Orthodontic Tooth Movement in MicroRNA-29 Sponge Transgenic Mice

BY

ANGELA LU
B.A., Barnard College, Columbia University New York, NY, 2009
D.M.D., University of Connecticut School of Dental Medicine, Farmington, CT, 2010

THESIS

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Defense Committee

Phimon Atsawasuwan, Chair and Advisor
Salvador Nares, Periodontics
Xiaofeng (Charles) Zhou, Periodontics
Grace Viana, Orthodontics

This thesis is dedicated to my parents, Yifeng Lu and Aihua He, without whom it would never have been accomplished. Thank you for all your love and prayers.

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LIST OF ABBREVIATIONS

3’-UTR	3’-Untranslated Regions
BMP	Bone morphogenetic protein
cFos	c-Fos protein (oncogene)
CLCN7	Chloride Channel 7 gene
COL1A1	Collagen, type 1, alpha 1
COL3A1	Collagen, type 3, alpha 1
COL5A1	Collagen, type 5, alpha 1
DGCR8	DiGeorge syndrome chromosomal region 8
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
ECM	Extracellular Matrix
FGF	Fibroblast growth factor
Fra-1	Fragile site 1,
H&E	Hemotoxylin and Eosin staining
IGF1	Insulin-like growth factor 1
IL1β	Interleukin-1 beta
MAPK	Mitogen-Activated protein kinase
mRNA	Messenger RNA
miRNA	MicroRNA
miR-29	MicroRNA 29
MMP2	Matrix metalloproteinase-2
NF-kB	Nuclear factor kappa beta

NFATc	Nuclear factor of activated T cells transcription complex
NFIA	Nuclear Factor I/A
OTM	Orthodontic Tooth Movement
OPG	Osteoprotegerin
PCR	Polymerase Chain Reaction
Pdcd4	Programmed cell death 4
PDL	Periodontal ligament
PDLCs	Periodontal Ligament Cells
PGE2	Prostaglandin E2
PI3K	Phosphoinositide 3-kinase
pUBC	promoter ubiquitin C
RANKL	Nuclear Factor- κB Ligand
RANK	Nuclear Factor- κB receptor
Ran-GTP	Ran-GTP binding protein
RISC	RNA-Induced Silencing Complex
RNA	Ribonucleic acid
RT	Reverse Transcriptase
SD	Standard Deviation
TGF-β	Transforming Growth Factor-Beta
TGIF2	Tumor growth inhibiting factor 2
TNFα	Tumor Necrosis Factor-Alpha
TRAF6	Tumor necrosis factor receptor-associated factor 6

TRAP	Tartrate-Resistant acid phosphatse
UIC	University of Illinois at Chicago
VEGF	Vascular endothelial growth factor
WT	Wild-type

Summary

Orthodontic tooth movement (OTM) occurs when a mechanical load is applied to a tooth through an appliance. Changes in these adaptive responses to orthodontic force depend on activities of periodontal ligament cells, osteoblasts, osteocytes and osteoclasts, which are influenced by cellular expression of regulatory genes at the right location for a given time. This is a sophisticated process that involves bone resorption at the sites of pressure and bone deposition on the tension side with a tight regulation at post-transcriptional and post-translational stages. To gain an insight into mechanisms that regulate the tooth movement at a molecular level will lead to an effective approach for clinicians to improve orthodontic care.

MicroRNAs (miRNAs) are short non-coding RNAs that are emerging as important regulators of post-transcriptional gene expression in osteoblastogenesis and osteoclastogenesis. Emerging evidence has indicated that specific miRNAs play a crucial role in osteoclastogenesis and osteoclast differentiation. In this study, we focus on the miR-29 family, which expresses in different patterns corresponding to different orientations of forces in human periodontal ligament cells. In addition, miR-29 family plays important roles in osteoblasts/clasts differentiation and functions.

The aim of the study is to evaluate the tooth movement phenotype of miR-29 sponge mice (miR-29 underexpressor) and gain insights into the possible mechanisms of how miR-29 family plays a role in tooth movement via osteoclast function; and to study the histological change and cellular effects from miR-29

loss of function. The tooth movement was achieved using a 5-cN expansion spring on a mandibular first molar of miR-29 sponge mice. After 2 weeks, the distance of tooth movement was evaluated using Faxitron® radiograph and a standardized gauge in microcomputed tomogram, H&E and TRAP staining were performed to investigate the activity of osteoclasts during tooth movement. In addition, RT-realtime PCR was performed to evaluate the level of expression of miRNA in each animal. The average distance of tooth movement in the miR-29 sponge transgenic mice were significantly less than the one in the wildtype control mice. The expression of miR-29 family in the miR-29 sponge mice were 0.2-0.6 folds lower than the ones in the control mice. In addition, the numbers of osteoclasts in the miR-29 sponge mice were less than in the control mice. The findings of this study reveal a previously unknown realm that a miRNA can modulate OTM via osteoclast function.

1. INTRODUCTION

1.1 Background

The concept of orthodontic tooth movement (OTM) was first published by Sandstedt in 1904 (Sandstedt, 1904). More than 100 years later, the knowledge of bone remodeling and the regulation of OTM has evolved with the discovery of new molecules and new experimental techniques. Orthodontic theory and practice requires a thorough understanding of bone biology. The relationship between molecular structural and functional changes, along with the communication of signaling proteins allows us to understand OTM in new ways. Discoveries in molecular biology of bone physiology allow the elucidation of the complex regulatory system of OTM. The relationship between mechanical stress and bone resorption is a highly intricate process of coordinated reactions that extends from molecular to mechanical responses, making tooth movement possible (Masella et al., 2006).

The periodontium, alveolar bone, gingiva, and PDL, is important in tooth movement. The local alveolar bone remodeling during orthodontic tooth movement contains tightly controlled regulatory mechanisms underlying osteoclastic and osteogenic differentiation. Physiologic bone remodeling is the balance of consists of osteocyte, osteoclast, and osteoblast functions. Once the force is applied to the brackets and relayed to tooth and supportive tissues during tooth movement, the imbalance of osteocyte, osteoclast and osteoblast function leads to bone resorption and tooth starts to move following the direction of force. After tooth moves, the repair process occurs to allow bone and mineral

homeostasis. Specifically, the tension side of tooth movement consists of bone deposition of alveolar wall; whereas the pressure side of tooth movement features resorbed alveolar bone with multinucleate osteoclasts in Howship's lacunae (Meikel, 2006). The interplay of these two sides produces tooth movement. Thus, the pressure-tension hypothesis plays an important role in organizing the concepts and advancing the understanding of this complicated biological process.

Bone tissue reacts to pressure by changing its underlying architecture with both resorption and deposition of bone occurring simultaneously. Orthodontic tooth movement results from bone remodeling that includes several important factors, the nuclear factor-κB ligand receptor (RANK) and nuclear factor- κB ligand (RANKL) system, osteoprotegerin (OPG) and ECM proteins working together. The process is initiated by mechanical load, growth factors and cytokines (Raggatt et al., 2010). Changes in the ECM load environment lead to cells responding mechanical forces by secreting inflammatory factors such as TGF-β, IGF1, IL-1B and VEGF, PGE2 (Iwasaki et al., 2006; Kaku et al., 2008; Chen et al., 2015). The relay of mechanical loading to biological signals by periodontal ligament and alveolar bone cells occurs (King et al., 2001). The cellular and molecular events in the PDL and alveolar bone cells are strictly controlled at transcriptional, post-transcriptional and translational levels and interference of these events affects the rate of tooth movement.

Each cell that makes up a living organism has the ability to change its DNA structure and sequence. The epigenetic mechanisms, including DNA

methylation, histone modification, chromosome remodeling and small interfering RNAs, function in cooperative and regulatory networks to control biological changes (Irwandi et al., 2016). Found in 1993, microRNAs (miRNAs, miRs) have recently discovered to be an important class of regulatory genes that control development and tissue remodeling in mammals, including the regulation of osteogenesis and osteoclastogenesis (Almeida et al., 2011; Lee et al., 1993; Park et al., 2010). It is estimated that miRNA account for 1-5% of the human genome and govern at least 30% of genes (Rajewsky, 2006; Liu, 2007; Stanczyk et al, 2008).

MicroRNAs are important post-transcriptional modulators of gene expression. The genes encoding miRNAs are found on every chromosome in humans (Ebert et al., 2010). They negatively regulate gene expression by binding to specific sequences to the target mRNA. These non-coding RNAs act together in families to control gene expression networks that is important for cellular function, thus, fine-tuning in responses to changes in the cell's environment.

The miRNA biogenesis pathway includes transcription, primary miRNA (pri-miRNA) processing, transporting to cytoplasm, precursor miRNA (pre-miRNA) processing, targeting mRNA binding sites and ultimate repression of translation (Ebert et al., 2010). MiRNAs bind to target mRNAs and form RNA-induced silencing complex (RISC), thereby, negatively repressing gene expression.

miRNAs have emerged as powerful regulations of a wide range of biological processes by binding to the 3'-untranslated region of mRNA by perfect complementarity to target, leading to mRNA cleavage. Furthermore, complementarity with imperfect base pairing will cause repression of translation of proteins. In the extracellular matrix, miRs control gene expression through cell cycle regulation, matrix secretion, cell survival and death (Ameres et al, 2013). The miRNA-mRNA interactions form complex gene regulatory networks. MicroRNAs, as part of integrated regulatory networks of controlling biological changes provide a way to define specificity of gene expression in living organisms.

1.2 Significance

Recent studies report that members of the miR-29 family are key modulators in ECM synthesis and remodeling. ECM of periodontium responds to stress and strain leading to periodontium remodeling during the tooth movement. The expression of miR-29 in periodontal ligament cells responded to the orientation of applied forces. In addition, miRNA-29 plays a significant role in osteoblast/clast differentiation. Therefore, it is hypothesized that underexpression of miR-29 family in a mouse would affect the tooth movement phenotype. Tooth movement is a complicated process and *in vivo* model is a valuable way to studying functions of critical genes regulating tooth movement.

1.3 Specific Aims

The objective of the present study was to evaluate the tooth movement phenotype in miR-29 sponge mice (miR-29 underexpressor) compare to wildtype

control mice and to study the cellular effect of miR-29 underexpression in the mouse at microscopic level. We hypothesized that miR-29 family was a critical regulator of bone remodeling during tooth movement.

