upregulated and downregualted associated with compressive and tensile strain, as summarized in the table, with the net outcome of resorption in compression and bone apposition in tension.

As discussed, orthodontic loading alters blood flow in the PDL and regional hypoxia develops. Reduction in O_2 tension stabilizes Hypoxia Inducible Factor-1 (HIF-1), a transcription factor that activates Vascular Endothelial Growth Factor (VEGF) and RANKL expression in PDL fibroblasts and osteoblasts; osteoclast differentiation is also increased, favoring resorption in areas of compression[10-11]. With mild hypoxia, HIF-1 stimulates cell proliferation and angiogenesis downstream of VEGF, promoting regeneration of the PDL and its blood supply[12]. Hypoxia is a critical initiator for orthodontic tissue remodeling that acts in concert with loading-induced fluid flow, another activator of signaling. The fluid flow hypothesis focuses on osteocyte and fibroblast response to strain due to fluid displacement in canaliculi[13]. Force application initiates a sequence of events including: 1) matrix strain and fluid flow; 2) cell strain; 3) cell activation and differentiation; and 4) tissue remodeling[14]. Mechanoreceptor cells that detect strain are present in the bone, as osteocytes, and in the PDL, as fibroblasts. Loading causes remodeling of mineralized tissue (bone) and non-mineralized paradental tissues (the PDL, gingiva and neurovascular supply)[15]. When teeth are loaded, interstitial fluid is forced through the canaliculi and around osteocytes, causing strain on the cell surface and extracellular matrix (ECM). The ECM is a network of fibrous structural proteins embedded in a polysaccharide gel surrounding cells. Fluid flow applies shear stress to the ECM and cell membrane, perturbing Integrins and activating signaling cascades in osteocytes[13,16] (Figure 1.3). Integrins are transmembrane proteins that tether a cell's external ECM to its internal cytoskeleton. Integrin stimulation on the cell surface causes release of intracellular molecules that alter osteocyte gene expression, promoting differentiation of osteoblasts and osteoclasts to form and resorb bone. Intracellular calcium rises, increasing phospholipase A activity which releases arachidonic acid, the precursor to prostaglandins; cyclooxygenase (COX) enzymes then convert arachidonic acid

to prostaglandins, key inflammatory mediators [7,13,16]. Second messengers cAMP and cGMP are elevated downstream of calcium, prompting phosphorylation events and subsequent gene expression change with release of autocrine and paracrine signals initiating bone turnover

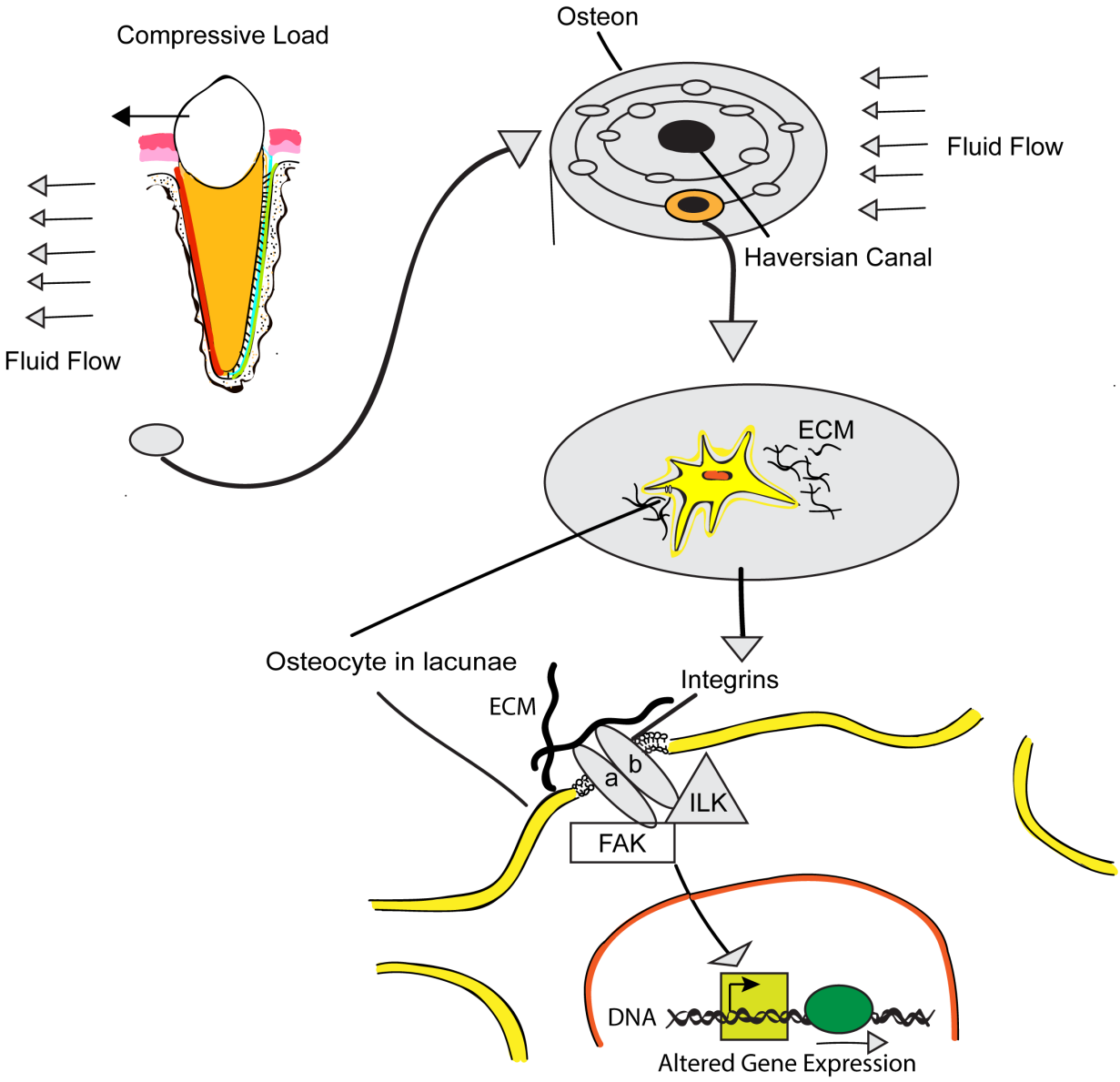


Figure 1.3. Role of fluid flow in orthodontic tooth movement. Tooth loading causes flow of interstitial fluid around osteocytes, resulting in strain on the extracellular matrix (ECM) perturbing membrane- bound Integrins. Integrins activate focal adhesion kinase (FAK) and an intracellular signaling cascade culminating in altered gene expression and tissue remodeling.

(discussed below) [16,17]. While osteocytes are mechanosensors that couple strain from orthodontic loading to tissue remodeling[15], fibroblasts serve a similar mechanosensor function in the PDL and gingiva. When Strain perturbs the ECM in the PDL and gingiva, like osteocytes, fibroblasts express transmembrane Integrin receptors. Stress from mechanical loading is transmitted intracellularly from the ECM via Integrins, which induces a signaling cascade through focal adhesion kinases (FAK) to alter gene expression, cytoskeletal organization, proliferation and differentiation, and ultimately tissue remodeling[18-19].

Role of inflammation in Orthodontic Tooth Movement

Fluid-induced strain and hypoxia synergistically promote bone and PDL remodeling, by inducing an aseptic inflammatory response devoid of bacteria. Tooth loading causes areas of tension and compression of the PDL, its associated nerve endings and blood vessels. PDL nerve endings are tightly associated with blood vessels. When nerve endings are distorted, they release vasoactive neurotransmitters, e.g. substance P and CGRP, which interact with vascular endothelial cells causing vasodilation and increased permeability with plasma leakage[7,16]. The activated endothelium binds and recruits circulating leukocytes, monocytes, and macrophages to the PDL, signifying the onset of acute inflammation[15,20]. Leukocytes elaborate cytokines, prostaglandins, growth factors and colony-stimulating factors (CSF) that promote tissue remodeling[21,22]. After several days, inflammation transitions from acute to a chronic and proliferative process involving fibroblasts, endothelial cells, osteoblasts and osteoclasts.

