The Role of NF-kB in Mesenchymal Stem Cell during Maxillary Expansion

2022 Biomedical Research Awards (BRA)

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FollowUp Form

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Title of Project:*
The Role of NF-kB in Mesenchymal Stem Cell during Maxillary Expansion

Award Type
Biomedical Research Award (BRA)

Period of AAOF Support
July 1, 2022 through June 30, 2023

Institution
The Trustees of the University of Pennsylvania

Names of principal advisor(s) / mentor(s), co-investigator(s) and consultant(s)
Hyeran Helen Jeon (PI)/ Chider Chen (co-investigator)

Amount of Funding
$30,000.00

Abstract

(add specific directions for each type here)

Specific Aims

Maxillary transverse deficiency is a common skeletal deformity of the craniofacial region with about 21% of children in the primary dentition. It leads to occlusal disharmony, facial asymmetry, and breathing problems. To address this, the maxillary expansion procedure has been used for more than 50 years, including the separation of two maxillae and allowing new bone to fill in the midpalatal suture area for about six months. Even after a long retention period, relapses are frequently observed, and the underlying mechanisms of how the mechanical force converts into biochemical responses are largely unknown. Mesenchymal stem cells (MSCs) in the midpalatal suture have been suggested to proliferate and differentiate into osteoblasts when the suture is expanded. MSCs maintain craniofacial bone homeostasis and interact with osteoclasts and osteoblasts in the bone remodeling process. The Gli1+ cells within the suture mesenchyme are considered as the main MSC population for craniofacial bones, whereas their ablation leads to craniosynostosis and arrest of skull growth, demonstrating the importance of these cells. The nuclear factor kappa B (NF-kB) is a central regulator of inflammation and bone homeostasis and controls cell proliferation, apoptosis, and differentiation. Interestingly, a recent study supports that the sterile inflammation-induced bone resorption must precede the anabolic response to tensile force during the maxillary expansion. Our preliminary data demonstrate a substantial increase in the number of Gli1+ cells during maxillary expansion. Moreover, palatal expansion stimulates the increased expression of NF-kB and IL-1β. Thus, we propose a central hypothesis that the NF-kB activation in MSCs is essential in tensile force-induced bone remodeling by i) increasing the proinflammatory cytokines, which initiates osteoclastogenesis and subsequent coupled new bone formation, and ii) increasing the number of MSCs through its effect on proliferation and/or apoptosis. We will test this hypothesis by Gli1+ cells lineage tracing and lineage-specific deletion of NF-kB activation in MSCs using the genetically modified mice.

Aim 1: To examine the lineage tracing of Gli1+ cells in the tensile force-induced bone remodeling. We will examine Gli1-CreERT2+.ROSA26/Ai9 mice. In those mice, MSCs are genetically altered to permanently express TdTomato with tamoxifen administration, allowing us to map these cells' fate in vivo. To induce Cre activity, we will administer tamoxifen orally to 4-week-old Gli1-CreERT2+-ROSA26/Ai9 mice for 5 consecutive days. For control we will give corn oil only instead of tamoxifen in the same manner. One week after the last administration we will perform maxillary expansion with 6-week-old mice. An opening loop will be bonded to the first and second maxillary molars on both sides, providing an initial force of 25 g. Mice with dead opening loops (no expansion force) will serve as another control. Mice will be euthanized on day 3, 7, and 14 (n = 10 for each group).

a. To determine the number and activity of the Gli1+ cells during maxillary expansion
b. To examine the osteogenic differentiation and identify osteoblasts during maxillary expansion

Aim 2: To investigate the effect and mechanism of NF-κB inhibition in MSCs on bone remodeling during maxillary expansion. IKKβ is required for activation of the classical NF-kB pathway and mediates the primary inflammatory responses. We will examine the experimental Gli1-CreERT2+.ROSA26/Ai9.IKKβL/L and control Gli1-CreERT2+.ROSA26/Ai9 mice as proposed in Aim 1. These experimental transgenic mice carry the ROSA26/Ai9 reporter transgene in addition to the floxed IKKβ gene.

a. To determine the effect of NF-kB inhibition in MSCs on bone remodeling during maxillary expansion
b. To determine whether NF-kB inhibition in MSCs affects osteoclastogenesis through the IL-1β and/or RANKL regulation
c. To examine the effect of NF-kB inhibition on the proliferation and apoptosis of MSCs

Future Research Plans

In this proposal, we will determine the role of NF-kB in MSCs during tensile force-induced bone remodeling. Our findings will form the basis of future treatment not only for the maxillary expansion but also for craniofacial deformity patients. Currently, orthodontists have observed the different responses after expansion according to the patients' age. For the next step, I will focus on the role of NF-kB in MSC in aging. It will help develop the strategies for precision treatment to expedite new bone formation and reduce relapse in older patients.