1.4 Hypothesis

H1 (Null): There is no significant mean difference in distance of tooth movement, bone parameter, osteoclast number and osteoclast activity between control animal group and experimental animal group

H2 (Alternative): there is a significant mean difference in distance of tooth movement, bone parameter, osteoclast number and osteoclast activity between control animal group and experiment animal group.

2. REVIEW OF LITERATURE

2.1 Orthodontic Tooth movement

Orthodontic tooth movement (OTM) is a highly sophisticated process involving dynamic tissue response in the extracellular matrix and changes within the periodontal ligament and alveolar bone cells, followed by bone remodeling. Biologic response to orthodontic tooth movement lead to alveolar bone remodeling governed by osteoblastic and osteoclastic activity (Huang et al., 2014). In the periodontal ligament and alveolar bone, tensile strains up-regulate the expression of osteogenic genes resulting in bone formation, while compressive strains cause catabolic tissue changes causing bone resorption (Feller et al, 2015). The biologic events that occur within the PDL and alveolar bone in response to orthodontic force are the basis to facilitate tooth movement.

Bone turnover is a physiological process balanced between bone resorption and formation. Bone tissue responds to mechanical forces and undergoes bone remodeling through the activities of bone-resorbing osteoclasts of hematopoietic lineage and bone forming osteoblasts of mesenchymal origin. Cells secrete cytokines and neurotransmitters, including BMPs, FGF, TGF-β into local microenvironment, inducing bone remodeling (Krishnan et al, 2015). These cells are tightly controlled by key signaling pathways, including nuclear factor-κB ligand receptor (RANK) and nuclear factor-κB ligand (RANKL) system, osteoprotegerin (OPG) pathway. These ligand-receptor interactions allow a crosstalk between the two lineages of cells to regulate the balance between bone resorption and formation.

Osteoclasts, the primary cell type to resorb bone in the human body, originate from hematopoietic origins. The process of osteoclast differentiation is controlled by cytokines, growth factors, and hormones, including RANKL (Boyce et al, 2007). RANKL, a soluble ligand, is expressed by osteoblast precursors. RANKL binds to membrane receptors RANK on osteoclast precursors inducing their differentiation by activating downstream signaling pathways, such as MAPK, PI3K and NF-kB pathways. Furthermore, this signaling pathway regulates the expression of important transcription factors, including, c-fos, NFATc1,and Fra-1 (Asagiri et al 2007; Leibbrandt et al 2008). On the other hand, OPG prevents excessive bone resorption by binding to RANKL and preventing it from binding to its receptor, RANK. These pathways modulate osteoclast differentiation, maturation and function. Investigation of the molecular mechanisms that coordinate osteoclastic differentiation and regulation of function will contribute to better understanding of bone resorption in tooth movement.

At least twenty-four genes and sixty proteins are implicated in the regulation of osteoclastogenesis and osteoclast function, along with transcription factors (Teitelbaum, 2000). Studies have shown that the rate-limiting agents of osteoclast differentiation and function include OPG, cathepsin K, and chloride channel 7 (CLCN7) (Roberts et al, 2004). Thus, bone resorption is the rate-limiting step in OTM.

The importance of bone pathophysiology in treatment outcomes requires the orthodontists to be an expert bone specialist. The biology of craniofacial bone and therapeutic implications sets orthodontists apart as forerunner health

professionals rather than technicians. Discoveries in molecular biology and genetics of bone physiology allow clinicians to appreciate the complexity and regulatory sophistication of OTM. The link between clinical orthodontics and mainstream molecular-genetic research allow clinicians to maximize bioadaptability of orthodontic force application.

2.2 Role of miRNA in Bone Remodeling

MicroRNA (miRNA, miR) is a class of endogenous non-coding RNAs approximately 18-25 nucleotides long, controlling gene expression at the post-transcriptional level (Ambros et al., 2007). miRNAs are evolutionarily conserved. Hundreds of miRNAs have been found in humans and more than 3% of the genes in humans have been found to encode for miRNAs. They silence target mRNAs by binding to complementary sequences in the 3'untranslated region (UTR) of mRNA and block protein translation (Carthew et al., 2009). They are key post-transcriptional repressors of gene expression, providing a sophisticated way to gene regulation by inhibiting mRNA translation. We are only now beginning to understand how this group of regulatory RNA is being generated and assembled into functional units to regulate gene expression.

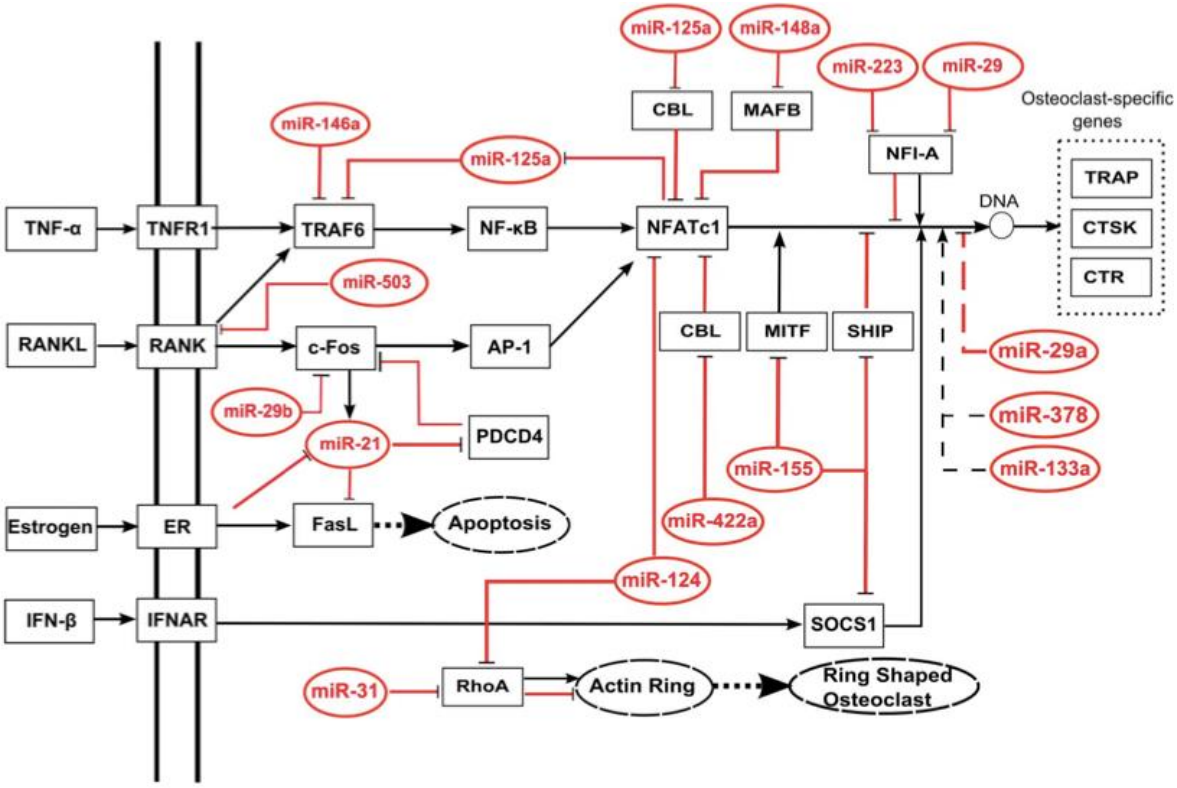

Figure 1. "Regulatory networks of miRNAs in osteoclast differentiation. miRNAs can directly or indirectly repress transcription factors and affect osteoclast differentiation, or phenotype. Bold dotted lines indicate the promotion of cellular processes; dotted boxes indicate osteoclast specific genes, and dotted ovals indicate cellular processes or phenotypes." (Tang et al., 2014)

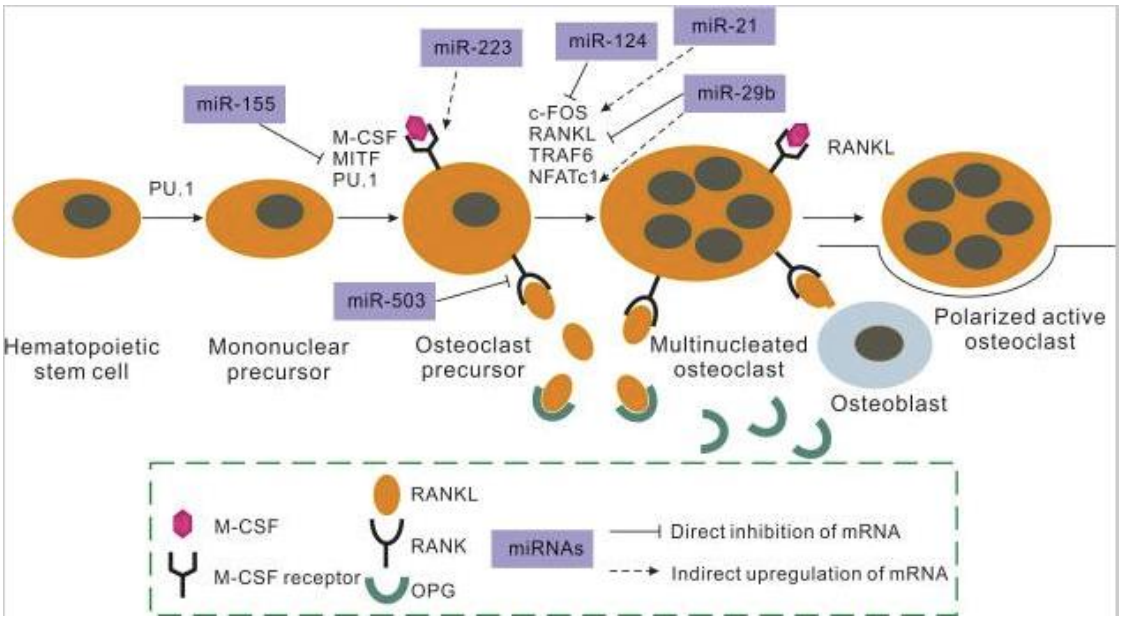

Figure 2. "miRNAs on osteoclast differentiation. This figure indicates the major activities of miRNAs influencing osteoclast commitment. miRNAs affect different molecular mechanisms related to this commitment, such as RANK or M-CSF receptor, among others. miRNA expression leads to alterations in osteoclast activity *in vitro* and changes in bone resorption *in vivo*. M-CSF, macrophage colony-stimulating factor; OPG, osteoprotegerin; MITF, microphthalmiaassociated transcription factor; RANK, receptor activator for nuclear factor κB; RANKL, RANK ligand; NFATc1, nuclear factor of activated T-cell calcineurin-dependent 1." (Jing et al, 2015 miRNA in bone remodeling).