Native paradental cells, leukocytes, and platelets release a milieu of inflammatory factors initiating functional units to remodel bone and paradental tissues; factors include cytokine IL-1β, IL-6, IL-10, Nitric Oxide (NO), Tissue necrosis factor-α (TNF-α), tissue growth factor β (TGF-

β), macrophage colony-stimulating factor (M-CSF), Prostaglandins, OPG, and RANKL. Compression and tension zones are associated with specific mediators regulating resorption and deposition, respectively (Figure 1.2). Compression is associated with elevated Cycloxygenase-2 (COX-2) that catalyzes production of prostaglandins, including PGE_2, from arachidonic acid[23]. Prostaglandins can stimulate both osteoclastic and osteoblastic activities, serving as suitable mediators for tooth movement. They act on osteoclasts, increasing intracellular cAMP concentrations and boosting their resorptive activity[16]. PGE_2 can also stimulate osteoblast differentiation and expression of RANKL and OPG[23,16]. An increase in RANKL and M-CSF and a decrease in OPG release by osteoblasts collectively favor osteoclast differentiation and bone resorption. Release of cytokines IL-1β and TNFα induces osteoclast differentiation, function and survival while increasing inflammation and matrix metalloprotease (MMP) levels[23,16,24]. Cathepsins and MMPs including collagenase, degrade PDL ECM and the boney organic matrix, allowing osteoclast attachment for resorption[14]. Compression also activates inducible Nitric Oxide Synthase (iNOS) to produce nitric oxide (NO), which mediates inflammation-induced bone resorption[25]. These factors recruit and activate osteoclasts to form resorptive lacunae in the compressive zone[25]. Tooth movement begins once necrotic tissue is removed by osteoclasts, followed by osteoblasts creating osteoid with new periodontal fibrils embedded in the alveolar bone wall and root cementum (Figure 1.2). Compression-induced bone morphogenic protein (BMPs) and Runx2 expression potentiate osteoblast differentiation and bone mineralization, while proliferating and active fibroblasts upregulate ECM fiber production[7,23,26]. The compressed bone and PDL are disassembled and then rebuilt.

Under tension, alveolar bone deposition predominates, with an increase in osteoblast numbers and activity. Tensile strain stimulates osteoblast progenitor proliferation in the PDL and

activates endothelial Nitric Oxide Synthase (eNOS) to increase NO to mediate bone formation[25]. Cytokine IL-10 increases in areas of tension, boosting OPG and reducing RANKL production by osteoblasts; there is an overall reduction in RANK signaling, favoring bone deposition through inhibition of osteoclast formation, activity and survival[23]. TGF-β is also enriched under tension, and induces proliferation and chemotaxis of PDL cells, upregulates Col-I (collagen gene), recruits osteoblast precursors, induces their differentiation, downregulates MMPs and upregulates tissue inhibitors of metalloproteases (TIMPs)[15,27]. MMPs and their inhibitors, TIMPs, act in concert to regulate remodeling and have localized expression patterns, suggesting careful coordination of turnover [7,26]. The cumulative result is increased osteoblast and reduced osteoclast activity, with production of bone and remodeled PDL fibers on the side opposite tooth movement (Figure 1.2).

Inflammatory factors are central to tissue remodeling for tooth movement, yet many orthodontic patients take non-steroidal anti-inflammatory drugs (NSAIDs) for pain relief that inhibit COX enzymes and their production of PGs, slowing rates of tooth movement. Acetaminophen (Tylenol), on the other hand, acts centrally rather than peripherally and is the preferred pain reliever for orthodontics. In rabbit studies, the rate of orthodontic tooth movement was unaffected by Acetaminophen, while Ibuprofen and Aspirin resolve pain but slow tooth movement[28,29]. Pharmacological inhibition of inflammation is associated with retarded tooth movement, underlying the importance of inflammation in orthodontic tissue remodeling.

While the inflammatory cascade is critical for OTM, unregulated or excessive inflammation is problematic. For example, remodeling of tissue and orthodontic-induced inflammatory root resorption (OIIRR), or simply known as root resorption, should be limited to the bone and paradental tissues, excluding the cementum and tooth. However, in 1-5% of

orthodontic patients, excessive root resorption is observed, with loss of greater than 4mm or a third of the original root length[17,30]. Reducing root length diminishes the crown-to-root ratio of affected teeth, with potentially great clinical significance. The cellular mechanism of OIIRR is similar to osteoclastic bone resorption and correlates with elevated concentrations of RANKL and reduced OPG in the PDL[16,31,32]. In patients with excessive root resorption, regulation of bone remodeling is compromised due to excess elaboration of cytokines and pro-resorptive ligands[33]. This signifies the importance of regulation of inflammation: while inflammation is necessary for orthodontic tooth movement, if uncontrolled, it leads to tooth destruction, similar to uncontrolled periodontal disease serving as an anti-bacterial defense while causing collateral tissue damage[33]. Orthodontic treatment in patients suffering from periodontal disease is particularly dangerous, as the combination of aseptic inflammation and periodontal-related inflammation cause accelerated attachment loss and disease progression. Orthodontists must carefully screen for periodontal disease to avoid worsening periodontal status via braces due to excessive inflammation, particularly in adult patients.

Connection between Inflammation and Autophagy

While initiation of inflammation promotes necessary host defense upon mechanical strains during OTM, it is equally important to ensure a timely resolution of such inflammation to maintain homeostasis. In normal conditions, inflammation occurred during OTM appears to be well controlled, as the PDL space remains fairly constant in healthy individuals during treatment. In contrast to extensive studies on the mechanisms underlying inflammation initiation, little research had focused on mechanisms in regulation of inflammation, in particular in inflammation resolution during OTM. Inflammation is an energy-intensive process; consequently, metabolism

is closely associated with immune functions including inflammatory responses. Autophagy plays a role in metabolism by providing energy in conditions of cellular stress in order to maintain homeostasis. Increasing evidence has suggested a close relationship between autophagy and inflammation during both physiological and pathological conditions [34].

