

Developing new strategies against orthodontic relapse using microRNAs

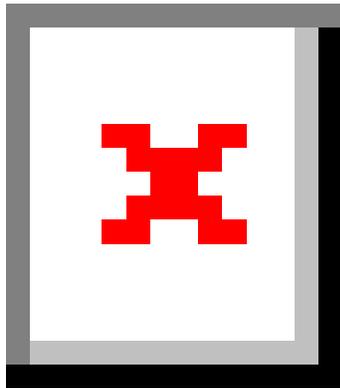
2022 Biomedical Research Awards (BRA)

Dr Christine Hong

yeumin@gmail.com
O: 617-872-1766

FollowUp Form

Award Information



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

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

Title of Project:*

Developing new strategies against orthodontic relapse using microRNAs

Award Type

Biomedical Research Award (BRA)

Period of AAOF Support

July 1, 2022 through June 30, 2023

Institution

The Regents of the University of California, San Francisco

Names of principal advisor(s) / mentor(s), co-investigator(s) and consultant(s)

Christine Hong, Tamara Alliston, Sunil Kapila

Amount of Funding

\$30,000.00

Abstract

(add specific directions for each type here)

Orthodontic relapse, in which orthodontically moved teeth rebound to their original position, remains a major challenge for up to 90% of patients.^{1,2} Despite being a substantial socio-economic burden, the causes of relapse are unclear³, as are strategies to prevent or cure it.

Recent studies have examined the role of osteocytes working through various biological mediators such as the TGF β pathway as vital mechanosensing regulators of bone homeostasis during orthodontic tooth movement (OTM). To understand the effect of osteocytic TGF β in OTM, we generated and tested a transgenic mouse line with the specific deletion of TGF β receptor II in osteocytes (T β RIIoc β -/-). Our findings demonstrated that osteocyte-intrinsic inhibition of TGF β signaling has significant skeletal consequences including impaired mechanosensitivity and decreased RANKL production by osteocytes.^{4,5} More importantly, our first results showed that defective TGF β signaling may decrease OTM rate in T β RIIoc β -/- mice, highlighting the importance of osteocyte TGF β signaling in mechanomodulation of alveolar bone remodeling and pointing to the TGF β pathway as a potential therapeutic target in preventing post-orthodontic relapse. However, due to TGF β 's broad and complex role in bone metabolism and OTM, a TGF β inhibitor itself may not be the optimal clinically relevant therapy to reduce relapse.⁶⁻⁹

Recently, microRNAs (miRs) have gained recognition as ideal candidates for targeted therapies as they integrate distinct pathways to generate a unified biological response.^{10,11} However, the role of miRs in OTM or relapse is largely unknown. Using an unbiased small RNA-seq approach to profile mechanosensitive miRs in osteocytes, we have identified miR-100 as a crucial mechanosensitive regulator downstream of TGF β signaling in osteocytes.¹² We have also shown that this miRNA is downregulated in OTM. This raises the intriguing possibility of precisely intervening in this pathway to prevent mechanosensitive relapse following OTM. Therefore, we hypothesize that miR-100 is an important regulator of osteocyte-specific mechanoregulation of OTM and post-orthodontic tooth stability such that mimicking its activity will mitigate orthodontic relapse. Specifically, we aim to:

AIM 1: Characterize the role of miR-100 and other mechanosensitive miRNAs in OTM

We will identify the functional role of mechanosensitive miRs and particularly miR-100 during OTM and identify the miRNAs that are key regulators of OTM in a mouse model. Besides verifying the postulated key role of miR-100 in this process we will perform a comprehensive profiling of mechanosensitive miRs following OTM and assess these identified miRs and miR-100 using spatiotemporal transcriptomics.

AIM 2: Determine the effects of an miR-100 mimic in orthodontic relapse

We will define the impact of miR-100 mimic treatment in limiting relapse in a mouse model of orthodontic relapse. miR-100 mimic or control miR mimic will be administered locally to orthodontically moved teeth prior to undergoing relapse. The effects of miR-100 mimic treatment in alveolar bone and PDL remodeling will also be examined. Molecular and cellular events will be evaluated by longitudinal MicroCT analysis, histology, and spatiotemporal transcriptomics.