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".
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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".

Due to the large file size, the PDF version of the published manuscript will be sent separately via email.
[PMID: 40324615] Jeon, H.H.†*, Contreras Salas, M.C., Park, K., Fisher, L., Ha, S., Palmer, C., Chan, F., Graves, D.T. Comparison of the Bone Remodeling in the Midpalatal Suture during Maxillary Expansion between Young and Middle-Aged Mice. Bone. 2025 Aug:197:117512.

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

The results from Aim 1 have been published. We are currently analyzing the Aim 2 data and preparing a manuscript for submission. In addition, based on the preliminary data generated with support from the BRA, I plan to submit an R21 application during the October funding cycle.

Were the results published?*

Yes

Have the results of this proposal been presented?*

Yes

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

The AAOF BRA provided critical support for generating data that contributed to two manuscripts. In addition, the preliminary findings obtained through this funding have formed the basis of an R21 application that I am currently preparing for submission in the October funding cycle.

The AAOF BRA also supported conference attendance, enabling me to present our research findings at national meetings, including the 2025 and 2026 AADOCR Annual Meetings.

Accounting: Were there any leftover funds?

$0.00

Published

Citations*

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1. Alghamdi, B., Jeon, H.H., Ni, J., Qiu, D., Liu, A., Hong, J., Ali, M., Wang, A., Troka, M., Graves, D.T. Osteoimmunology in Periodontitis and Orthodontic Tooth Movement. Curr Osteoporos Rep. 2023 Apr;21(2):128-146.

2. Yu, W., Jeon, H.H. (co-corresponding author)*, Kim, S., Dayo, A., Mupparapu, M., Boucher, N.S. Correlation between TMJ Space Alteration and Disc Displacement: A Retrospective CBCT and MRI Study. Diagnostics (Basel). 2023 Dec 25;14(1):44.

3. Kang, K.†, Jeon, H.H. (co-first and corresponding author)†*, Shahabuddin, N. Does Aligner Refinement Have the Same Efficiency in Deep Bite Correction?: A Retrospective Study. BMC Oral Health. 2024 Mar 15;24(1):338.

4. Jeon, H.H.†, Huang, X.†, Cortez, L.R.†, Sripinun, P., Lee, J.M., Hong, J., Graves, D.T. Inflammation and mechanical force-induced bone remodeling. Periodontol 2000. 2024 Dec 30.

5. Kim, L., Jeong, J., Setzer, F.C., Chung, C.H., Jeon, H.H.* Cephalometric Norms for African Americans with Normal Occlusion in the Greater Philadelphia Region: A Retrospective Observational Study. J Orofac Orthop. 2025 Jul 15.

6. Jeon, H.H.†*, Contreras Salas, M.C., Park, K., Fisher, L., Ha, S., Palmer, C., Chan, F., Graves, D.T. Comparison of the Bone Remodeling in the Midpalatal Suture during Maxillary Expansion between Young and Middle-Aged Mice. Bone. 2025 Aug:197:117512.

7. Tucker, B.†, Kang, J.†, Hudson-Boyd, C., Kadan, S., Saconi, B., Keenan, B.T., Schwab, R.J., Chung, C.H., Jeon, H.H.* Skeletodental and Soft Tissue Changes Following Treatment with Herbst and Pendex Appliances: A Retrospective CBCT Study. Clin Oral Investig. 2026 Jan 12;30(1):48.

8. Kyle, W.P.†, Li, J.M.†, Tucker, B., Kadan, S., Wiemken, A.S., Saconi, B., Keenan, B.T., Schwab, R.J., Chung, C.H., Jeon, H.H.* Effects of Herbst and Pendex Appliance Treatment on the Upper Airway: A CBCT Analysis. Am J Orthod Dentofacial Orthop. 2026 Jun;169(6):798-813.

Was AAOF support acknowledged?