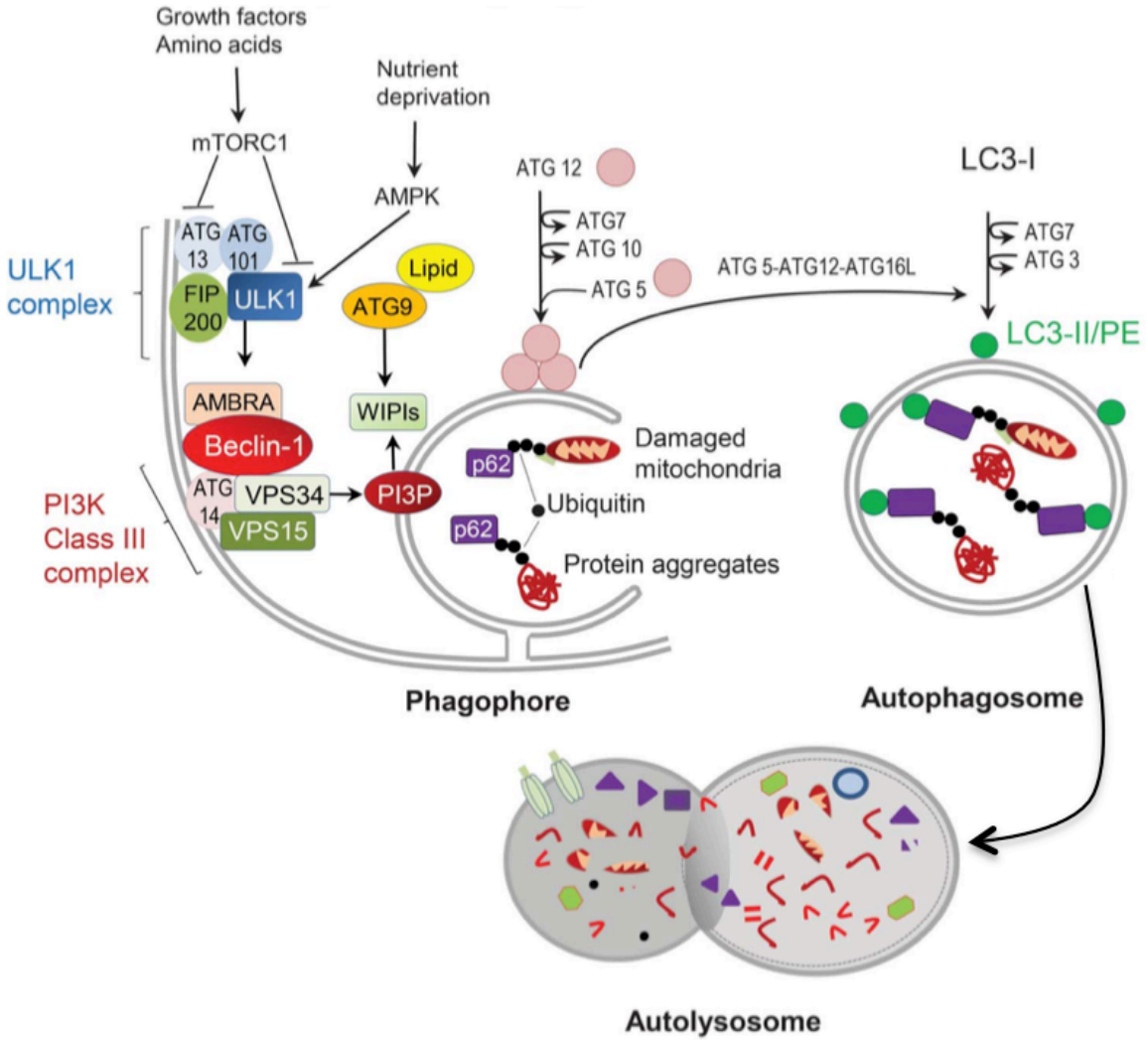


Figure 1.4. (Macro)autophagy pathway. Autophagy is normally inhibited by mTORC1. Under stressful conditions, decreased mTORC1 and increased AMPK activities lead to activation of ULK1, which phosphorylates several downstream proteins and ultimately induces phagophore formation. Phagophore engulfs damaged cytosolic components to form autophagosome. During this process, microtubule-associated proteins 1 light chain 3 (LC3) undergoes phosphatidyl-ethanolamine (PE) conjugation to be located to the autophagosome, facilitating the closure of the phagophore. Autophagosome fuses with lysosome, allowing sequestered contents to be broken down by lysosomal hydrolase (Modified from Kenney DL et al. (2015) Neurology 85(7): 634-645[35])

There are several forms of autophagy, among which the most important and relevant is macroautophagy (henceforth referred to as autophagy). It is a highly conserved and sensitive intracellular catabolic pathway that can be induced in a wide range of stressful conditions, such as hypoxia, starvation, toxin accumulation and damaged organelles. Under basal condition, the central autophagy inhibitor mTOR (mammalian/mechanistic target of rapamycin) complex 1 (mTORC1) associates with ULK1/2-ATG13-FIP200 complex and inhibits autophagy activity via phosphorylation of ULK1/2 (unc-51 like autophagy activating kinase 1/2) and ATG13. Under stressful condition, mTORC1 is inhibited and dissociated from the ULK1/2-ATG13-FIP200 complex and adenosine monophosphate activated kinase (AMPK) is activated, allowing ULK1/2 to become activated and phosphorylate several downstream proteins that leads to activation of autophagy. Autophagy activation results in the formation of phagophore, a double-layer membrane that engulfs the damaged cytosolic components. During this process, microtubule-associated proteins 1 light chain 3 (MAP1LC3 or LC3) undergoes phosphatidylethanolamine (PE) conjugation to facilitate the closure of phagophore. Complete sequestration of these components results in the formation of autophagosome, which then fuses with the lysosome, allowing the sequestered contents to be broken down by lysosomal hydrolases and subsequently released in the cytosol for reuse [35] (Figure 1.4 and 1.5).

In addition to homeostasis and cell survival, autophagy has also been identified as a key player in host defense through several mechanisms, including: innate immunity and direct elimination of invading pathogens, induction of innate immune memory, control of adaptive immunity through regulation of antigen presentation, and interaction with inflammation. Several mediators of inflammation have shown to regulate autophagy activity. Engagement of various pattern recognition receptors (PRRs), such as TLRs (toll-like receptors) and NLRs (NOD-like

receptors have been reported to induce autophagy through pathways via mTORC1 and AMPK. In addition to PRRs, several Th1 type/proinflammatory cytokines, such as TNFα and IL-1β, have been shown to induce autophagy[36]. By contrast, Th2 type/ anti-inflammatory cytokines, such as IL-4, IL-10 and IL-13 generally suppress autophagy induction via activation of mTOR and stimulation of PI3K/Akt signaling pathway[36,37]. Effects of IL-6 (a Th2 type proinflammatory cytokine) on autophagy seem to vary depending on tissue types. IL-6 enhanced autophagy in mouse pancreatic tumor cells and myeloid cells[38,39]. However, overexpression of IL-6 suppressed autophagy in human bronchial epithelial cells and enhanced autophagy counteracted the effect of IL-6[40]. Recently, compelling evidence demonstrates another layer of autophagy regulation by inflammatory response through control of transcription factors. For instances, NFκB (nuclear factor-κB) has been shown to upregulate transcription of Beclin 1 and Sequestosome 1/p62[41,42]. Other inflammation-related transcription factors capable of regulating proteins involved in autophagy pathway include HIF-1, JUN, STAT 1 and STAT 3 (signal transducers and activators of transcription) [43].

While inflammation affects autophagy activities in several ways, the opposite is also true, i.e. autophagy can also regulate inflammation. Autophagy has been shown to negatively regulate IL-1β- inflammasome activation and IL-1β and IL-18 production. Mice deficient in Atg16l1, essential molecule for autophagy, showed higher levels of IL-1β and IL-18 in response to LPS stimulation or during colitis[44]. Inhibition of autophagy in human primary cells also led to increased production of IL-1β[45]. Conversely, in macrophages treated with TLR, further activation of autophagy by rapamycin induced degradation of pro-IL-1β and blocked mature cytokine production[46]. In addition to its inhibitory effect on inflammasome, autophagy also reduces NFkB activation by selective degradation of Bcl 10 complexes [47].

Taken together, compiling evidence suggests that inflammation and autophagy are closely related. Derangement of the crosstalk between these two processes can have deleterious consequences. Indeed, defective autophagy activities are associated with a wide range of disease conditions in which hyperinflammatory conditions prevail, such as metabolic disorders (diabetes and obesity), Crohn disease, Chronic Granulomatous Diseases, and neurodegenerative diseases (Alzheimer's and Parkinson diseases) etc. The relationship of autophagy with inflammation can sometimes be quite circuitous. For instances, in metabolic disorders, suppression of autophagy in diseased tissues leads to increased inflammation with overexpression of cytokines, which in turn results in an activation of autophagy[48]. One may suspect that that autophagy may contribute to its positive role on health by restraining the detrimental side effects of (chronic) inflammation. Interestingly, autophagy efficiency is decreased during aging, concomitantly to an increase in the basal inflammation level [49,50]. This further supports the notion that autophagy potentially protects cells from excessive long lasting inflammation in order to maintain healthy state.