The long-term goal of this study is to improve the understanding of miR involvement in OTM and develop future innovations in orthodontics. These studies may reveal a precise and clinically relevant intervention targeting a microRNA, in particular miR-100, to limit relapse.

Respond to the following questions:

Detailed results and inferences:*

If the work has been published, please attach a pdf of manuscript below by clicking "Upload a file".

OR

Use the text box below to describe in detail the results of your study. The intent is to share the knowledge you have generated with the AAOF and orthodontic community specifically and other who may benefit from your study. Table, Figures, Statistical Analysis, and interpretation of results should also be attached by clicking "Upload a file".

AAOF final report FINAL.pdf

Please see the attached file.

Were the original, specific aims of the proposal realized?*

Yes. Please see the attached file.

Were the results published?*

No

Have the results of this proposal been presented?*

No

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

Certainly! Here's an expanded version of your text, providing more detail and a narrative flow:

The American Association of Orthodontists Foundation (AAOF) has played an integral role in shaping my career, particularly in the area of orthodontic research. The support provided by AAOF has been instrumental in enabling me to pursue groundbreaking studies that address critical questions in our field. Leveraging the

preliminary data generated with AAOF funding, I applied for an R21 grant and received a favorable score, positioning the project strongly for resubmission.

I am profoundly grateful to the AAOF for empowering researchers like me to explore areas that hold significant relevance to the orthodontic profession. Through this partnership, I have been able to contribute meaningfully to the advancement of evidence-based orthodontic practices and the development of cutting-edge therapeutic modalities for our patients.

Accounting: Were there any leftover funds?

\$2,000.00

Not Published

Are there plans to publish? If not, why not?*

Yes, we plan to submit to JDR in Feb-March 2025.

Comment: The AAOF commends you on your research accomplishments and goals to pace these works in the public domain that advances our specialty of orthodontics. We thank you for your efforts and look forward to the fruits of your labor among published works in orthodontics and craniofacial biology.

Not Presented

Are there plans to present? If not, why not?*

Yes, we plan to present at IADR. The abstract is due in Jan. Therefore, I would like to use the remaining \$ for travel. \$2000. I would like to request extension to use this fund after IADR travel 2025 June. UCSF reimburses for trips after the completion of travel. Thank you!

Internal Review

Reviewer comments

Reviewer Status*

Approved

File Attachment Summary

Applicant File Uploads

- AAOF final report FINAL.pdf

FINAL REPORT

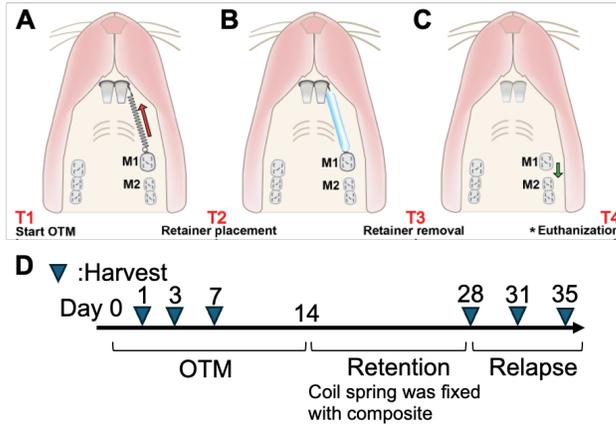


Fig 1. Schematic diagrams of the mouse model used in this experiment and the experimental schedule. (A) OTM (B) Retention (C) Relapse (D) experimental schedule. Orthodontic tooth movement (OTM) was performed for 14 days, followed by a 2-week retention period during which the coil spring was secured with composite resin. Afterward, all devices were removed, and a 7-day relapse period was observed.

Specific Aim 1 has been completed.