Studies have shown that miRNAs are novel regulatory factors of osteoclastogenesis (Lian et al., 2012; Xia et al., 2011). A lack of miRNAs in osteoblast and osteoclast pathway led to defective osteoblastic and osteoclastic function and differentiation (Krzeszinski et al., 2014). miRNAs function in tissue-specific transcription factors in osteoblasts and osteoclasts, providing new insights into the regulatory networks of bone deposition and turnover (Lian et al, 2012).

The biogenesis of miRNA begins in the nucleus as a primary miRNA (pri-miRNA) transcribed by RNA polymerase II, which is a long primary transcript with a local stem-loop structure that contains the hairpin structure encoding miRNA sequence (**Figure 3**). The pri-miRNAs is then processed by the double-stranded

RNA-binding protein coded by DiGeorge syndrome critical region gene 8 (DGCR8) and the nuclear RNase III enzyme Drosha into a stem-loop-structured pre-miRNAs (Bushanti et al., 2007). Once cleaved to a pre-miRNA, it is translocated into the cell's cytoplasm by exportin-5 (Lund et al., 2004). The pre-miRNA is then processed into the double-stranded miRNA by a second DNase III endonuclease Dicer (Lian et al, 2012). This biogenesis pathway generates a mature, double-stranded miRNA, ready to bind to target mRNA. The miRNA is incorporated into a protein complex containing Argonaute 2 protein, called RISC, RNA-induced silencing complex (Gregory et al., 2004). When the double-stranded miRNA is functional, one strand is selected as the mature miRNA and the other is degraded. Mature miRNAs in complex with RISC can then target mRNAs through base-pairing (Ha et al., 2014). The mature miRNA regulates gene expression through repression of translation into proteins or mRNA cleavage, which depends on the complementarity between the miRNA and its target mRNA. If the target mRNA has perfect base-pairing to the RISC, the mRNA is degraded. If the target mRNA has imperfect complementarity, suppression of mRNA translation will occur, resulting in reduced expression of proteins (**Figure 3**).

MicroRNA, as part of the integrated regulatory networks of controlling biological changes provide a way to define specificity of gene expression in living organisms. Previous studies demonstrated that numerous miRNAs have found to be active in the regulation of bone homeostasis and bone remodeling. In the extracellular matrix, miRNAs control gene expression through cell cycle

regulation, matrix secretion, cell survival and death (Ameres et al, 2013). The ECM of periodontium contain periodontal ligament cells and alveolar bone cells that respond to stress and strain from orthodontic forces by expressing and secreting biological factors including IL1β, TGF-β, IGF-1, VEGF, and PGE2, RANKL and OPG (Iwasaki et al., 2006; Kaku et al., 2008; Riddle et al., 2009; Meikle, 2006).

Studies have demonstrated the important regulatory functions of miRNAs in human PDLs involving bone remodeling including an increase of miR-132 by fluid shear stress, and exogenous miR-146a, which can stimulate human PDL cell proliferation and osteogenic differentiation (Qi et al., 2014; Hung et al., 2010).

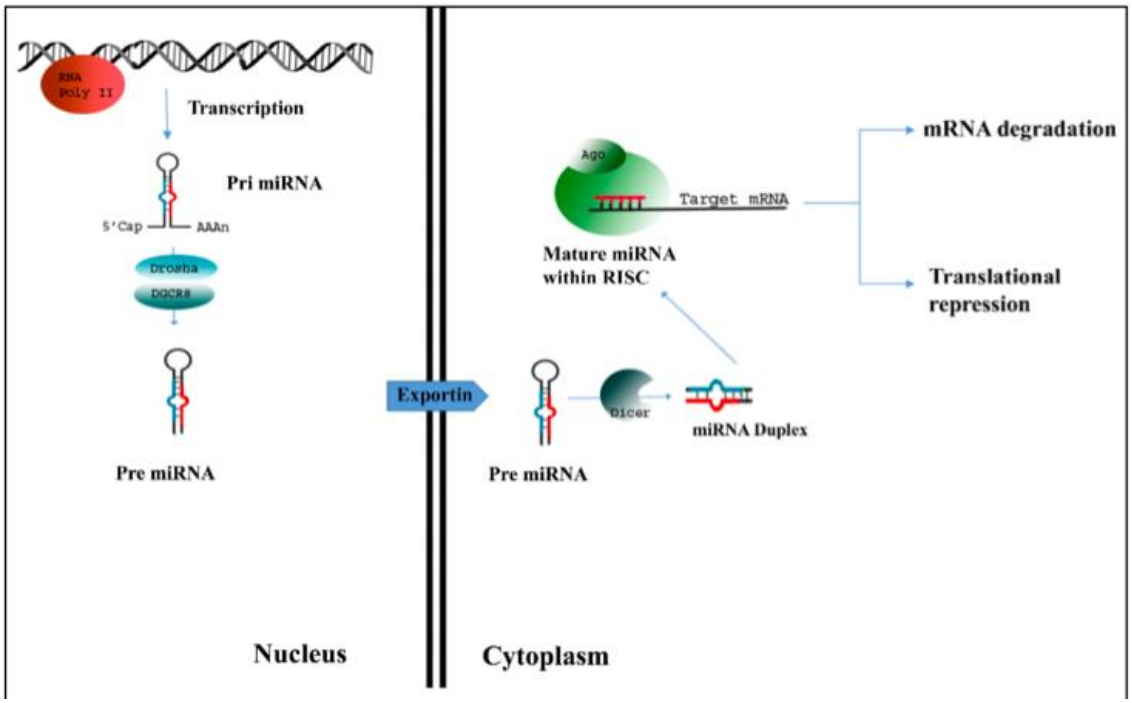

Figure 3. Biogenesis of miRNAs. In the nucleus, miRNAs are first transcribed into pri-miRNA. Pre-miRNA is then processed by Drosha into a stem loop structure called pre-miRNAs. The PremiRNA is transported into the cytoplasm with the escort of Exportin5. In the cytoplasm, Dicer enzyme processes pre-miRNA into a double-stranded mature miRNA. The miRNA is then incorporated into RISC. One strand of the double-stranded miRNA is rapidly removed and degraded, while the other strand is chosen as the guide strand. The guide strand, or mature miRNA lead to translation repression or degradation of mRNA depending on the degree of complementarity with the target mRNA. (from Paul Lazari's unpublished thesis manual at UIC Department of Orthodontics)

2.3 The Role of miRNA in Osteoclast Lineage and Bone Resorption

Osteoclasts, originated from hematopoietic lineage cells, are formed in the bone marrow and are led to the bloodstream through cytokines and other inflammatory factors. Studies have shown that DGCR8-dependent miRNAs are indispensable for osteoclastogenesis, and gene silencing studies of Dicer and DGCR8 have elucidated role of miRNAs in osteoclast function (Mizoguchi et al., 2010; Sugatani et al., 2009; Sugatani et al., 2014). Therefore, miRNAs influence osteoclastogenesis on a molecular level.

In the process of osteoclast formation, RANK ligand, which are expressed on the surfaces of osteoblasts, osteocytes and T-cells, bind to membrane protein RANK expressed on osteoclast precursors to activate the formation of osteoclast. Tumor necrosis factor (TNF) receptor-associated factors, can bind to RANK to activate NF-kB and JNK/AP-1 pathway (Jing et al., 2015). During osteoclastogenesis, the expression level of miRNAs can be regulated by RANKL. To attenuate this system, a natural factor that also binds to receptor for RANKL, OPG, can compete with RANK to prevent osteoclast maturation (Boyce et al., 2007). Other important factors include c-Fos, which is a transcriptional factor in osteoclast functions and can improve osteoclast differentiation.

Orthodontic tooth movement is characterized by increased expression of anabolic genes such as collagen I, III, and V on the stretch side and decreased expression of matrix proteins on compression side, allowing alveolar bone remodeling and subsequent tooth movement (Meikle, 2006; Xu et al., 2014).

According to recent studies by Chang et al in 2015, there are 32 miRNAs differentially expressed between cyclic tension force-stimulated human PDL cells and control cells. Furthermore, Wei et al in 2014 found that specific miRNAs function in cyclic mechanical stretch-induced osteoblast differentiation, specifically in human PDL cells and alveolar bone cells.

Microarray data have demonstrated that 53 microRNAs are expressed in human PDL stem cells (Wei et al., 2014). It is established that miRNAs play important roles in the regulation of osteoclastogenesis. Specifically, a lack of miRNAs in osteoblast and osteoclast led to defective osteoblastic and osteoclastic function and differentiation (Krzeszinski et al., 2014). MicroRNAs could be mechanosensitive and studies have just beginning to reveal its critical role in osteoclastogenesis.

Krzeszinski et al found that miR-34a plays a role in the inhibition of osteoclastogenesis, and has been shown to target TGIF2, which leads to inhibition of osteoclast differentiation (Quinn et al, 2001). Furthermore, the authors found that miR-34a knockout mice exhibit decreased TRAP mRNA and TRAP positive cell numbers when compared to wild-type mice, indicating miR-34a suppressed osteoclastogenesis *in vitro* (Quinn et al., 2001).

Numerous studies have shown there are several miRs active in periodontal ligament cells (Li et al., 2012; Liu et al., 2011; Qi et al., 2014; Sipert et al., 2014). To note, miR-21 deficient mice showed inhibited alveolar osteoclastogenesis when compared with wile-type mice (Chen et al., 2016). During orthodontic tooth movement, miR-21 deficiency blocked alveolar bone

resorption in compressive and tensile sides. The authors of the 2016 study had found that targets of miR-21 include pdcd4 and a deficiency of miR-21 removed the blockage pathway of Pdcd4 in mRNA and protein levels in the periodontium, resulting in up-regulation of downstream effector C-fos. As a conclusion, authors reported that miR-21 deficiency blocked lipopolysaccharide-induced maxillary bone loss. Furthermore, miR-223 promotes osteoclast formation through inhibition of NFIA (Irwandi et al., 2016).