Autophagy and bone remodeling

There is emerging evidence that autophagy is involved in bone remodeling. Studies in murine rheumatoid arthritis have shown that TNFα induced autophagy in osteoclasts, and activation of autophagy by overexpression of Beclin-1 promoted osteoclastogenesis and enhanced resorptive capacity of cultured osteoclasts[51]. Nollet et al. investigated the role of autophagy in osteoblasts and found that autophagy was induced in osteoblasts during mineralization under oxidative stress. Osteoblast-specific autophagy-deficient mice had 50% reduction in trabecular bone mass; osteoblasts with defective autophagic activity showed increased oxidative stress and secretion of RANKL, favoring generation of osteoclasts and bone

resorption[52]. Moreover, Sambandam et al. studied autophagy under microgravity conditions and found microgravity induced autophagic activity in preosteoclast cells, and increased autophagy in turn enhanced osteoclast differentiation[53].

Conclusions

A better understanding of biological mechanisms underlying tooth movement helps guide our efforts towards new approaches to solve current challenges of orthodontic treatment. Specifically, although the role of inflammation during OTM has been widely studied, its regulation, especially how inflammation is kept under control, has remained poorly understood. Autophagy has been shown to have a close relationship with inflammation in other biological systems and disease conditions, and it also appears to be involved in bone remodeling via its activation in osteoclasts and/or osteoblasts. In our present study, we hypothesize that autophagy may play an important role in modulating inflammatory responses, likely in a negative feedback fashion, which is critical during the course of OTM. We are hopeful that our study will provide insights for future translational and clinical research that will ultimately lead to improvement of the quality, rate and stability of tooth movement in orthodontic practice.

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THE ROLE OF AUTOPHAGY DURING ORTHODONTIC TOOTH MOVEMENT

Introduction

Orthodontic tooth movement (OTM) is a pathophysiologic process that combines the alveolar bone adaptation with minor reversible injury to the periodontium in response to mechanical strains. Under healthy conditions, such a movement is achieved by highly efficient and coordinated bone remodeling, involving a net bone resorption by osteoclasts at the compression side and bone formation by osteoblasts at the tension side. This process is regulated by a dynamic aseptic inflammatory response that is characterized by the release of inflammatory mediators, such as prostaglandins and cytokines. Shortly after the application of external force, inflammatory mediator prostaglandins (mainly prostaglandin E [PGE]) are released from cells under mechanic deformation [1]. PGE can stimulate not only the number and activity of osteoclasts [2,3,4], it can also induce osteoblast differentiation and new bone formation [5], suggesting its ability to coupling the two aspects of bone remodeling. In addition to mechanical force-induced release of inflammatory mediators, abundant evidence has demonstrated neurovascular mechanisms also play important roles in initiating inflammation during OTM. Following mechanic loading, there is an initial reduction in the diameter and number of PDL blood vessels at the compression side within the PDL [6,7]. PDL neurotransmitters (substance P, CGRP, VIP etc.) act on endothelial cells to cause vasodilation and increased vasopermeability, promoting the circulating leukocytes to migrate into the paradental extracellular matrix. The migratory leukocytes, together with the residential paradental cells

(fibroblasts and osteoblasts), activate an aseptic inflammatory reaction by producing ample amounts of chemokines and cytokines, notably IL-1β, IL-6, TNFα, IL-8, and IL-11 [8-12]. These inflammatory cytokines act on PDL cells, promoting osteoclastogenesis through upregulation of Receptor Activator of Nuclear Factor Kappa-B Ligand (RANKL) [13,14,15]. Increased RANKL together with a decreased release of Osteoprotegerin (OPG) by osteoblasts favors osteoclast differentiation and bone resorption. Release of cytokines IL-1β and TNFα also induces osteoclast differentiation, function and survival [16,17,18]. At the tension side, IL-10 level is increased, boosting OPG and reducing RANKL production by osteoblasts, which favors bone deposition through inhibition of osteoclast formation[16]. TGFβ level is also elevated under tension, which induces proliferation and chemotaxis of PDL cells as well as recruitment of osteoblast precursors and osteoblast differentiation [19,20].

While initiation of inflammation promotes necessary host defense upon mechanical strains during OTM, it is equally important to ensure a timely resolution of such inflammation to maintain homeostasis. In normal conditions, inflammation occurred during OTM appears to be well controlled, as the PDL space remains fairly constant in healthy individuals during treatment. In contrast to extensive studies on the mechanisms underlying inflammation initiation, little research had focused on mechanisms in regulation of inflammation, in particular in inflammation resolution during OTM. To identify such potential regulatory mechanisms, we found that autophagy, an evolutionarily conserved self-protecting process, has important effects on the modulation of inflammatory response. Defective autophagy has been shown to contribute to many disease conditions in which hyperinflammatory conditions prevail [21].

There are several forms of autophagy, among which the most important and relevant is macroautophagy (referred to as autophagy in our study). It is a highly conserved and sensitive

intracellular catabolic pathway that can be induced in response to a wide range of stressful conditions (e.g. starvation, hypoxia, toxin accumulation, damaged organelles). Autophagy activation results in the formation of autophagosome, a double-layer membrane structure that sequesters the damaged cytosolic components. The autophagosome then fuses with the lysosome, allowing the sequestered contents to be broken down by lysosomal hydrolases and subsequently released in the cytosol for reuse [22]. In addition to homeostasis and cell survival, increasing evidence has identified autophagy as a key player in host defense, in particular through its crosstalk with inflammation. Several Th1 type/proinflammatory cytokines, including IL-1β and TNFα, have shown to induce autophagy [23], whereas Th2 type/ anti-inflammatory cytokines such as IL-4, IL-10 and IL-13 generally suppress autophagy induction via activation of mTOR (mammalian/mechanistic Target Of Rapamycin), a central inhibitor of autophagy [23,24]. Effects of IL-6 (a Th2 type proinflammatory cytokine) on autophagy seem to vary depending on tissue types. IL6 enhances autophagy in mouse pancreatic tumor cells and myeloid cells [25,26]. However, overexpression of IL6 suppresses autophagy in human bronchial epithelial cells and enhanced autophagy counteracted the effect of IL-6 [27]. Autophagy can also modulate inflammatory reaction, primarily through its inhibitory effects on inflammasome activation, and IL-1β and IL-18 production [28,29]. In addition to its inhibitory effect on inflammasome, autophagy has also been shown to reduce the activation of NFκB (nuclear factor of kappa light polypeptide gene enhancer in B-cells) by selective degradation of Bcl 10 complexes[30], thereby indirectly affecting genes involved in inflammation that are controlled by NFκB.

In our present study, we aimed to investigate the potential role of autophagy pathway in regulating inflammatory response after force loading using a well-established mouse model of

orthodontic tooth movement (OTM). We hypothesized that autophagy may negatively regulate inflammation, which is critical for normal OTM.

Materials and Methods

Mouse model for studying OTM

Orthodontic force application in mice is a well-accepted model in OTM studies. Optimal conditions have been previously characterized[31]. Briefly, mice were anesthetized by intraperiotoneal injection of solution containing xylazine (10mg/kg) and ketamine (100mg/kg). Thirty grams (=0.3N) of force was delivered to the maxillary right first molar in the mesial direction, by bonding a nickel-titanium (NiTi) closed coil spring (American Orthodontics, Cat# 855-181, length shortened to adapt to each mouse's mouth) between the maxillary right first molar and incisors with light-cured resin (Transbond Supreme LV, 3M Unitek, Morovia, Calif) (Figure 2.1 A and B). No reactivation of spring was performed during the experimental period. All procedures and animal care followed the ethical regulations for animal experiments, defined by Institutional Animal Care and Use Committee (IACUC) of the University of North Carolina at Chapel Hill. All mice were monitored daily and given softened food after spring loading, and there was no significant weight loss during the entire experiment period.