A total of 65 mice underwent successful Orthodontic Tooth Movement (OTM, Fig. 1A). Pilot studies were first conducted to validate the efficacy of the OTM, retention (Fig. 1B), and relapse (Fig. 1C) models and to determine the appropriate experimental timepoints. To investigate miRNA expression changes, animals were sacrificed at designated timepoints: OTM (1, 3, and 7 days) and relapse (28, 31, and 35 days). Maxillae were dissected to extract small RNAs from bone tissue (n = 10–12, Fig. 1D).

The expression profiles of mechanosensitive miRNAs known to influence bone remodeling, including miR-100, miR-200a, miR-181a, and miR-181b, were analyzed during OTM and relapse. Among these, miRNA-200a negatively regulates bone remodeling by inhibiting osteogenic differentiation of mesenchymal stem cell through downregulation of *OCN*, *RUNX2* and *OPN* gene expression by targeting *GLS*.¹ The results showed that miR-100 expression levels decreased significantly at Day 3 and Day 7 during OTM and also during relapse at Day 31 and Day 35. In contrast, miR-200a expression was significantly upregulated at Day 7 of OTM and Day 28 of relapse. miR-181a was downregulated at Day 7 of

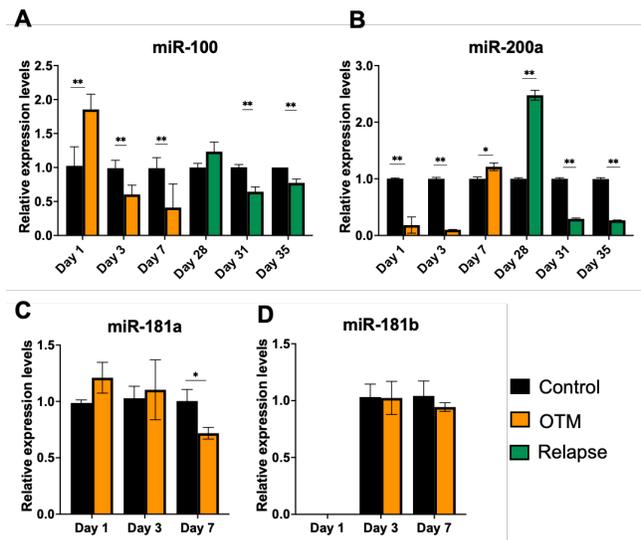


Fig 2. Expression analysis of mechanosensitive miRs during OTM and Relapse (A) miR-100 (B) miR-200a (C) miR-181a (D) miR-181b. n=3 for each group. * $p < 0.05$ ** $p < 0.01$ *** $p < 0.001$.

OTM, while miR-181b levels remained unchanged throughout the study. These findings suggest that mechanosensitive miRNAs undergo dynamic changes during OTM and relapse phases, indicating their potential roles in modulating alveolar bone remodeling processes.

To comprehensively identify miRNAs with differential expression during OTM and relapse, we utilized the Nanostring nCounter gene expression assay, which is capable of detecting 577 miRNAs, based on recommendations from the UCSF core services. RNA samples from 18 mice (7-day OTM and 3-day relapse following a 14-day retention period; n = 9 per group) were analyzed. Nanostring analysis confirmed that miR-100 was downregulated during OTM, consistent with earlier gene expression findings (data not shown). The most significantly upregulated and downregulated miRNAs during OTM and relapse are shown in Fig. 3. These data facilitated the identification of novel miRNAs as potential therapeutic targets to prevent relapse following OTM.