Studies have shown that miR-503 target RANK and the inhibition of miR-503 promotes RANKL-induced osteoclastogenesis and bone resorption in vitro and in vivo (Chen et al., 2014). Furthermore, TRAF6 is a direct target of miR-125a. In addition, activated by NF-kB, NFATc1 bind to the promoter of miR-125a and inhibit the suppression of TRAF6 (Guo et al., 2014). miR-124 can also regulate osteoclast differentiation, proliferation and migration by suppressing NFATc1 (Lee et al., 2013). C-Fos lead to the expression of miR-21, which can down-regulate *pdcd4* protein levels to activate c-Fos (Sugatani et al., 2011). Sugatani and colleagues concluded that miR-21 was a mediating factor in RANKL-induced osteoclastogenesis. Other studies have found similar results, concluding that miR-21 promotes osteoclast differentiation and target pdcd4 (programmed cell death domain 4) mRNA (Irwandi et al., 2016).

Many miRNA have been found to be important in osteoclastogenesis. Nevertheless, the mechanisms by which specific miRNAs can modulate orthodontic tooth movement and mediate alveolar bone remodeling *in vivo* need to be investigated and revealed.

2.4 MicroRNA-29 Expression Dynamics in Tooth Movement

MicroRNAs are emerging as critical regulators of ECM homeostasis and essential contributors in controlling ECM synthesis and remodeling (Rutnam et al., 2003; Luna et al., 2009). miR-29 has previously been reported *in vitro* to mediate stretch-induced PDL and support osteoclast differentiation (Chen et al., 2015).

Specifically, miR-29 family members have been found to contribute to ECM homeostasis and regulate proteins including collagens and elastin, and thus have ability to control ECM remodeling (Luna et al., 2009; Kriegel e al., 2012; Mouw et al., 2014; Wang et al., 2012; Yang et al., 2013). miR-29 family includes miR-29a, miR-29b, miR-29c. In a recent review, Franceschetti et al concluded that miR-29 family members sustain the migration and commitment of the precursors to osteoclastogenesis. Furthermore, miR-29 expression increased osteoclastic differentiation and miR-29 knockdown impaired migration, commitment and formation of osteoclasts. Therefore, it is evident that miR-29 regulates osteoclastogenesis by downregulation of osteoclast negative differentiation factor (Franceschetti et al., 2013). The authors suggested that miR-29 family plays a positive role in osteoclastogenesis. Franceschetti et al also demonstrated that miR-29 family members are positive controllers of osteoclast formation by targeting mRNAs encoding NFIA, as well as calcitonin receptors. These receptors are important for commitment and survival of osteoclast. Studies confirm that the expression of miR-29 family increases during osteoclast differentiation and inhibition of miR-29 family suppresses formation of TRAP-positive multinucleated osteoclasts (Tang et al., 2014).

Furthermore, miR-29b can promote osteogenesis by down-regulating osteogenic inhibitors (Suh et al., 2013). miR-29 is a positive regulator of osteoblast differentiation and controls the expression of collagens in differentiated osteoblasts (Li et al., 2009). Several miRNAs have been shown to be important in osteoblast differentiation and bone formation. However, studies need to be done to elucidate the regulatory role of miRNAs in osteoclastogenesis, including miRNA-29 family.

Recent study by Chen and colleagues characterized the miR-29 family in the expression of periodontal ECM genes and showed that levels of miR-29b inversely influenced the expression levels of major ECM genes in periodontal ligaments. Authors of the study confirmed the direct interactions of miR-29 and ECM genes and transient transfection of a miR-29b mimic in mouse PDL cells down-regulated ECM genes (COL1A1, COL 3A1, and COL5A1) while the transfection of miR-29b inhibitor up-regulated these genes, indicating that target ECM genes are directly responding to altered level of miR-29b. This is the only current literature that link microRNA to orthodontic tooth movement. The present study takes a step further in investigating the *in vivo* function of miR-29 in biologic tooth movement. Based on previous studies, the possible roles of miR-29 family as modulator for ECM homeostasis during orthodontic tooth movement is established. These findings give important insights into the regulatory loop of tooth movement.

Studies in rats have revealed that a gain-of-function of miR-29a reduced glucocorticoid-induced osteoclast differentiation in vitro; while knockouts of miR-

29a accelerated bone resorption by osteoclasts, cortical bone porosity and bone fragility (Wang et al., 2013). Thus, miR-29 functions to inhibit GC-induced osteoclast differentiation, relating to glucocorticoid-induced bone loss (Wang et al., 2013). Rossi et al in 2013 had reported that target genes of miR-29b include c-fos and MMP2, and endogenous miRNA expression lead to downregulation of RANKL induced osteoclastogenesis and inhibits osteoclast differentiation, which is contradictory to previous studies (Rossi et al., 2013).

In the present study, we investigate the function of miR-29 in the osteoclastic lineage, which is not well understood. Quantitative RT-PCR showed that all miR-29 family members increased during osteoclast differentiation with markers (Franceschetti et al., 2013). miR-29 family have been found to be positive regulators of osteoclast formation and targets RNAs that are important for cytoskeletal organization and osteoclast function.

Bone metabolism involves miRNAs that influence cell interaction and signaling at a post-transcriptional level. Periodontal ligament cells secrete RANKL and inflammatory factors that can be modulated by miRNAs. Further investigation is to come in determining the function of miRNAs in orthodontic tooth movement through periodontal homeostasis and bone remodeling. Knowledge from future studies will be beneficial in developing therapeutic approaches to provide patients with sound biological tooth movement.

The present study is the first time report that will reveal previously unrecognized mechanism that miR-29 can modulate OTM and alveolar bone

remodeling in *vivo.* The study is designed to determine how altered expression levels of miR-29 family members influence orthodontic tooth.

The application of orthodontic force induces alveolar bone remodeling, but whether microRNAs respond to orthodontic force and its mechanism in contribution to OTM is unknown. The *in vivo* function of miR-29 in the regulation of bone remodeling, in response to orthodontic force, remains unknown. Here we have decided to characterize the role of miR-29 family in orthodontic tooth movement relating to bone remodeling. The present study is the first time reports on the expression of miR-29 family member and its potential effects on orthodontic tooth movement relating to bone resorption. Our study paved the first step in developing miRNA-based approaches to manipulate OTM or prevent unnecessary alveolar bone loss.

2.5 Sponge Mouse Model

Loss-of-function studies are superior because they can elucidate functions of physiological miRNA. The miRNA sponge method, introduced in 2007 by Ebert and Sharp, is a way to create a method of loss of function in cell lines. Sponge RNAs contain multiple complementary binding sites to miRNA of interest. Sponge's binding sites are specific to miRNA seed region, allowing them to block a whole family of miRNA.

The advantages of sponge studies include the ability to inhibit miRNA over long durations and generating transgenic sponge-expressing animals to continuously inhibiting miRNA of interest for the lifetime of the animal in the study. Moreover, the advantages of sponges RNA over chemically modified

antisense oligonucleotide inhibitors include that antisense inhibitors are specific for one miRNA because they depend on high level of sequence complementarity (David et al., 2006; Esau, 2008). Therefore, it requires the delivery of a mixture of perfectly complementary oligonucleotides to inhibit a family of miRNAs. Also, cells *in vitro* and *in vivo* can be resistant to the uptake of oligonucleotides, whereas, the sponge transgene can be delivered by a viral vector (Ebert et al., 2007). In addition, we can make the sponge promoter tissue-specific for the tissue of the study by including regulatory elements in the promoter, whereas antagomir oligonucleotides that is injected into the mouse cannot access all tissues and can accumulate in the liver (Krutzfeldt et al., 2005). Most importantly, oligonucleotide inhibitors require repeated administration in large doses to inhibit a miRNA over long time, and transgenic sponge-expressing animals can be generated to continuously inhibit the miRNA of interest for the lifetime of the animal (Ebert et al., 2010).

There are limitations to the miRNA sponge technique including that optimized sponges may exhibit different degrees of inhibition in different contexts (Ebert et al., 2010). In cells expressing a significant amount of endogenous targets for miRNA family of the study, there would be less free miRNA available, then a lower concentration of sponge RNA should be enough to provide a strong inhibition. Furthermore, determining whether a sponge transgene is inhibiting the miRNA of interest is more challenging than genetic deletion method, which results in loss of mature miRNA. In order to validate the efficacy of sponge constructs, assays for expression of known targets genes can be done. For

example, a luciferase reporter fused to miRNA binding sites or confirmed target 3'UTR is measured in the presence of the miRNA sponge (Ebert et al., 2010). The sponge transgenic model is a powerful tool for uncovering new areas of miRNA biology and achieves great promises as therapeutic agents.

3. MATERIALS AND METHODS

3.1 Animal Model

The miR-29 sponge transgenic mice were generously provided by Professor Xingxu Huang, Department of Genetics, Model Animal Research Center of Nanjing University, China. All mice were housed with good ventilation in the animal facility at University of Illinois at Chicago, College of Dentistry. The mice were kept in the facility under light and dark cycle conditions. All animal procedures were approved according to UIC University Committee on Use and Care of animals. The animal protocol number is 14-063. Briefly, the miR-29 sponge transgenic mice were generated with miR-29 transgenic sponge construct. Four repeats of miR-29 Sponge (anti-sense oligonucleotide sequences of mature miR-29) was designed as 5'-GAAGACACACCGTAACCGATTCCAATGGTGCTACTATTAACCGATTCCAATGGTGCTAACCGGTTAACCGATTCCAATGGTGCTACACTAACCGATTCCAATGGTGCTACTTTTTCTCGAG-3' and cloned into the transgenic expression vector pUBC. After testing the efficacy of miR-29 Sponge over-expression in HEK 293T cells, the construct was linearized for pronuclear microinjection. The parental mice creates continuous loss-of-function in cell lineage. Each mouse was genotyped using a specific pair of primers: For transgenic miR-29 sponge mouse: Forward primer: 5'-AAGTGAGGCGTCAGTTTCTTTGG-3' and reverse primer: 5'-AACAACAGATGGCTGGCAACTAG-3' were used. Ten-week-old transgenic sponge and wild-type mice (n=16) were used for tooth movement experiment. The mandible sections of pUBC-GFP transgenic mice at postnatal

day 21 (Jackson Laboratory, Sacramento, California) were used to demonstrate the expression of pUBC in alveolar bone and PDL.

3.2 Tooth Movement and Mechanical Force Application

Tooth movement in a mouse model: A two-arm expansion spring was installed on the mouse's mandible with one arm placed on the left first molar and the other arm rested on the lingual plate of alveolar bone on the right side. The width between two arms were measured and calibrated (1.8 mm, 5 cN) before installation **(Figure 4).**

Figure 4. Installation of spring ligated to mandibular incisor by composite resin and to the lingual surface of right and left sides of the mandible.