For histomorphometric analyses, i.e. OTM distance measurement, detection of autophagy activity and TRAP staining, we utilized a GFP-LC3 reporter mouse line. This mouse line carries a GFP-LC3 fusion protein that is normally associated with isolated membrane without autophagy activity. When autophagy is activated, GFP-LC3 fusion protein is relocated to the autophagosome and begins to give off GFP signal[32]. Thirty GFP-LC3 adult male mice (8-9 weeks old, in C57BL/6 background) were subdivided into 6 groups (n=5 for each group), and

were sacrificed at different time points (days 1, 3, 5, 7, 10 and 14) after spring loading. For molecular analyses, i.e. mRNA/qRT-PCR and Western Blot, 60 wild-type adult male mice (C57BL/6, 8-9 weeks old, obtained from Jackson Laboratory, Bar Harbor, ME) were subdivided into 6 groups, half (n=5) for mRNA/RT-PCR and half (n=5) for Western Blot experiments. Except for group 1-control mice (without spring loading) that were sacrificed at day 0, the other groups were sacrificed at different time points (days 1, 3, 5, 7 and 10) post spring loading.

For rapamycin injection experiments, intraperiotoneal injection of rapamycin (6mg/Kg/day) or rapamycin vehicle (control) solution were given to mice daily beginning on the day of spring placement and until the mice were sacrificed. Rapamycin solution was made as previously described[33]. Briefly, a 20mg/ml stock solution of rapamycin (LC Laboratories, Woburn, MA) was prepared in ethanol. The stock solution was diluted to 1.2 mg/ml in vehicle solution (saline containing 5% polyethylene glycol 400 and 5% Tween-80). The vehicle solution was prepared under the same conditions without inclusion of rapamycin. Sixty GFP-LC3 adult male mice (8-9 weeks old, in 57BL/6 background) were subdivided into 6 groups (n=10 for each group, half injected with rapamycin and half injected with rapamycin vehicle solution), and were sacrificed at different time points (days 1, 3, 5, 7, 10 and 14) after spring loading. For molecular analysis, 10 wild-type adult male mice (C57BL/6, 8-9 weeks old, from Jackson Laboratory, Bar Harbor, ME), half injected with rapamycin and half injected with rapamycin vehicle solution, were sacrificed 1 day post spring loading for mRNA/RT-PCR experiment.

OTM distance measurement

Occlusal view of the maxilla was imaged using a stereomicroscope (SMZ18, Nikon Instruments, Melville, NY, USA) and an adapted digital camera (Nikon Instruments, Melville, NY, USA) (Figure 2.1C). NIS-Elements Basic Research imaging software was used for distance

measurements, by measuring the distance between two parallel lines tangent of the most convex regions distal of the first molar and mesial of the second molars. Both the right/experiment (E) and left/control (C) sides were measured. The OTM distance measurement of the upper right first molar was determined by subtracting the measurement of the C side measurement from the E side measurement, i.e. OTM distance = E side value – C side value. For each time point measuring OTM distance, 5 mice were used.

Tissue preparation, GFP imaging and histological staining

Adult male mice were sacrificed with CO_2 asphyxiation. Tissue preparation procedures were similar to what was previously described with slight modification[34]. The maxillae including part of scalps were dissected free of most adherent tissue and placed in a processing/embedding cassette (Fisher Scientific, Cat #15-182-706). Samples were fixed in freshly prepared 4% paraformaldehyde at 4 °C for 3 days, followed by decalcification in 14% EDTA solution at 4 °C for 4 days (both solutions were replenished daily). After several washes in 1XPBS for 1-2 hours at room temperature, samples were equilibrated in 30% sucrose (dissolved in 1XPBS) overnight at 4°C. Each maxilla was cut in halves, and each half (E vs. C sides) was embedded in OCT compound (Fisher Healthcare, Cat # 23-730-571) containing base mold (Fisherbrand, Cat # 22-363-553). The embedding media was flash frozen by placing the molds on a metal platform that was prechilled in a dry ice-ethanol bath. Once the media were frozen, the molds were wrapped in aluminum foil and stored at -80°C until they were sectioned.

Cryosectioning was performed on Leica CM 1520 at 6μm thickness, and sections were collected consecutively on Superfrost Plus slides (Fisherbrand, Cat # 22-037-246). Slides were kept in dark at -20 °C until being further processed.

For GFP imaging, frozen sections were briefly washed with 1XPBS and nuclei were counterstained with DAPI solution (Sigma Aldrich, Cat # D9542-10MG). Slides were mounted using VectaShield Mounting Medium (Vector Laboratories, Cat # H-1000) with coverslips. Images were obtained with a Nikon Eclipse Ti-U inverted microscope, using NIS-Element Basic Research Imaging software (Nikon Instruments Inc., Melville, NY, USA). FITC and DAPI filters were applied for GFP and nuclear visualization, respectively, at 20X magnification with an average exposure time of 100ms.

Tartrate-resistant acid phosphatase (TRAP) staining was performed as described[35]. Sections were counterstained with Mayers/Harris Hematoxylin for 1-2 minutes, dehydrated, and mounted with coverslip using VectaShield Hard Set mounting medium. Images were obtained with a Nikon Eclipse Ti-U inverted microscope, using NIS-Elements Basic Research imaging software (Nikon Instruments Inc., Melville, NY, USA). Brightfield (BF) was used for cellular visualization at 20x magnification with an average exposure time of 100ms.

RNA isolation and quantitative Real-Time PCR (qRT-PCR)

RNA extraction and qRT-PCR were performed for each animal separately (for each time point, n=5). After sacrifice of the adult male mice, maxillary first molars and their surrounding PDL/alveolar bone were extracted and collected individually. Tissue total RNA was isolated with TRIzol (Invitrogen) according to manufacturer's instructions. The resultant DNA-free RNA was diluted in RNase-free water and quantified by Nanodrop (Thermo) at 260 nm. RNA samples were stored at −80°C until use. Total RNA was reverse transcribed using iScript™ cDNA Synthesis kit (Bio-Rad, Cat # 170-8891). The iTaq™ Universal SYBR Green Supermix Kit (Bio-Rad, Cat # 172-5120) was used for quantitative real-time RT-PCR analysis. The primers were designed using Primer Express (Applied Biosystems) and synthesized by Invitrogen. The

primer sequences are listed in Table 1. Relative differences in gene expression between groups were determined from cycle time (Ct) values. The values were normalized to B2M in the same sample (ΔCt) and expressed as fold-change over day 0 control ($2^{-\Delta\Delta Ct}$) [36]. Real-time fluorescence detection was carried out using an ABI StepOnePlus Real-time PCR system (Applied Biosystems).