If so, please describe:

Yes, AAOF support was acknowledged in the publication as follows:

"This work was supported by the Biomedical Research Award from the American Association of Orthodontists Foundation (HHJ)."

Presented

Please list titles, author or co-authors of these presentation/s, year and locations:*

1. Park, K.J., Contreras Salas, M.C., Jeon, H.H. Effect of Aging on the Tensile Force-induced Mesenchymal Stem Cells Response: Proliferation and Apoptosis (Poster presentation). PDM Research Day, Philadelphia, PA, 2024.

2. Kim, L., Jeon, H.H., Jeong, J., Setzer, F.C. Cephalometric Norms for African Americans with an Ideal Occlusion in Philadelphia (Poster presentation). PDM Advances in Clinical Care and Education Day, Philadelphia, PA, 2024.

3. Kyle, W., Jeon, H.H., Tucker, B., Hudson-Boyd, C., Kadan, S., Chung, C.H. Upper Airway Changes After Herbst or Pendex Appliance Treatment in Class II Patients: a CBCT Study (Poster presentation). PDM Advances in Clinical Care and Education Day, Philadelphia, PA, 2024.

4. Hudson-Boyd, C., Tucker, B., Jeon, H.H., Kadan, S., Chung, C.H. Perception of Profile Changes After Herbst or Pendex Appliances in Class II Patients (Poster presentation). PDM Advances in Clinical Care and Education Day, Philadelphia, PA, 2024.

5. Wan, A.G., Jeon, H.H., Kim, S. Three-dimensional Upper Airway Anatomy for Korean Population with Mild to Severe Obstructive Sleep Apnea: an MRI Study (Poster presentation). PDM Advances in Clinical Care and Education Day, Philadelphia, PA, 2024.

6. Jeon, H.H., Contreras Salas, M.C., Park, K.J., Fisher, L., Ha, S., Chan, F., Graves, D.T. Comparison of the Osteogenic Potential of Mesenchymal Stem Cells during Maxillary Expansion between Young and Middle-Aged Mice (Oral presentation). Angle East Annual Meeting, Boston, USA, 2024

7. Jeon, H.H., Kyle, W., Li, J.M., Tucker, B., Kadan, S., Wiemken, A.S., Saconi, B., Keenan, B.T., Schwab, R.J., Chung, C.H. Treatment Outcomes Comparison on the Upper Airway Between Herbst and Pendex Appliances: A CBCT Study (Poster presentation). AADOCR/CADR Annual Meeting, New York, USA, 2025

8. Park, K.J., Contreras Salas, M.C., Jeon, H.H. Effect of Aging on the Tensile Force-induced Mesenchymal Stem Cells Response: Proliferation and Apoptosis (Poster presentation). AADOCR/CADR Annual Meeting, New York, USA, 2025

9. Fisher, L., Contreras Salas, M.C., Jeon, H.H. Effect of NF-kB in Mesenchymal Stem Cells during Mechanical Force-induced Bone Remodeling: MicroCT Analysis (Poster presentation). PDM Research Day, Philadelphia, PA, 2025

10. Park, K.J., Kim, C.S., Contreras Salas, M.C., Jeon, H.H. The Role of NF-kB in Mesenchymal Stem Cells during Mechanical Force-induced Bone Remodeling: MSC Proliferation (Poster presentation). PDM Research Day, Philadelphia, PA, 2025

11. Bohm, S., Contreras Salas, M.C., Jeon, H.H. The Expression of NF-kB in Mesenchymal Stem Cells during Tensile Force-induced Bone Remodeling (Poster presentation). PDM Research Day, Philadelphia, PA, 2025

12. Gong, G., Contreras Salas, M.C., Jeon, H.H. Effect of NF-kB in Mesenchymal Stem Cells on Osteoclast Formation During Maxillary Expansion (Poster presentation). PDM Research Day, Philadelphia, PA, 2025

13. Palmer, C., Contreras Salas, M.C., Jeon, H.H. Comparison of The Osteogenesis in the Midpalatal Suture During Maxillary Expansion Between Young and Middle-aged Mice (Poster presentation). PDM Research Day, Philadelphia, PA, 2025

14. Kyle, W., Jeon, H.H. Three-Dimensional Evaluation of Glenoid Fossa Remodeling Following Herbst Appliance Treatment in Growing Class II Patients (Poster presentation). PDM Advances in Clinical Care and Education Day, Philadelphia, PA, 2025