After placement, the expansion spring was bonded to mandibular incisor with composite resin to provide 5cN orthodontic force on the left mandibular first molar to allow the tooth movement while there was no loading force on the right mandibular first molar. No tooth movement was generated on the right mandibular first molar. After 2 weeks tooth movement following spring installation, mandibles of the mice were dissected on ice and the experimental side was fixed with 10% formaldehyde buffer for histological studies. The other side of the mandibles were kept in -80°C for RT-realtime PCR.

3.3 Tooth Movement Measured by Faxitron® radiography

Faxitron® radiograph was used for determination of the distance of tooth movement of the left mandibular first molar. Using a finely focused electron beam to provide a tiny X-ray source so that projective enlargement to fivefold was employed when taking image of the mouse's mandible. Faxitron® radiographic unit was calibrated by running four flat dark field images using time set at 10s and voltage of 26kV. The hemimandible samples were placed at the 5X magnification in Faxitron® unit. The parameters of Faxitron® radiographic settings were set to time of 10 seconds and voltage of 33kV. Orientation of samples was set upright so the radiographic views were projected as an occlusal view of the mandibles. The distance of tooth movement was measured from the most buccal surface of the first mandibular molar to the alignment of other mandibular molars. In addition, the other side (passive no tooth movement side) of the same mandible were subjected to Faxitron® radiography and compared. Distance from 1st molar lingual surface to the tangent line from the alignment of the remaining molars was measured using Adobe Photoshop (Adobe, Version 12.5) **(Figure 5).** A standardized control gauge was subjected to the Faxitron® radiography and used for calculation of the distance in μm.

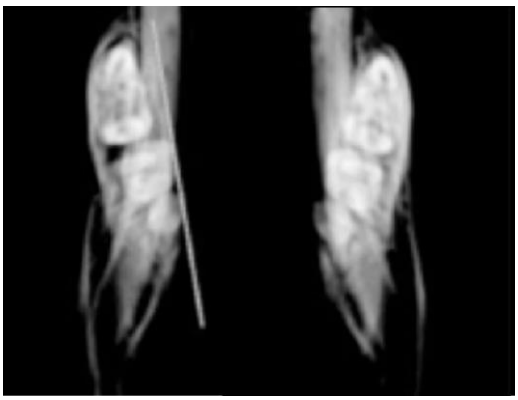

Figure 5. The tooth movement measurement was performed using Faxitron® radiograph. The right side of mandible (passive side) showed no tooth movement and good alignment.

3.4 Microcomputed Tomography

To quantify mineralized tissues in the mandibles of control and transgenic sponge mice, microcomputed tomography (micro-CT) was used. Micro-CT was performed using a Scanco μCT 40 systems (Scanco Medical, Brüttisellen, Switzerland) applying 70 kV, 114 μA, 300 ms integration time and 10μm voxel size to compare bone mineral density, volume and tooth movement in the miR-29 transgenic sponge and wildtype control mice's mandibles. 3D X-ray CT images were acquired **(Figure 6)**. Bone histomorphometry analysis was performed and the designated volume included the bone volume around mandibular first molar from the alveolar bone crest to the apical tip of the first molar roots.

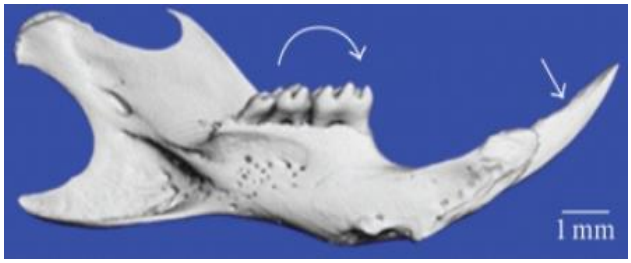

Figure 6. 3D X-ray CT images were acquired and reconstructed as example shown here.

3.5 Histology

Tartrated resistant acid phosphatase (TRAP) and Hematoxylin and Eosin (H&E) staining techniques were performed. The left mandible samples were fixed, demineralized and paraffinated. The sections were microtomed to 5μm and stained with H&E staining. Adobe Photoshop (Version 12.0, CS 5.0) was used to quantify the amount of bone matrix at the interradicular area and alveolar bone from mesial surface of the first molar mesial root to mesial surface of second molar mesial root. Color pixels with tolerance of 20 were used to differentiate and measure the amount of bone matrix and total bone volume. The following

parameters were measured: bone volume/total volume (BV/TV in %). Bone histomorphometry analysis was conducted using Adobe Photoshop version 12.5, CS 5.0.

Another group of the sections were stained with tartrate-resistant acid phosphatase (TRAP) staining kit (Sigma, St. Louis, MO) to visualize osteoclasts. The numbers of TRAP positive (TRAP+) multinucleated cells were counted on the designated area (mesial surface of the mesial root of first and second molars (alveolar wall) and in the bone marrow cavities immediately adjacent to the alveolar wall and in the interradicular bone. The slides were counterstained by fast green stain (Sigma). Osteoclasts numbers were compared between the experimental and control groups. Osteoclastic activity was quantified using Adobe Photoshop version 12.5, CS 5.0.

Immunohistochemistry of pUBC-GFP transgenic mouse was performed to elucidate the expression of pUBC in alveolar bone and PDL. The deparaffinated mandible sections of pUBC-GFP transgenic mice (Jackson laboratory, Sacramento, California) were subjected to anti-mouse GFP antibody (Abcam, Cambridge, Massachusetts) and biotinylated goat polyclonal anti-mouse GFP antibody **(**Abcam**).** The signal were obtained by the addition of horseradish peroxidase kit (Invitrogen, Carlsbad, California) and counterstained with hematoxylin staining.

3.6 Quantitative Real-Time PCR Procedure

Total RNA was extracted from the other sides of hemimandible and humerus bones of the mice. Total RNA with small RNAs was isolated with miRNeasy kit (Qiagen, Valencia, CA), following the manufacturer's instruction.

Quantitative real-time PCR for mature miRNA from the samples of miR-29 sponge and control mice were performed using Taqman microRNA assay (Thermo Fisher Scientific, Carlsbad, CA) described in **Table 1**.

3.7 Statistical Analysis

The distribution of the raw data was investigated by Shapiro-Wilk test. Student Independent t-tests were performed. Statistical significance was set at $p<0.05$ and the tests were done in SPSS for windows version 22.0 (IBM Corp., Armonk, NY).

TABLE I. TaqMan™ miRNA Assays

Assay Name	Assay Number	Species
U6 snRNA (control)	001973	Human, Mouse, Rat
Hsa-miR-29a	002112	Human, Mouse, Rat
Hsa-miR-29b	000413	Human, Mouse, Rat
Hsa-miR-29c	000587	Human, Mouse, Rat

4. RESULTS

4.1 Animals and microRNA Expression

The transgenic miR-29 sponge mice and their control wild-types (WT) were viable and their growth rate was comparable. The morphology and cranial shape of the sponge mice are comparable to the wildtype mice **(Figure 7)**.

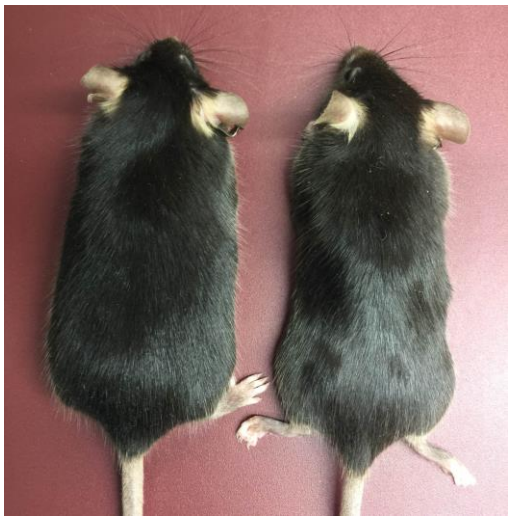

Figure 7. Gross anatomy of control wild-type on the left and miR-29 sponge transgenic mice on the right show similar growth rate and morphology.

Both males and females were reproductive. Genotyping with specific sets of primers were performed when the transgenic mice were at the age of 3 weeks. For transgenic miR-29 sponge mouse: Forward primer: 5'-AAGTGAGGCGTCAGTTTCTTTGG-3' and reverse primer: 5'-AACAACAGATGGCTGGCAACTAG-3' were used and the PCR product size was 445bp.

The animals' behaviors after spring installation was normal and their weight was not decreased less than 10% of the original weight before the spring

installation. Each mouse's hemimandible and humerus bones were used for the miRNA expression determination. The expression of pUBC in mouse mandible was shown in **Figure 8.** The expression of pUBC was in periodontal ligament fibroblasts, osteoblasts and osteocytes (brown color). The expression level of miR-29a, b and c was shown in **Figure 9**. The expression of miR-29a was decreased to 0.44±0.11 folds, miR-29b was decreased to 0.29±0.23 folds and miR-29c was decreased to 0.53±0.17 folds compared to the samples from the control WT **(Figure 9)** ($P<0.05$). U6 small RNA was used as an internal control for normalization. Overall, there was less miR-29 family expression in sponge mice. This indicates that miR-29 may contribute to OTM.

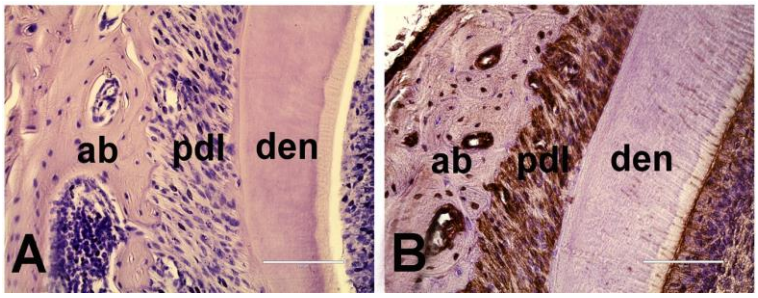

Figure 8 GFP expression under pUBC promotor in mandibles of Tg(UBC-GFP) at P21 **(A)** and control mouse **(B)** demonstrated the localization of pUBC in PDL, osteocytes and osteoblasts (brown color); ab:alveolar bone, pdl:periodontal ligament, den:dentine

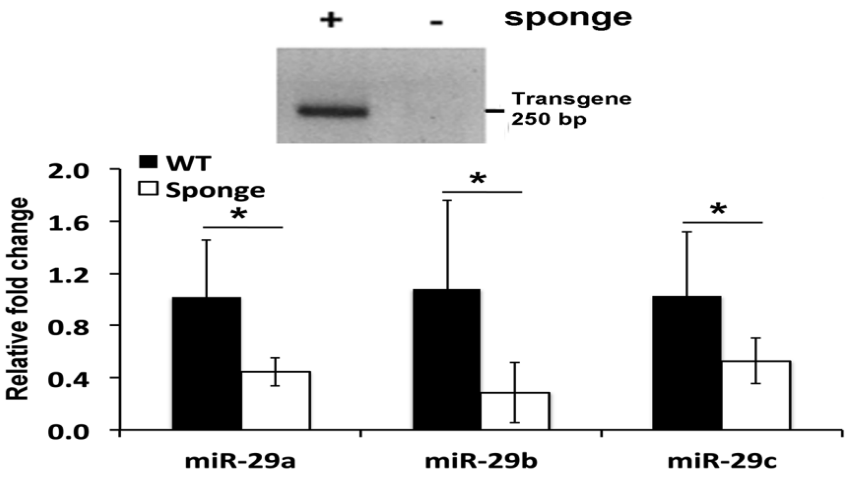

Figure 9 Differential expression levels of miR-29 family including miR-29a, miR-29b, miR-29c, compared to WT and sponge transgenic mice.