Table 1. Primer sequences for RT-qPCR

Gene	Forward (5' to 3')	Reverse (5' to 3')
BECN1	ATGGAGGGGTCTAAGGCGTC	TCCTCTCCTGAGTTAGCCTCT
ATG5	TGTGCTTCGAGATGTGTGGTT	GTCAAATAGCTGACTCTTGGCAA
LC3	GACCGCTGTAAGGAGGTGC	CTTGACCAACTCGCTCATGTTA
IL-1β	TTCAGGCAGGCAGTATCACTC	GAAGGTCCACGGGAAAGACAC
IL-6	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
TNFα	CCCTCACACTCAGATCATCTTCT	GCTACGACGTGGGCTACAG
B2M	TTCTGGTGCTTGTCTCACTGA	CAGTATGTTCGGCTTCCCATTC

Tissue preparation and Western Blot

At the time of sacrifice, for the same time point, maxillary first molars (n=5) and their immediately surrounding PDL tissue were extracted and polled together from the experimental (E)/loading side or the control (C)/nonloading side. Maxillary first molars without loading were collected as Day 0 control. Samples were stored in liquid nitrogen until use. Specimen were incubated on ice for 20minutes with RIPA buffer containing protease inhibitors (Sigma-Aldrich, cOmplete mini protease inhibitor cocktail, Cat #11836153001), PMSF (1mM) and E64 (2μg/ml). During incubation, pre-chilled pestles (Fisherbrand, Cat 12-141-364) were used to physically grind the tissues. After centrifugation to remove cell debris, supernatants were transferred to a new pre-cooled tube and heated with 1X SDS buffer at 95°C for 5 minutes. Samples were either directly loaded to SDS-PAGE gels or saved at -20°C until ready for Western blot.

Western blot analysis was performed essentially as previously described [37]. For each time point, 45μL lysates per lane were loaded to 4-20% Criterion TGX Stain-Free Proteingels (BioRad, Cat #5678093). Primary antibodies for the analysis include: phopho-ULK1 (Ser 555) (Cell Signaling Cat #5869), p62 (Cell Signaling Cat #5114), LC3b (Cell Signaling Cat #2775), IL-1β (ThermoFisher Cat #MM425B) and Actin (Santa Cruz, Cat # sc-1616). All primary antibodies were used at 1:1000 dilutions.

Statistical analysis

The results in each group were expressed as the mean ± SEM. The data sets were normally distributed; therefore, comparison among different groups from different time points were analyzed by two-way Analysis of Variance (ANOVA), followed by effect and contrast test. The significance level was set to p=0.05.

Results

We first evaluated if the device placement system for our mouse model recapitulated as previously described [31]. By measuring the tooth movement amount at different time points (days 1-14) post force loading, we found that OTM amount gradually and steadily increased over time from day 1 to day 14 (Figure 2.1D). There is a linear correlation between the OTM amount and the time during the examination period (R^2=0.98).

To examine whether autophagy is activated during OTM, we took advantage of a GFP-LC3 reporter mouse line. This mouse line contains a genetically engineered GFP-LC3 fusion protein that is normally associated with isolated membrane without autophagy activity. When autophagy is activated, GFP-LC3 fusion protein is relocated to the autophagosome and begins to give off GFP signal [32]. Therefore, this reporter line is routinely used to detect endogenous

autophagy activity. Upon force loading, we found that LC3-GFP signal was activated as early as day 1 in the compression side, located more apically close to the root tip. The activity increased gradually in the compression side from day 3 to day 7 and appeared to extend more coronally. Such GFP signal appeared to be peaked at day 7, then gradually reduced later on and returned to base line at day 14 after loading (Figure 2.2, top panel). By contrast, no autophagy activity was present in the non-loading control (C) side during the time frame examined (Figure 2.2, bottom panel). During the same time frame, by staining the consecutive sections with TRAP staining to detect osteoclasts, we found that TRAP positive osteoclasts appeared as early as day 3 on the compression side, and highest level of TRAP staining on the compression side were detected at days 7-10, and TRAP staining returned to baseline level at day 14 post force loading (Figure 2.3, top panel). No appreciable differences in TRAP staining were found on the compression in the non-loading control (C) side (Figure 2.3, bottom panel).

To determine if there is correlation between autophagy and inflammatory response after orthodontic force loading, we next examined mRNA expression of inflammatory cytokines (IL-1b, IL-6 and TNFa) and key genes involved in autophagy pathway (Becn1, Atg5 and LC3) by qRT-PCR. We found a sharp spike of IL-1b mRNA expression at day 1, and a slight upregulation of IL-6 mRNA expression that seems stable from day 1 to day 10. We didn't detect appreciable upregulation of TNFa in our experiment setting. We also didn't find much change in mRNA expression of Becn1, Atg5 or LC3 from day1 to day 14 after force loading (Figure 2.4).

To evaluate if changes of autophagy activity occurred at translational/post-translational level, we performed Western blotting and found that levels of phospho-Ulk1 and p62 were reduced as early as day 1 after loading (Figure 2.5). These two proteins are components of autophagy pathway upstream of autophagosome formation, and their levels are inversely

correlated with autophagy activity[38]. Therefore, these western results indicate activation of autophagy pathway as early as day 1 after force loading, consistent with what's shown by GFP-LC3 activity (Figure 2.2).

Based on the above data, it appears autophagy activity correlates well with inflammation during OTM. In order to gain more insight into the mechanism, we want to manipulate autophagy activity by giving male mice daily injection of rapamycin, a well-known autophagy inducer. We reasoned that if autophagy plays a role in modulating inflammatory response during OTM, by giving rapamycin injection to induce autophagy activity, we should expect alteration in inflammation which in turn impact overall tooth movement. While we are still in the process of colleting data, we found that at 7 and 10 days after force loading, there was a statistically significant reduction in tooth movement amount in rapamycin injection group compared to either no injection or vehicle injection groups (Figure 2.6, red asterisks, $p<0.05$). There is no statistically significant difference between vehicle and no injection groups for tooth movement amount (Figure 2.6).

Discussion

Like in many biological processes, controlled inflammation plays a critical role during normal orthodontic tooth movement. While there is abundant evidence demonstrating how inflammatory response is initiated during tooth movement, little is known on how such an important process is regulated. In our present study, we have presented data, for the first time, demonstrating that autophagy activity is closely correlated with inflammatory signal during tooth movement after force loading. Our data also suggest that the changes of autophagy may occur at the translational/post-translational level, but not at the transcriptional level, similar to what has

been found previously in other systems [39,40]. To further understand the underlying mechanism, ie. to determine if autophagy plays a role in modulating inflammation during OTM, we administered rapamycin, an autophagy inducer, to force loaded mice. The rationale is that if autophagy does affect inflammation, given inflammation is critical for normal orthodontic tooth movement, by slowing down inflammation via increased autophagy activity, one should expected reduced tooth movement. Indeed, we observed reduced OTM amount at day 7 and day 10 after force loading, indicating that autophagy may negatively regulate inflammation during OTM. More molecular and cellular analyses are needed to elucidate the underlying mechanism that governs the regulation of inflammation by autophagy pathway.

Studying autophagy pathway not only fulfills an important fundamental question related to inflammation control during OTM, it also has significant clinical implications. According to the 2015 AAO Economics of Orthodontics Survey, the estimated total number of patients in active treatment is 5.419 million, 27% of which are adults (age 18 or older). On average, one orthodontist is treating 125 adult patients in 2014, vs. 41 adult patients in 1989, a dramatic increase especially in recent years [41]. As tooth movement is considerably slower in adults compared to that in adolescents, treating more adults may increase the average length of treatment, potentially adding extra overhead to one's practice. Besides adult patients, there is also a general demand for accelerated orthodontic treatment. Both parents and orthodontists are willing to pay 20% more for faster treatment. Various ways have been explored to expedite the treatment time; however, none have been completely satisfactory [1]. Studying the role of autophagy, in particular by testing pharmacologic modulation of autophagy during OTM, may provide a novel and feasible approach that's directly translational toward accelerated treatment.

Conversely, our study involving autophagy inhibition may allow us to discover new measures to facilitate retention, when tooth movement is undesirable.