15. Choi, J.S., Wan, A.G., Chen, V., Gong, G., Dayo, A., Kim, J.Y., Wiemken, A., Schwab, R.J., Kim, S., Jeon, H.H., Shin, C. Association of Upper Airway Morphological Phenotypes with Obstructive Sleep Apnea in Middle-Aged Korean Adults (Oral and poster presentation). 2025 World Sleep Congress, Singapore, 2025

16. Kyle, W., Jeon, H.H. 3-Dimensional Assessment of Glenoid Fossa Remodeling After Herbst Appliance Treatment (Oral presentation). IADR/AADOCR/CADR, San Diego, CA, 2026

17. Jeon, H.H., Kim, L., Kim, S., Evans, M. Effectiveness of Customized MARPE in Adults Over 30: CBCT Study (Oral presentation). IADR/AADOCR/CADR, San Diego, CA, 2026

18. Kyle, W., Jeon, H.H. Three-Dimensional Assessment of Skeletal and Dental Changes Following Pendex Appliance Treatment in Growing Class II Patients (Poster presentation). Advances in Clinical Care and Education Day, Philadelphia, PA, 2026

Was AAOF support acknowledged?

If so, please describe:

Yes, AAOF support was acknowledged in the publication as follows:

"This work was supported by the Biomedical Research Award from the American Association of Orthodontists Foundation (HHJ)."

Internal Review

Reviewer comments

The publication that was a result of the support by the AAOF's 2022 BRA is attached in the documents tab for the final report reviewer's reference-GR

Reviewer Status*

Approved

Comment: *The PI has been prolific with the AAOF award project. Congratulations on a job well done!*

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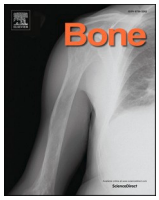

Full Length Article

Comparison of the bone remodeling in the midpalatal suture during maxillary expansion between young and middle-aged mice

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ABSTRACT

Maxillary expansion is a common orthodontic procedure for treating maxillary transverse deficiency. However, the cell responses to mechanical force may vary across different age groups, suggesting the need for age-specific treatment protocols. To compare the age-related responses to the mechanical force, we examined the 6-week- and 12-month-old mice undergoing maxillary expansion with 0.012-in. stainless steel orthodontic wire bonded to the maxillary first and second molars (25 g force). Mice were euthanized on days 0, 3, 7, and 14 for analysis. MicroCT analysis, tartrate-resistant acid phosphatase (TRAP) stain, and immunofluorescence/immunohistochemistry stain using antibodies to RUNX2, alkaline phosphatase (ALP), Gli1 and Ki67 along with the TUNEL assay, were conducted to evaluate suture width, osteoclast activity, new bone formation and mesenchymal stem cell (MSC) proliferation and apoptosis. Both 6-week- and 12-month-old mice exhibited successful midpalatal suture opening, but young mice demonstrated earlier and more intense osteoclast activity, along with higher expression of RUNX2 and ALP. Young mice also exhibited a higher percentage of Gli1+Ki67+ immunopositive cells, while middle-aged mice showed a higher percentage of Gli1+TUNEL+ positive cells on day 3 after maxillary expansion. Our findings suggest that aging negatively impacts mechanical force-induced bone remodeling by reducing osteoclastogenesis, osteogenesis, and MSC proliferation while increasing MSC apoptosis.

1. Introduction

Maxillary transverse deficiency is a prevalent skeletal deformity affecting approximately 23.3 % of children in primary dentition and 30 % of adult orthodontic patients [1,2]. This condition can result in occlusal disharmony, facial asymmetry, and breathing difficulties. To address this issue, the maxillary expansion procedure has been utilized for over five decades. During maxillary expansion mechanical force separates the two maxillae, creating a gap in the midpalatal suture. Initially, this gap is filled with blood and granulation tissue, followed by active new bone formation [3–5]. It is generally recommended to maintain an expander for 4–6 months to ensure adequate bone formation and remodeling, regardless of the patient's age. Until now, most research has focused on reducing resistance from the circummaxillary sutures or enhancing skeletal anchorage during maxillary expansion in adult patients. Bone formation and mineralization within the midpalatal suture are essential for the success and stability of maxillary expansion [6]. However, despite its critical role, few studies have evaluated and compared bone formation in the midpalatal suture across different age groups.