4.2 Tooth Movement

After 2-week of spring activation, the results elucidated that less tooth movement was evident in the miR-29 sponge mice (164±42 μm) was detected compared to the control wildtypes (211±33 μm) ($P<0.05$). **(Figure 10).** These results suggested that periodontal miR-29 induced by orthodontic force regulates OTM.

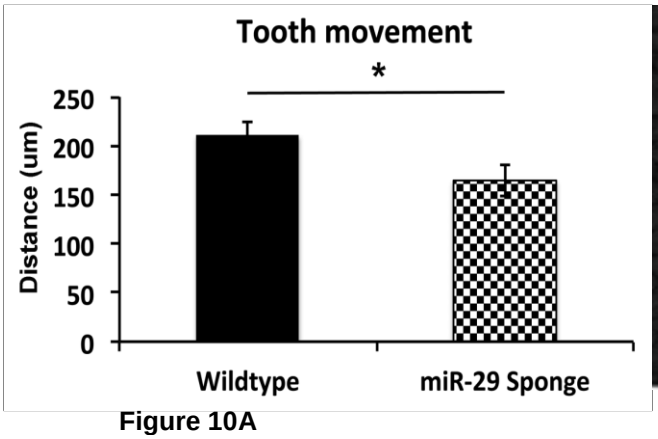

Figure 10A

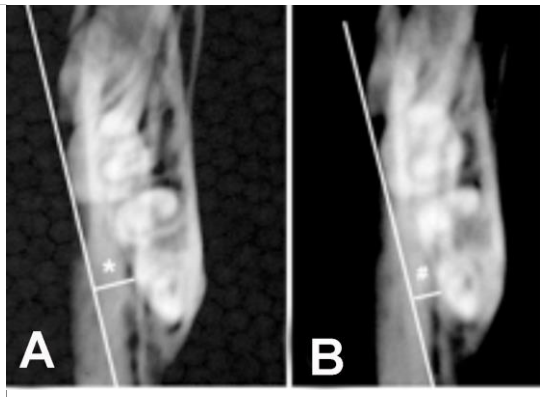

Figure 10B

4.3 Microcomputed Radiography

Three mandibles from each group were subjected to microcomputed radiographic analysis for evaluation of bone parameter: bone volume, trabecular number, trabecular thickness, bone tissue mineral density. The distribution of the raw data investigated by Shapiro-Wilk test indicated that the study variables demonstrated normally distribution. Student Independent *t*-test was then performed. Statistical significance was set at 0.05 and the tests were done in SPSS for Windows version 22.0 (IBM Corp., Armonk, NY). Overall, the test results revealed non-statistically significant mean differences ($p>0.05$) in bone volume, trabecular number, trabecular thickness, bone tissue mineral density, shown in **Figure 11A-D**. Overall, we did not detect a significant mean difference in bone morphology parameters between miR-29 transgenic sponge and WT control animals.

Figure 10. Tooth movement results. A)The results elucidated that significantly delayed tooth movement is evident in the miR-29 sponge mice (164±42 μm) and was detected compared to control WT (211±33 μm) ($P<0.05$). **Figure 10B.** Comparison of faxitron radiographs of distance of tooth movement is lesser in miR-29 sponge transgenic mice (B) than in WT (A).

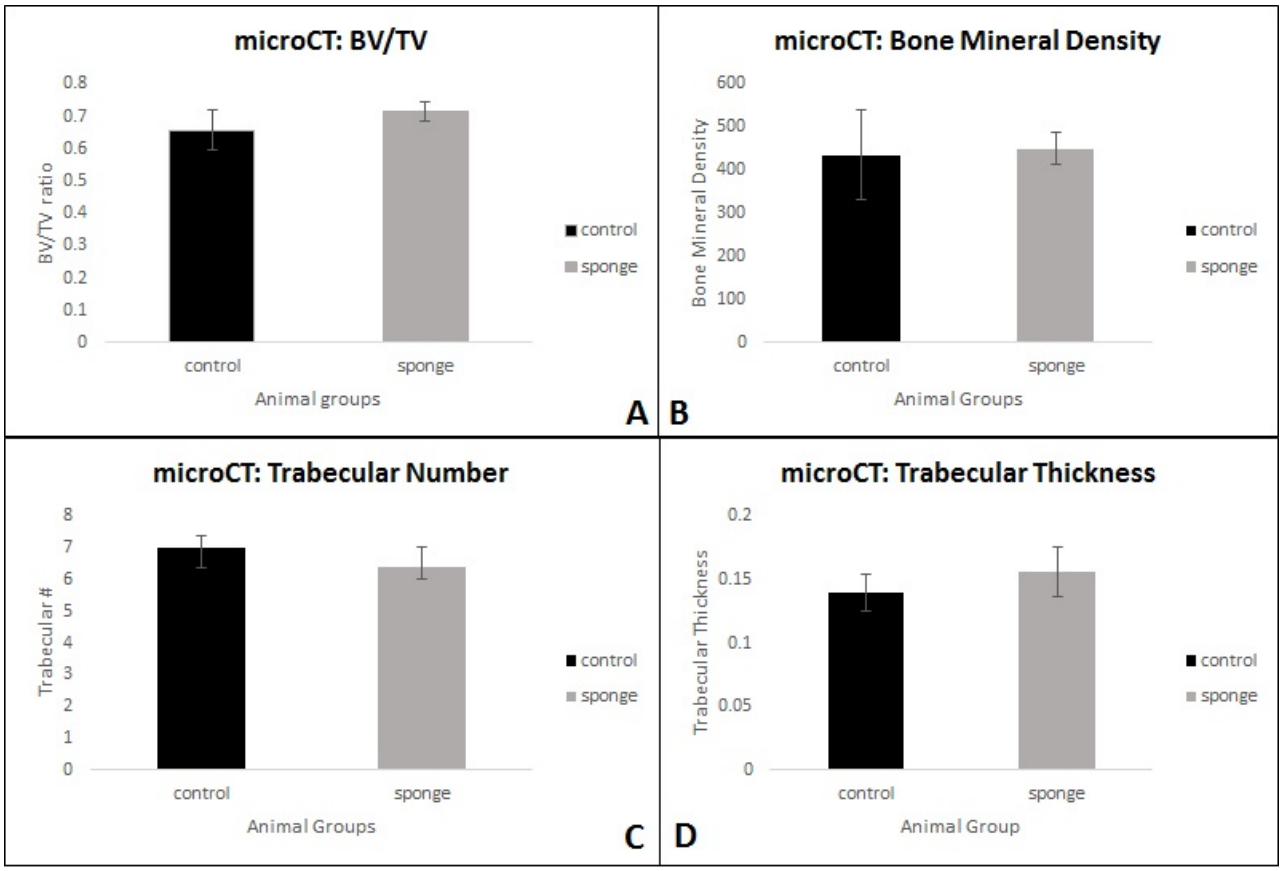

Figure 11A-D. Data from microCT analysis showed that non-statistically significant mean differences, P >0.05 for all variables including trabecular number, trabecular thickness, bone mineral density and BV/TV ratio. There is no difference in bone density or trabecular thickness or number between sponge transgenic and WT mice groups.

4.4 Histology Studies

The mandible samples were dissected and subjected to two different staining techniques. Hematoxylin & eosin (H&E) staining was stained to demonstrate the bone matrix and histomorphological appearances of alveolar bone under force from the spring. There were no difference in bone parameter: BV/TV, between transgenic and WT animal groups which confirmed the finding from microCT (data not shown).

H & E staining showed irregular PDL spaces on the buccal sides around the roots of first mandibular molars compared to the lingual sides indicating

active buccal tooth movement in both control and transgenic groups. There was evident scalloped appearance of the alveolar bone. There were disorganized PDL fibers indicating that the principal collagen fibers disappeared. At this stage of active tooth movement, the PDL consisted of loose connective tissue without clear and organized orientation. Blood vessels and fibroblasts werealso present. Localized areas of direct resorption on the buccal bone was evident as Howship's lacunae were accumulated in the area as tooth moved buccally with the expansion springs (**Figure 12**).