Conclusions

1. Autophagy activity is closely correlated with inflammatory signal during orthodontic tooth movement.
2. Rapamycin, an autophagy inducer, reduces tooth movement amount, indicating autophagy may negatively impact inflammation during orthodontic tooth movement.

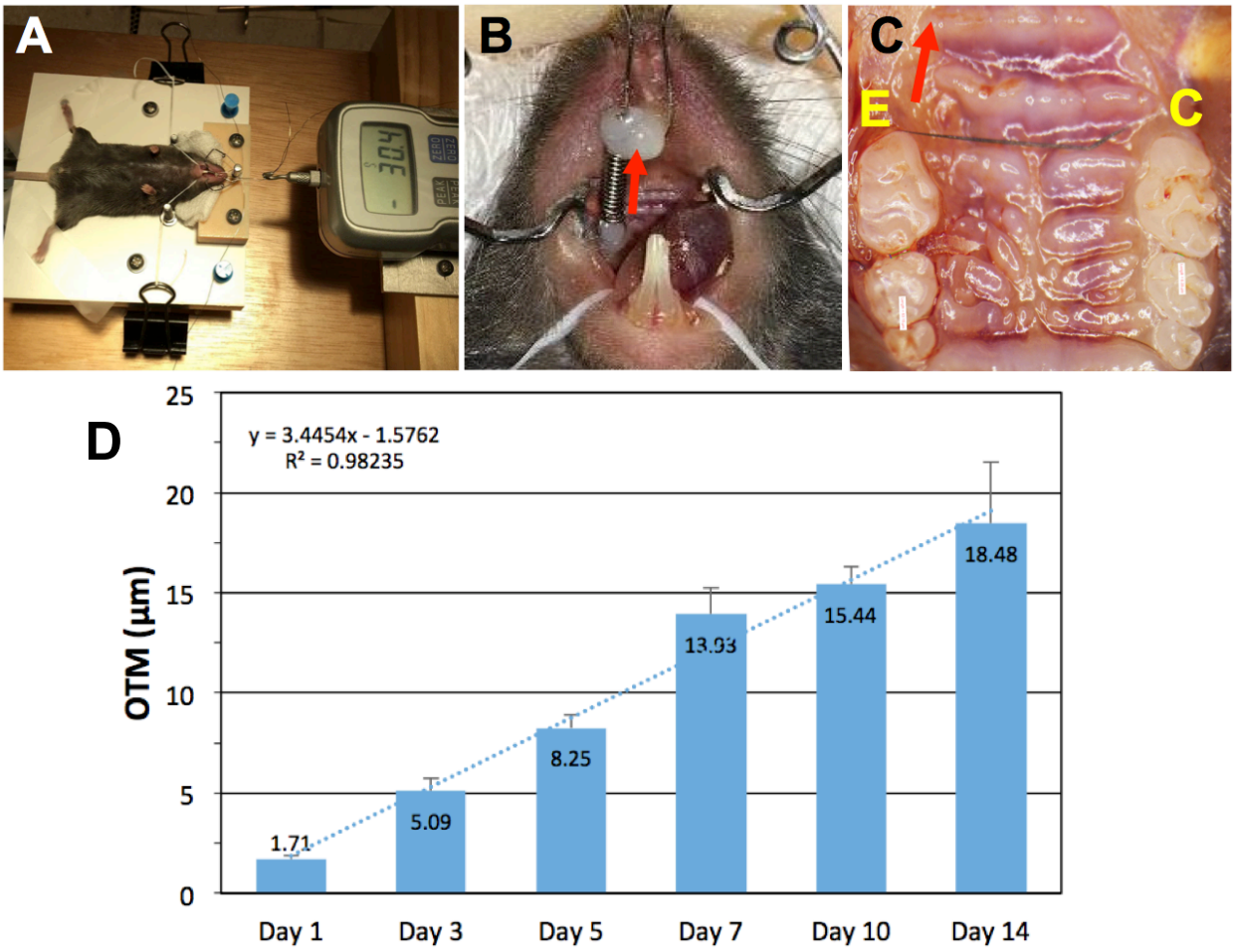

Figure 2.1. Demonstration of spring placement in mice and OTM distance measurement. **A**. Spring placement device, with anesthetized mouse positioned in the dorsal decubitus on the surgical table and a digital tension gauge attached to the surgical table showing 30 grams (=0.3N) of force loading. **B**. A NiTi closed coil spring is bonded between the maxillary right first molar and incisors to deliver the 30 grams of force in mesial direction (red arrow). **C**. An example of occlusal view of the maxilla showing the experiment/loading (yellow colored E) side and the control (yellow colored C) side. **D**. OTM measurement at different time points after force loading. For each time point, n=5. Time and OTM amount show linear correlation of coefficient, R^2=0.98.

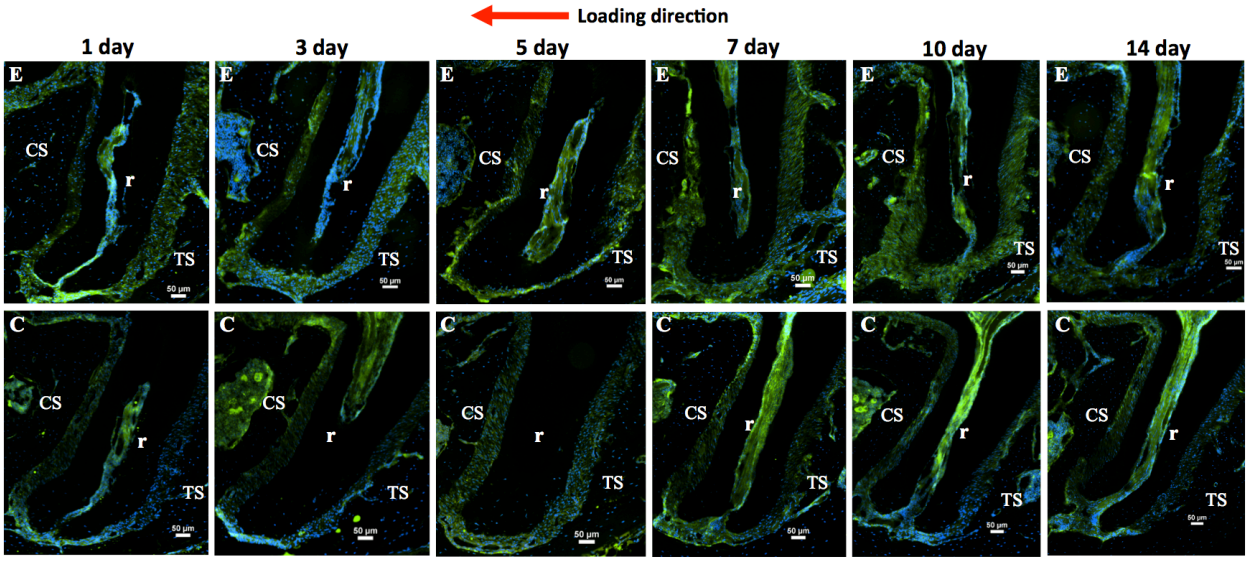

Figure 2.2. Representative images of GFP-LC3 activities around the mesial roots of the maxillary first molars at different time points (days 1-14) after force loading. TS: tension side, CS: compression side, r: root, E: Experiment side, C: control side. Red arrow points to loading direction. Green= GFP signal. Blue=DAPI (nuclei) staining. Bar=50μm.