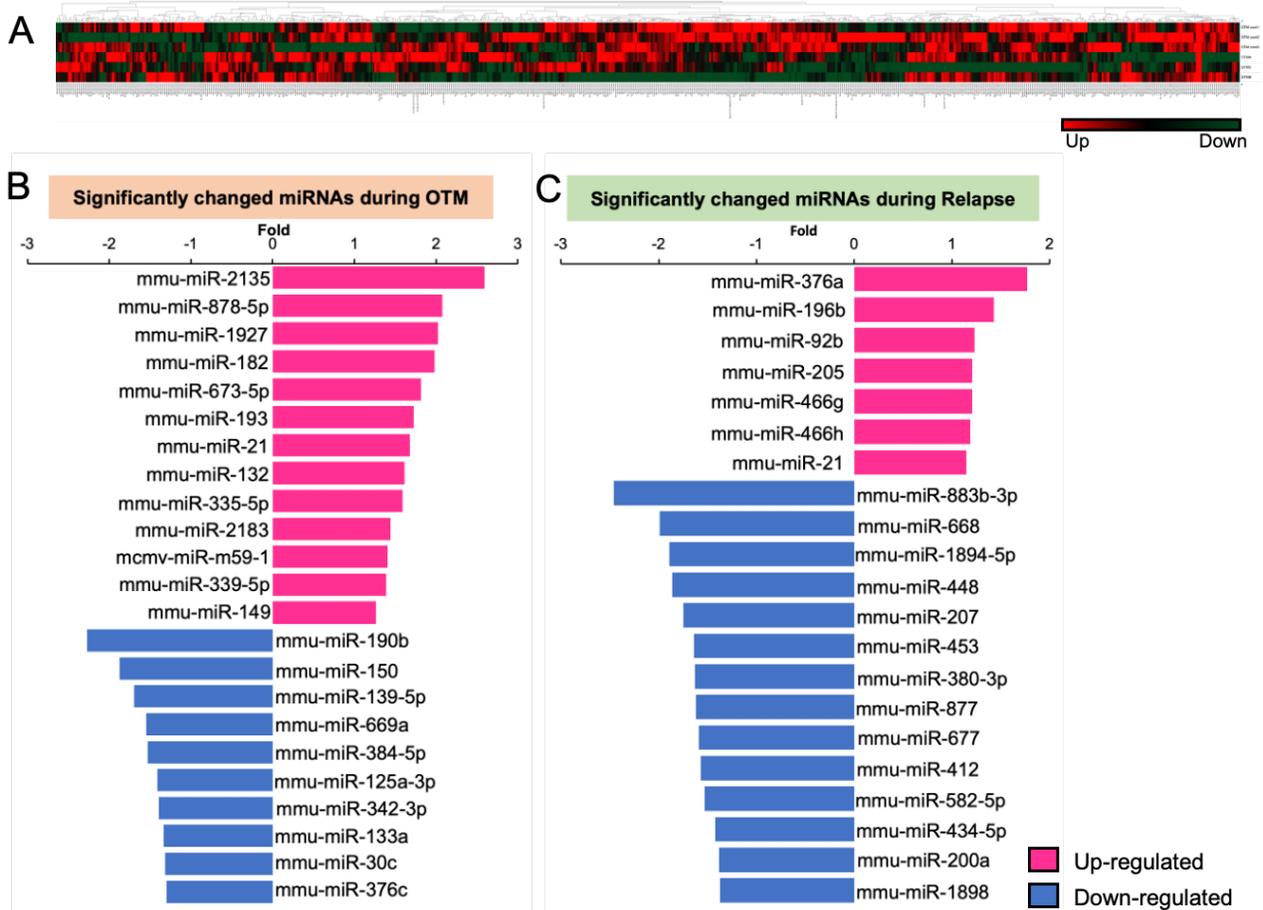


Fig 3. miR array of Nanostring nCounter assay data during OTM and Relapse. (A) Heatmap. 577 miRNAs were detected ($p < 0.05$). (B) Significantly changed miRNAs during OTM. (C) Significantly changed miRNAs during Relapse. n=9 for each group.

To further elucidate the functional role of miR-100, target prediction analysis was performed using the miRsystem² database, which identified 38 potential target genes. Among these targets, several genes demonstrated significant changes in expression during OTM and relapse. Nox4 (NADPH oxidase 4), a positive regulator of osteogenesis and osteoclastogenesis, was significantly upregulated at Day 7 and Day 31 during both OTM and relapse (Fig. 4A). Notably, Nox4 exhibited an inverse expression pattern to miR-100, suggesting it may be a direct target. Additionally, Fzd8 (Frizzled-8), a Wnt receptor, was upregulated at Day 7 and Day 31 (Fig. 4C), while Fzd5 (Frizzled-5), another Wnt receptor, showed significant upregulation at Day 31 (Fig. 4D). FGFR3 (Fibroblast Growth Factor Receptor 3) was significantly upregulated at Day 7 and Day 31 (Fig. 4B). Among these targets, the inverse relationship between Nox4 and miR-100 expression strongly suggests that miR-100 regulates Nox4-mediated pathways during OTM and relapse. These findings highlight the potential of miR-100 as a key modulator and therapeutic target in the context of bone remodeling associated with OTM.