TRAP staining was performed to demonstrate the osteoclastic activity in both groups during tooth movement. Under TRAP staining, osteoclasts possessed acid phosphatase and identified as purple multinuclear cells near the

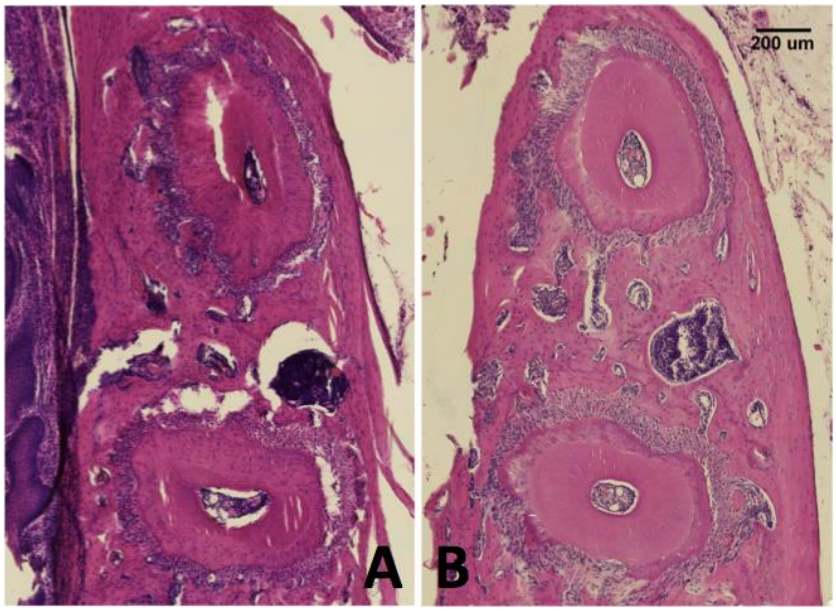

Figure 12. H & E stain of wildtype (**A**) and transgenic sponge (**B**) showing irregular PDL fibers indicating active buccal tooth movement in both control and transgenic groups. There is evident scalloped appearance of the alveolar bone indicating remodeling. Localized areas of direct resorption on the buccal bone is evident as Howship's lacunae is accumulated in the area as tooth is moved buccally with expansion spring.

alveolar bone **(Figure 13).** The number of TRAP-positive osteoclasts was quantified. Under TRAP staining, TRAP signal was localized within the ruffled border area. TRAP staining assay demonstrated that miR-29 sponge had significantly less numbers of osetoclasts during OTM while control WT had significantly more numbers of

osteoclasts present on the compression side towards the direction of tooth movement (buccal). There was decreased numbers of osteoclasts in sponge transgenic mice compared to control WT **(Figure 14).**

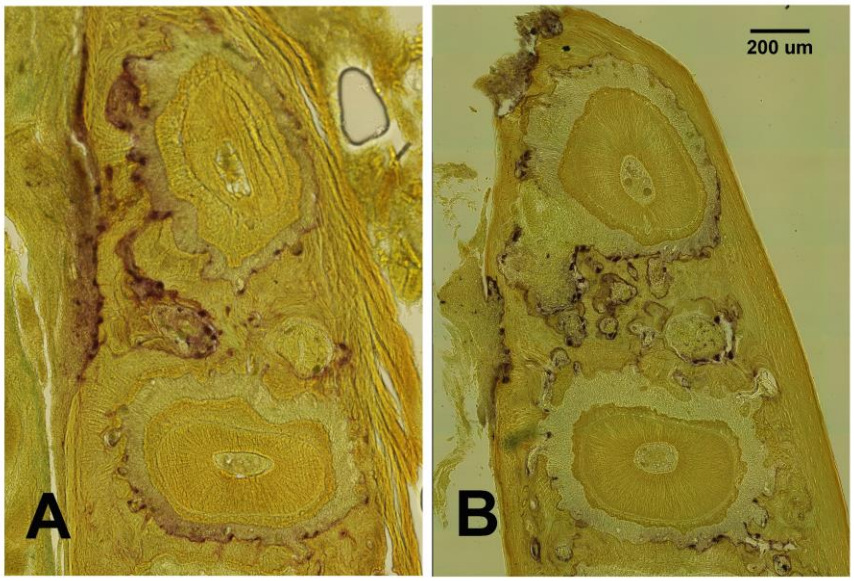

Figure 13. TRAP staining of control and miR-29 sponge mice tissue showing significantly increased # of osteoclast in control group (A). The TRAP-positive cells were identified as purple multinuclear cells near howship's lacuna of alveolar bone.

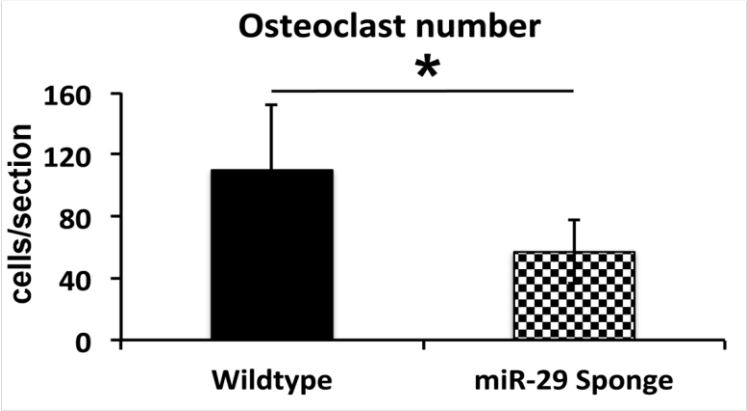

Figure 14. The TRAP staining showed that the number of osteoclasts was significantly higher in control wildtype (solid) than in sponge transgenic mice group (plaid) group *****P<0.05.

5. DISCUSSION

5.1 Discussion

The present study examines the difference in tooth movement phenotype between transgenic miR-29 sponge and wild-type control mice. The specific functions of each miRNA family in bone remodeling are just beginning to be discovered. Because miRNA is predicted to regulate hundreds of target mRNAs, there is a need to establish the best experimental approaches to create a loss of function in the miRNA of interest.

The present study was the first study to demonstrate how orthodontic tooth movement was affected by underexpression of miR-29 family *in vivo*. miRNA-29 family (a/b/c) was selected based on its potential involvement in molecular and cellular pathways associated with OTM (Chen et al., 2016). To address these questions, the transgenic miR-29 sponge mice were utilized for tooth movement and the mandibles were collected to compare with those of the wildtype controls.

In our study, we successfully generated a mouse model consisting of sponge transgenic underexpression lineage disrupting the activity of miR-29 family simultaneously. There were no significant anatomical differences of gross morphology between WT and transgenic sponge mice. The miR-29 sponge mice were fertile and survived. However, the average expression of miR-29 family in the sponge mice were around 0.2-0.6 folds of the wildtype control mice. The sponge transgenic was utilized in this study because it is time consuming and difficult to generate the knockout animals when the target genes are located on

different chromosomes (Ebert et al., 2010; Loya et al., 2009). The sponge transgene can be generated with the inserts of repeated complimentary seeding sequences of miR-29 family and created an underexpression model. The injection of antigomir oligonucleotide into the animal is not appropriate because the PDL and alveolar bone are protected underneath the gingiva and the access is limited. The injected amount of antigomir oligonucleotide needs to be optimized and might create liver toxicity (Krutzfeldt et al., 2005). When sponge is expressed at high levels, it inhibits the activity of a family of miRNAs sharing a common seed sequences at 3'UTR of target mRNAs, such as miR-29 in this study. Another major advantage of transgenic sponge to study functions of miRNA is that many miRs have seed family members encoded at different locations of chromosomes due to functional redundancy, these miRNAs would have to be knocked out individually and animals bred to generate the complete knockout strain (Ebert et al., 2010).

Constant expression of the sponge inhibitor makes it possible to examine long-term miRNA loss-of-function studies in *in vivo* model. Recent studies show that even partial miRNA inhibition can yield measurable and interesting phenotypes.

We have successfully generated mice with expression of miR-29 sponge transgenes through the promoter of gene encoding ubiquitin C (pUBC). The pUBC promotor was reported to direct high ubiquitous expression of transgenes in mice (Schorpp et al., 1996).

We detected differentially expressed miR-29 family members (a/b/c) shown in (**Figure 9**)**.** The sponge transgenic model is a powerful tool for uncovering new areas of miRNA biology. Using the sponge transgenic mice, we revealed a previously unknown important role for miR-29 during tooth movement.

The objective of the present study is to study the effect of underexpression of miR-29 on tooth movement. We used expansion spring to perform tooth movement study. The expansion spring displaces the left first molar in mice mandible and generates a light, continuous force for two weeks allowing bone remodeling to occur around the first molar. We are using this particular tooth movement model to generate a constant force during the application of mechanical force so we can study the cellular and molecular changes that occur relating to tooth movement. Previous beliefs stated that compression influences the pattern of bone resorption (Reitan, 1960). Previously, mouse knockout strains have helped us study gene and protein studies during OTM in mouse (Andrade Jr et al., 2007; Taddei et al., Yoshimatsu et al., 2006). We used this particular OTM model to trigger bone remodeling by applying mechanical load to the tooth using an expansion spring. An advantage of this model is that the forces can be transmitted to the surrounding alveolar bone, after application of mechanical force in the direction. During OTM, the alignment of PDL ligament fibers is disturbed. Furthermore, the alignment of PDL fibers may be important in the activation of osteoclasts, leading to resorption and tooth movement (Li et al., 2010).

Models of OTM are effective for the study of mechanical loading-induced bone remodeling (Verna et al., 2004). Achieving optimal force is important for performing maximum rate of OTM without harmful effects on the root, the PDL and the alveolar bone (Krishnan et al., 2006). When mechanical loading is provided by an orthodontic appliance, two different strains are generated in the PDL: compression site where the root is against the alveolar bone causing bone resorption and tension site, where PDL fibers are strained and bone is deposited (Taddei et al., 2012). Our tooth movement model provides calibrated 5cN force necessary to promote an adequate biological response from periodontal tissues. There is no consensus in the optimal amount of force for OTM in mice; The applied force has ranged between 0.10 and 0.35N in previous studies (Andrade et al., 2007; Pavlin et al., 2000; Yoshimatsu et al., 2006). Taddei et al (2012) had found that 0.35N is optimal force for OTM in mice, allowing differential expression of mediators that are involved in bone remodeling.

Other tooth movement models include the use of separators. Some disadvantages are that it is not continuous, cyclic force. Furthermore, it causes movement of multiple teeth and multiple surfaces. Our study time was two weeks long to generate of tooth movement, which would allow us enough time to observe changes that occur on tissue, cellular and molecular level in mouse model.

Orthodontic tooth movement occurs after applying force through an appliance. On a cellular level, the constant application of a mechanical force

leads to alveolar bone remodeling. Transgenic mice displayed less numbers of osteoclasts after tooth movement, resulting in less tooth movement **(Figure 10)**.

Our tooth movement results showed that the transgenic miR-29 sponge mice demonstrated less tooth movement than the control WT. According to TRAP staining results, there are significantly less osteoclasts present on the compression sides of the sections of transgenic sponge mice than the ones of WT controls. Previous studies have found that miR-29 family expressions increased during osteoclastogenesis, which is consistent with the expression of osteoclast markers TRAP and cathepsin K (Franceschetti et al., 2013). Furthermore, when miR-29 inhibited commitment and migration of pre-osteoclasts were repressed, there were no interference in cell viability, actin ring formation, or apoptosis in the mature osteoclasts (Franceschetti et al., 2013). Our studies are consistent with previous studies that demonstrated a loss of function of miR-29 has a negative impact on osteoclast formation, thus less osteoclasts are seen in transgenic sponge mice than control mice. All together, these findings suggest possible roles of miR-29 family as promoter for osteoclastogenesis process during orthodontic tooth movement.