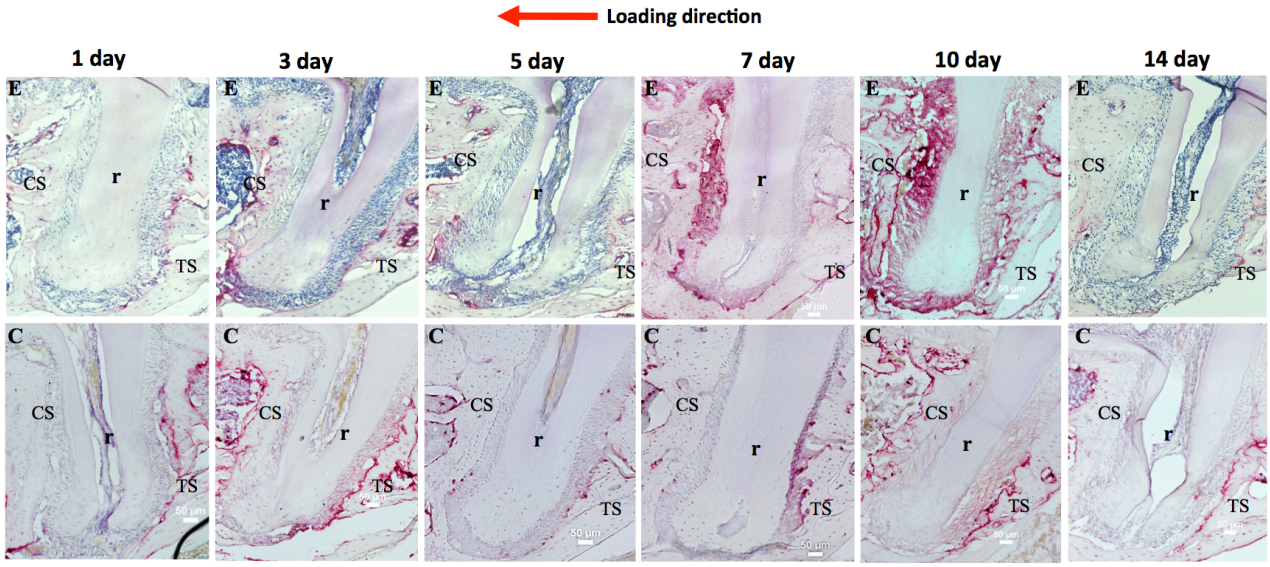

Figure 2.3 Representative images of TRAP staining around the mesial roots of the maxillary first molars at different time points (days 1-14) after force loading. TS: tension side, CS: compression side, r: root, E: Experiment side, C: control side. Red arrow points to loading direction. Bar=50μm.

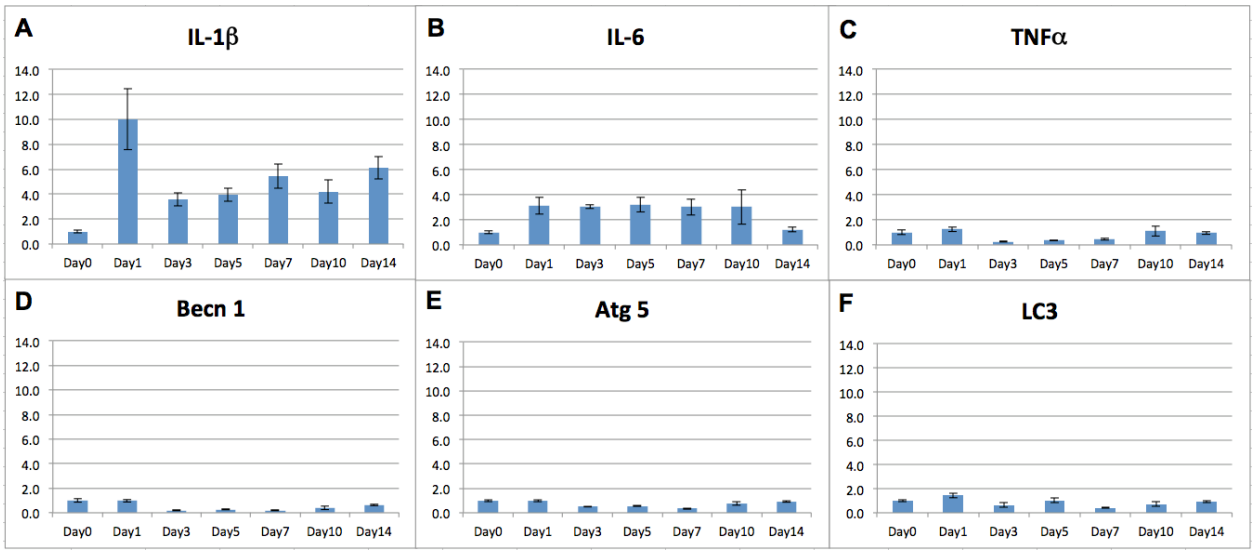

Figure 2.4 mRNA expression of inflammatory cytokines and genes involved in autophagy pathway at different time points after force loading (days 0-14). A. IL-1β; B. IL-6; C. TNFα; D. Becn 1; E. Atg5; F. LC3. For each time point, n=5, and normalized to day 0.

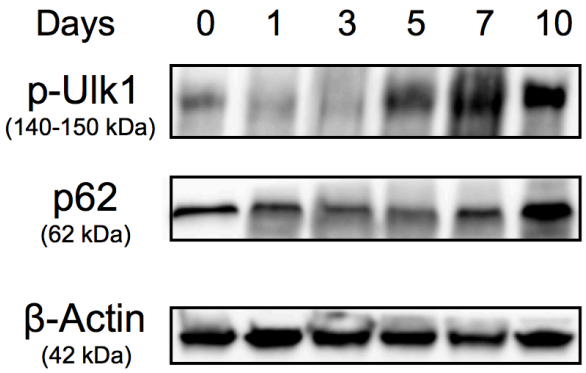

Figure 2.5 Alteration of protein levels at different time points (days 0 to 10) after force loading. p-Ulk1 and p62, whose levels are inversely correlated with autophagy activity, are reduced as early as day 1 after force loading, indicating increased autophagy activity. β-Action as internal loading control.

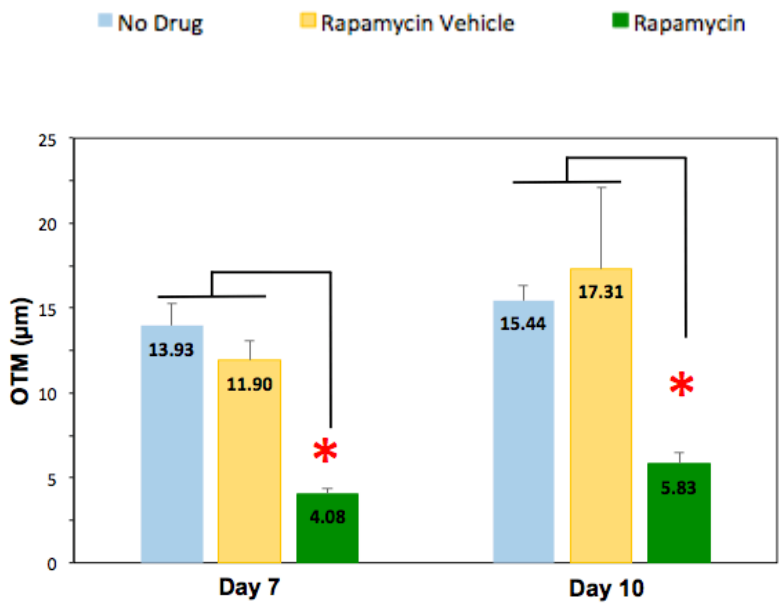

Figure 2.6 Effect of rapamycin injection on orthodontic tooth movement (OTM) amount. At days 7 and 10 after force loading, there is statistically significant reduction of OTM amount in rapamycin-injected group (green) when compared to either no drug injection (blue) or rapamycin vehicle injection (yellow), *=$p<0.05$. There is no statistically significant difference between vehicle and no drug injection groups. The significance level is set to $p=0.05$

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