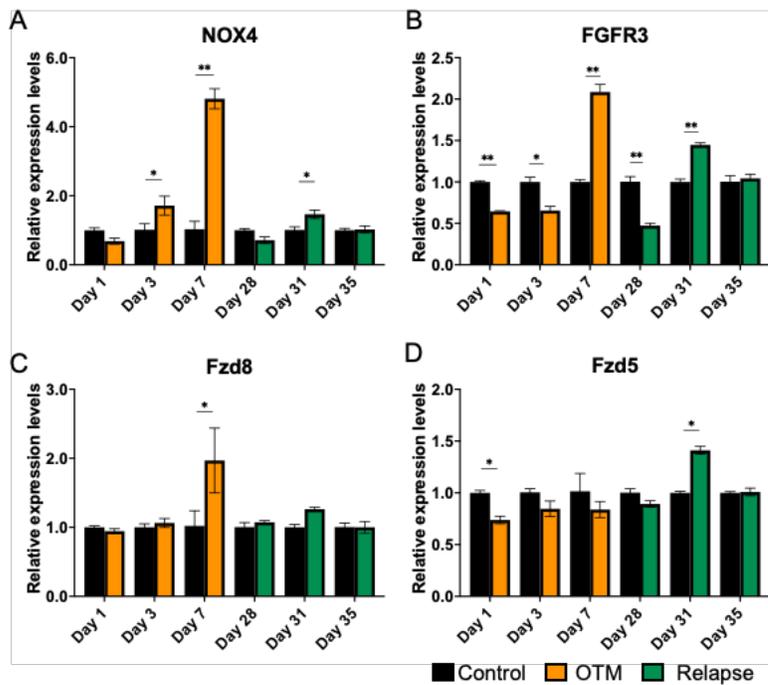


Fig 4. Expression analysis of mechanosensitive mRNAs predicted as targets of miR100 during OTM and Relapse. (A) Nox4 (B) FGFR3 (C) Fzd8 (D) Fzd5. n=3 for each group. * $p < 0.05$ ** $p < 0.01$.

Specific Aim 2 has been completed.

The role of miR-100 and miR-200a modulation in Orthodontic Tooth Movement (OTM) and relapse was investigated using targeted molecular approaches. For OTM, 2 μ l of a miR-100 inhibitor (100 μ M) or 2 μ l of a negative control inhibitor (100 μ M) was injected at the mesial, palatal, and buccal sides of the first molar (n = 4 per group). These injections were performed one day prior to the initiation of OTM and were repeated every other day throughout the experiment. Administration of the miR-100 inhibitor significantly increased the distance of the first molar movement compared to the control inhibitor group. (Fig 5A)

To investigate relapse, a miR-100 overexpression plasmid (P_{sil}-miR-100), a miR-200a inhibition plasmid (P_{mis}-miR-200a), and a control plasmid were utilized. These plasmids were developed by Dr. Amendt in Iowa University³ and are known to remain stable in vivo for several days without requiring a carrier. Injections were performed at Days 27 and 30,

corresponding to the relapse phase. Prior studies have demonstrated that miR-200a inhibition using Pmis-miR-200a significantly increases osteogenic biomarkers in human embryonic palatal mesenchyme cells and promotes bone regeneration in rat tooth socket defects³.

The expression of Nox4, identified as a candidate target of miR-100, was significantly increased in the negative inhibitor-injected group compared to non-OTM samples (Fig. 5B). Furthermore, Nox4 expression was significantly elevated in the miR-100 inhibitor group compared to the negative inhibitor group (Fig. 5B). These findings align with the predicted inverse relationship between miR-100 and Nox4 expression and suggest that miR-100 directly modulates Nox4 expression during OTM and relapse.