Furthermore, Kagiya et al found that miR-29b, among other miRs, was highly expressed during osteoclast differentiation in TNF-a/RANKL-treated cells compared to RANKL-treated cells, indicating miR-29b's involvement in TNF-a-regulated osteoclast differentiation (Kagiya et al., 2013). This study is consistent with the result of our study.

Recently study in 2016 had revealed miR-21's role in promoting osteoclastogenesis (Chen et al. 2016). The authors found that miR-21 deficiency inhibited OTM by inhibiting alveolar osteoclastogenesis and blocking alveolar bone resorption. Furthermore, mice with miR-21 deficiency showed normal skeletal phenotype and similar rate in alveolar bone formation rate to wildtype mice (Chen et al., 2016). We know from previous study that miR-21 functions to block the transcription factors at the same maturation stage as miR-29 (Jing et al., 2015). We speculate that perhaps, miR-29 and miR-21 may share the same mechanism in promoting osteoclastogenesis.

The present study is the first *in vivo* study investigating how miR-29 underexpression affect osteoclastogenesis relating to tooth movement. Previous *in vitro* studies only investigated the cellular effects without considering its microenvironment. *In vivo* study reflects activities of osteocytes, osteoclasts, as well as osteoblasts working together leading to tooth movement. *In vitro* studies do not replicate the precise cellular conditions of the topic of interest. *In vivo* study is a more powerful tool to examine the interaction of genes and cells functioning in networks (Saeidnia et al., 2015). The disadvantages of the *in vitro* procedures is that they are mostly performed on cell lines that have abnormal function and the absence of biokinetics *in vitro* methods may lead to misinterpretation of the data (Saeidnia et al., 2015) whereas *in vivo* animal studies are more reliable than *in vitro* tests (Saeidnia et al., 2015). Our *in vivo* animal studies provided the overall effects of miR-29 underexpression on osteoclastogenesis, relating to tooth movement.

Osteoclast is the principle cell type in the body capable to resorb bone. Recent studies have shown that miRNAs are involved in osteoclast proliferation, differentiation, apoptosis, and bone resorption (Ji et al., 2016). During the different timepoints in the stages of murine osteoclastogenesis, miRNA microarray analysis demonstrated 49 miRNAs were upregulated and 44 were downregulated (Franceschetti et al., 2013). Tooth movement occurs by force-induced alveolar bone resorption, which is prominent in the compressive side. Bone resorption is the rate-limiting step of orthodontic tooth movement. Our study demonstrated that miR-29 underexpression led to decreased number of osteoclasts during orthodontic tooth movement, compared to control wildtype. Furthermore, this signaling pathway regulates the expression of important transcription factors, including, c-fos, NFATc1, and Fra-1 (Asagiri et al 2007; Leibbrandt et al 2008). In addition, the target genes of miR-29 family were previously found to include calcitonin receptor, factors critical for cytoskeletal organization including Cdc42, and those associated with macrophage lineage including NFIA and Cd93 (Franceschetti et al., 2013). These pathways modulate osteoclast differentiation, maturation and function. Investigation of the molecular mechanisms that coordinate osteoclastic differentiation and regulation of function will contribute to better understanding of bone resorption in tooth movement.

In this study, we demonstrated an important role for miR-29 in the effects of tooth movement phenotype through its effects on osteoclasts differentiation. In this study, we established a transgenic mouse model and discovered the role of miR-29 during orthodontic tooth movement *in vivo*.

Our findings showed that miR-29 family plays important roles in osteoclast biology, suggesting that modification of expression of miR-29 leads to abnormal bone remodeling resulting in abnormal tooth movement *in vivo*.

5.2 Limitations of the study

To generate a line of sponge mice that expressed lower level of miR-29 family expression was painstaking and time consuming, resulting in a limited number of experimental animals in the study. Due to the limited numbers of animals, certain experiments such as bone volume density or bone mineral density did not show any significant difference. The expression of target genes was not investigated to confirm the effect of miR-29 family on the osteoclast number and differentiation.

Future directions

Future research should investigate overexpression of miR-29 relating to orthodontic tooth movement. Also transduction and communication pathways between miRNAs and cellular interactions in different stages of pathways of osteogenesis and osteoclastogenesis including targets of different miRNAs are important for future studies. Other studies can include effects of stress loading, microenvironment changes and miRNA functions in bone remodeling. Focus should be on treatments that lead to specific miRNAs function in different stages of bone turnover.

6. CONCLUSION

- A miR-29 sponge transgenic murine model is successfully generated and used to compare tooth movement phenotype with that of control wildtype.
- miR-29 sponge transgenic mice expressed lower levels (0.2-0.6 folds) of miR-29 family expression compared to WT control mice.
- miR-29 sponge transgenic mice demonstrated less tooth movement compared to the WT control mice.
- miR-29 sponge transgenic mice exhibited less osteoclast numbers compared to WT control mice after 2 weeks of orthodontic tooth movement.

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8. APPENDIX

APPENDIX A – Animal Protocol Renewed

UIC UNIVERSITY OF ILLINOIS AT CHICAGO

Office of Animal Care and Institutional
Biosafety Committee (M/C 672)
Office of the Vice Chancellor for Research
206 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612

November 16, 2016

Phimon Atsawasuwan
Oral Biology
M/C 841

Dear Dr. Atsawasuwan:

The modifications requested in modification indicated below pertaining to your approved protocol indicated below have been reviewed and approved in accordance with the Animal Care Policies of the University of Illinois at Chicago on *11/8/2016*.

Title of Application:	Role of MiRNA 29 in Peridonral Ligament and Alveolar Bone
ACC Number:	14-063
Modification Number:	01
Nature of Modification:	Personnel Added: Samra Hussain, Ali Al-Qaysi and Nisha Parthasarathy
Protocol Approved:	6/13/2014
Current Approval Period:	5/20/2016 to 5/20/2017.

Currently protocol NOT matched to specific funding source. Modification will need to be submitted prior to Just in time or acceptance of award to match protocol to external funding source. All animal work proposed in the funding application must be covered by an approved protocol. UIC is the only performance site currently approved for this protocol.

This institution has Animal Welfare Assurance Number A3460.01 on file with the Office of Laboratory Animal Welfare, NIH. **This letter may only be provided as proof of IACUC approval for those specific funding sources listed above in which all portions of the grant are matched to this ACC protocol.**

Thank you for complying with the Animal Care Policies and Procedures of UIC.

Sincerely yours,

John P. O'Bryan, PhD
Chair, Animal Care Committee
JPO/*ss*
cc: BRL, ACC File, Yinghua Chen

APPENDIX B – Animal Protocol Approved

UIC UNIVERSITY OF ILLINOIS AT CHICAGO

October 9, 2015

Phimon Atsawasuwan
Oral Biology
M/C 841

Office of Animal Care and Institutional
Biosafety Committee (M/C 672)
Office of the Vice Chancellor for Research
206 Administrative Office Building
1737 West Polk Street
Chicago, Illinois 60612

Dear Dr. Atsawasuwan:

The modifications requested in modification indicated below pertaining to your approved protocol indicated below have been reviewed and approved in accordance with the Animal Care Policies of the University of Illinois at Chicago on ***10/09/2015, with the following condition.***

Title of Application: Role of miRNA in Periodontal Ligament and Alveolar Bone

ACC Number: 15-076

Modification Number: ***02***

Nature of Modification: ***Personnel Addition: Angela Lu***

Condition of Initiation: ***New personnel added must complete facility orientation/zoonotic training and enter the UIC Occupational Health Program for Individuals with Animal Contact prior to initiation of any work with animals. For rodents, contact Dr. Jeanette Purcell (996-7051) for BRL, BBC, MBRB, COD, SES, and all satellites), Dr. Cynthia Adams for the mouse barrier in COMRB (996-9236), or Ms. Kelly Pavlik for BSB (996-7810). For large animal areas of the BRL and COMRB, contact Dr. Kelly Garcia (996-8619). For primates, contact Dr. Lisa Halliday (996-9453). Facility access will not be granted until this condition is completed.***

Protocol Approved: ***6/4/2015***

Current Approval Period: ***6/4/2015 to 5/19/2016. Protocol is eligible for 2 additional years of renewal prior to expiration and resubmission.***

Current Funding: ***Currently protocol NOT matched to specific funding source. Modification will need to be submitted prior to Just in time or acceptance of award to match protocol to external funding source. All animal work proposed in the funding application must be covered by an approved protocol. UIC is the only performance site currently approved for this protocol.***

This institution has Animal Welfare Assurance Number A3460.01 on file with the Office of Laboratory Animal Welfare, NIH. **This letter may only be provided as proof of IACUC approval for those specific funding sources listed above in which all portions of the grant are matched to this ACC protocol.**

Thank you for complying with the Animal Care Policies and Procedures of UIC.
Sincerely yours,

John P. O'Bryan, PhD
Chair, Animal Care Committee
JPO /*ss*
cc: BRL, ACC File

Phone (312) 996-1972 • Fax (312) 996-9088

9. VITA

Name: Angela W. Lu

Education:

Specialty certificate in orthodontics, University of Illinois at Chicago, Chicago, IL 2017.

Master of Science in Oral Sciences, University of Illinois at Chicago, Chicago, IL 2017

Doctor of Dental Medicine, University of Connecticut School of Dental Medicine, Farmington, CT 2014
Donald Greiner Scholarship Award for academic excellence

Bachelor of Arts, Barnard College – Columbia University, New York, NY 2009

Research Experience:

Department of Reconstructive Sciences, University of Connecticut Health Center, Farmington, CT (2011-2013)
Summer Research Project and poster: *Genetic Hyaluronan deficiency disrupts joint and articular cartilage Development.*

Department of Molecular Biophysics and Biochemistry, Yale University – School of Medicine, New Haven, CT (2009 – 2010) *Research Assistant*

Department of Neuroscience – Barnard College, Columbia University, New York, NY (2008 – 2009)
Senior Thesis: *The critical effects of the bed nucleus of stria terminalis (BNST) on neural systems involving Fear and Anxiety.*

Department of Psychology – Barnard College, Columbia University New York, NY 10027
Hughes Science Pipeline Project Research Internship: *Anxiogenic Action of Ghrelin within Discrete Regions of Hypothalamus*.

Professional Membership:

American Dental Association
American Association of Orthodontics
Illinois Society of Orthodontists
Chicago Dental Society