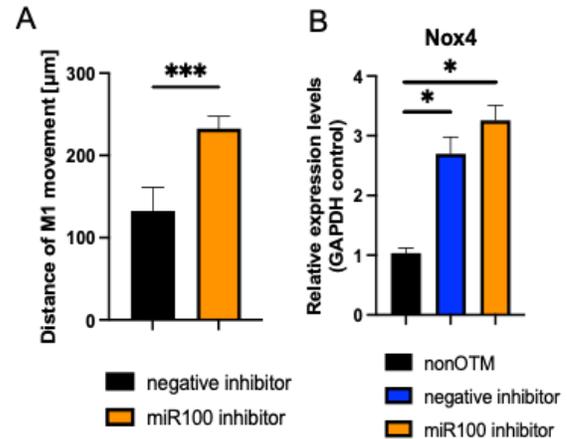


Fig 5. miR100 inhibitor effects on OTM . (A) The distance of M1 movement after 14 days of OTM. (B) Nox4 mRNA expression levels in the alveolar bone after injection of miR inhibitor. n=3 for each group. * $p < 0.05$ *** $p < 0.001$.

Next, the impact of miRNA modulation on the relapse of first molar (M1) movement was investigated using local injections of a miR-100 overexpression plasmid (Psil-miR-100) and a miR-200a inhibition plasmid (Pmis-miR-200a). A total of 4 µl of 0.2 µg/µl Psil-miR-100 or its respective control plasmid, and Pmis-miR-200a or its respective control plasmid, were injected into the gingiva surrounding M1 at Days 27 and 30 (n = 16). At Day 35, the

mice were sacrificed, and their maxillae were harvested for analysis. Imaging was performed at the UCLA Core Center for Musculoskeletal Biology and Medicine (CCMBM) MicroCT Core using the Scanco VivaCT40 (Scanco Medical, Brüttisellen, Switzerland) for live animal imaging and the Scanco MicroCT50 (Scanco Medical, Brüttisellen, Switzerland) for ex vivo specimen

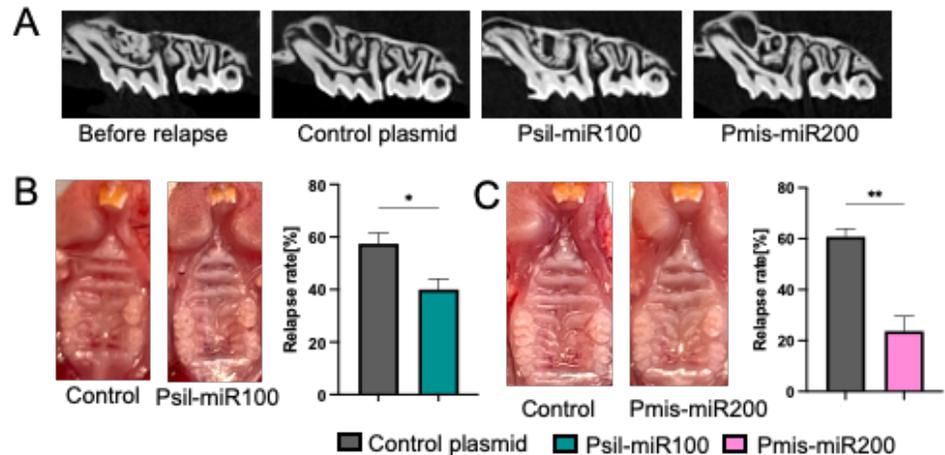


Fig 6. The relapse of the M1 was reduced by injecting miR100 overexpression and miR200a inhibition plasmids. (A) microCT images. From left to right, representative images just before device removal (prior to relapse), relapse after control plasmid injection, after Psil-miR100 injection, and after Pmis-miR200a injection. (B) Intraoral photos and the relapse rate of M1 after injecting Psil-miR100. (C) Intraoral photos and the relapse rate of M1 after injecting Pmis-miR200a. n=16. * $p < 0.05$, ** $p < 0.01$.

imaging. Ex vivo scans were conducted at a resolution of 10 μm with a 60 kV x-ray source, 166 μA current, and a 0.5 mm aluminum filter, using a 0.4-degree rotation per second.

MicroCT imaging revealed that Psil-miR-100 and Pmis-miR-200a treatments increased the distance between M1 and M2 compared to control plasmid injections during the relapse phase (Fig. 6A). The distance between M1 and M2 was measured, and the percentage relapse rate was calculated using the formula: $100 \times (1 - (\text{M1-M2 distance}/\text{OTM distance}))$. The relapse rate of M1 in Psil-miR-100- and Pmis-miR-200a-treated groups was significantly lower than that of the control plasmid group (Fig. 6B, C). These findings demonstrate that miRNA modulation effectively reduces relapse by maintaining greater separation between M1 and M2.

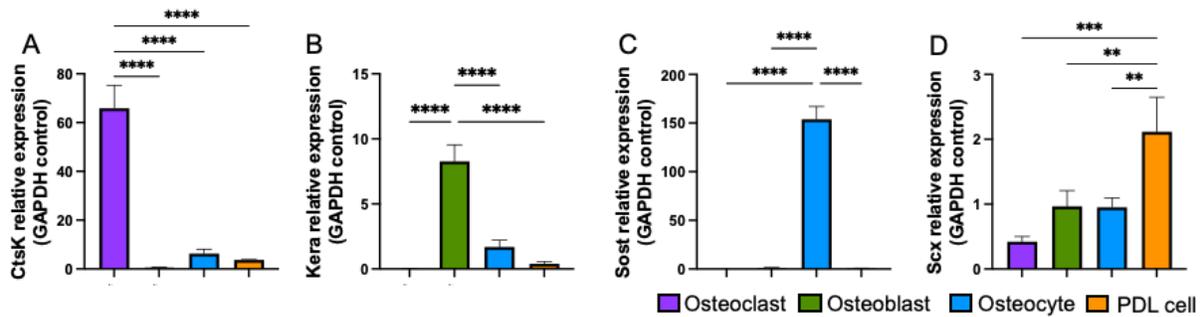


Fig 7. Osteoblast, osteocyte, and PDL cells were successful extracted from alveolar bone of the mice. (A) CtsK was the most highly expressed gene in the osteoclast. (B) Kera was the most highly expressed gene in the osteoblast. (C) Sost was the most highly expressed gene in the osteocyte. (D) Scx was the most highly expressed gene in the PDL cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. $n = 3$ for each group.

To determine the cellular localization of miR-100 and miR-200a expression in bone and periodontal tissues, maxillae were harvested from 12-week-old mice ($n = 3$). Teeth were extracted, and the periodontal ligament (PDL) cells were isolated by incubation with a solution containing 2% collagenase I and 1% dispase II for 40 minutes, followed by centrifugation. The pelleted cells were designated as PDL cells. The remaining maxillae, after tooth extraction, were further incubated with the same enzymatic solution, and fractions 2 and 4 were isolated and identified as osteoblasts and osteocytes, respectively, based on a previously established protocol. Bone marrow cells were cultured with 10 ng/ml M-CSF and 50 ng/ml RANKL for 7 days to differentiate them into osteoclasts. Cell fractions were confirmed by profiling

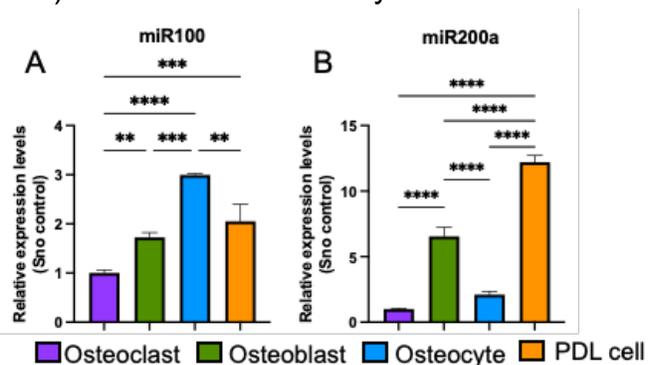


Fig 8. miR100 and miR200a expression levels in alveolar cells. (A) miR100 was expressed the most highly in the osteocyte. (B) miR200 was expressed the most highly in PDL cells. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. $n = 3$ for each group.

specific markers: Kera (osteoblasts), CtsK (osteoclasts), Scx (PDL cells), and Sost (osteocytes) (Fig 7).

Using the isolated cell fractions, the expression levels of miR-100 and miR-200a were analyzed. miR-100 expression was found to be significantly higher in osteocytes, whereas miR-200a expression was predominantly localized in periodontal ligament (PDL) cells among the alveolar bone-related cell types (Fig. 8A, B). These findings suggest that miR-100 and miR-200a have distinct cell-specific functions, with miR-100 primarily associated with osteocyte activity and miR-200a playing a central role in PDL cell function. This differential expression underscores the unique biological roles of these miRNAs in regulating bone and periodontal tissue dynamics during orthodontic tooth movement and relapse.

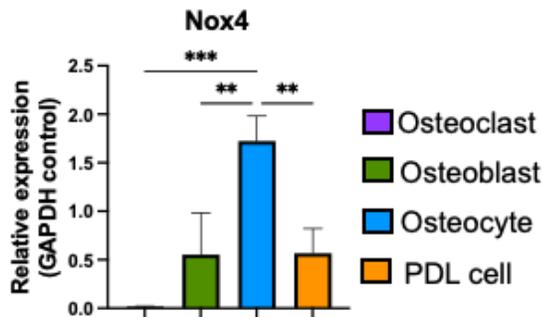


Fig 9. Nox4 expressed mainly in osteocytes where miR-100 expresses the most among alveolar bone cells. ** $p < 0.01$, *** $p < 0.001$. $n = 3$ for each group.

Additionally, Nox4 expression was highest in osteocytes among the bone-related cell types examined (Fig. 9). This observation supports the hypothesis that miR-100 directly targets Nox4 in osteocytes, contributing to its regulatory effects on orthodontic tooth movement and relapse. These findings provide mechanistic insight into the miR-100–Nox4 axis and its role in bone remodeling.

Subjects:

AIM 1: For OTM and relapse miR expression levels, 12-week-old male WT mice were divided into six groups: (1) OTM Day 1 (2) OTM Day 3 (3) OTM Day 7 (4) Relapse Day 28 (5) Relapse Day 31 (6) Relapse Day 35. For miR assay, 12-week-old male WT mice were divided into two groups: (1) OTM Day 14 (2) Relapse Day 31.

AIM 2: 12-week-old male WT mice were divided into 6 groups: (1) non-OTM (2) OTM with negative inhibitor (2) OTM with miR100 inhibitor (3) 14 days of OTM (4) Relapse with control plasmid (5) Relapse with miR100 overexpression plasmid (6) Relapse with miR200 inhibition plasmid.

Statistical analysis:

All statistical analysis was performed by using GraphPad Prism 10.0 (GraphPad Software). For multiple comparisons, one- or two-way ANOVA with Newman-Keuls test was used. For Nanostring miR array analysis, nSolver was used for the statistical analysis. Error bars in all figures represent standard error of the mean. Asterisks were assigned in the order $P^* < 0.05$, $P^{**} < 0.01$, $P^{***} < 0.001$, $P^{****} < 0.0001$ for statistically significant values, whereas exact P values were mentioned for statistically non-significant data sets.

Publications/presentations: It is planned to submit abstracts for IADR 2025 annual meetings. It is also planned to submit JDR for the publication in 2025.

References

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2. Lu TP, Lee CY, Tsai MH, Chiu YC, Hsiao CK, Lai LC, et al. miRSystem: an integrated system for characterizing enriched functions and pathways of microRNA targets. *PLoS One.* 2012;7(8):e42390.
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4. Shoji-Matsunaga A, Ono T, Hayashi M, Takayanagi H, Moriyama K, Nakashima T. Osteocyte regulation of orthodontic force-mediated tooth movement via RANKL expression. *Sci Rep.* 2017;7(1):8753.