Mesenchymal stem cells (MSCs) exist in the craniofacial suture, maintain bone homeostasis and interact with osteoclasts and osteoblasts in the bone remodeling process. These versatile cells can differentiate into various cell types, such as osteoblasts, chondrocytes, and adipocytes. In addition, MSCs are responsive to mechanical cues, with their differentiation fate being modulated by mechanical stimuli [7–9]. When subjected to tensile forces, MSCs tend to differentiate into osteoblasts, contributing to bone formation [10]. A study demonstrates that local injection of MSCs into the midpalatal suture after expansion enhances bone formation by augmenting the osteoblast population [11]. Notably, within craniofacial sutures, Gli1+ cells constitute the primary MSC population and exhibit rapid activation following expansion [4,12–14]. Conversely, depletion of Gli1+ cells results in craniosynostosis and stunts skull growth, underscoring their critical role in craniofacial bone

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development [12].

According to the American Association of Orthodontists, the proportion of adult orthodontic patients has risen significantly, comprising about 25 % of the total orthodontic patient population [15,16]. Consequently, there has been a corresponding increase in demand for maxillary expansion among adult patients. Interestingly, despite possible age-related differences in biological responses to tensile force, a uniform 4–6 months retention protocol has been applied clinically across all age groups. MSCs have garnered significant attention in tissue regeneration due to their self-renewal and multipotency. However, understanding the age-related impacts on MSC functions has produced conflicting results [17–22]. While some studies have found no discernible age-related differences in MSC functions, particularly in osteogenic differentiation and proliferation, others have reported diminished proliferative, anti-inflammatory, and multipotent potentials in older individuals. These inconsistencies warrant further investigation, especially concerning the age-related impacts of MSCs on osteogenesis during maxillary expansion.

Therefore, we aim to assess the effects of aging on osteoblast and osteoclast responses to mechanical force, as well as MSC proliferation and apoptosis in the short term, using 6-week-old and 12-month-old mice models.

2. Materials and methods

2.1. Mouse maxillary expansion model

Mouse experiments were approved by the University of Pennsylvania Institutional Animal Care and Use Committee. The maxillary expansion devices were applied to mice aged 6 weeks and 12 months under general anesthesia, administered via intraperitoneal injection of ketamine (80 mg/kg) and xylazine (5 mg/kg), following established protocols [10]. Mice aged 6 weeks correspond to early adolescence in humans, with both first and second molars fully erupted, while mice aged 12 months represent the middle-aged stage in humans [10]. Initially, a 0.012-in. stainless steel orthodontic wire forming an opening loop was affixed to the first and second maxillary molars bilaterally using light-cured adhesive (3 M Unitek, Monrovia, CA), applying an initial force of 25 g (Fig. 1). The initial width of the expander is set to 2.5 mm at the maxillary first molar level, and it is activated to 2 mm (0.5 mm activation with 25 g of force). Mice were euthanized on days 0, 3, 7, and 14 (n = 8 for each group, gender evenly distributed). Previous studies on maxillary expansion indicated that osteoclasts appeared by day 3 post-force application, peaking on day 5 and declining by day 7 [23]. New bone formation was first observed at the palatal bone edges on day 7, with continued sutural width increase until day 14 [10]. Throughout the experiment, mice were weighed every three days and housed in cages containing 2 to 5 individuals under standard conditions, with a 14-h light and 10-h dark cycle. All animals received powdered food (DietGel, ClearH2O, Westbrook, ME) and had unrestricted access to water, with continuous monitoring for their well-being.

2.2. MicroCT

Specimens were fixed in 4 % paraformaldehyde in PBS at 4 °C for 24 h before being subjected to microCT scanning (MicroCT35; SCANCO Medical, Bassersdorf, Switzerland) at settings of 55kVp and 145 μA intensity, with an integration time of 200 ms. The maxillary molar regions were scanned at a 20 μm isotropic voxel size. After reconstruction, all images were converted to DICOM format and imported into OsiriX (Pixmeo SARL, Bernex, Switzerland) for analysis. Suture width was measured at the nasal, middle, and oral thirds of the midpalatal suture at the midpoint of the palatal roots of the maxillary first molars using multi-planar reconstruction (Fig. 2). Intermolar width was determined as the distance between the palatal root apices, furcation and crowns at coronal heights of contours (Table 2).

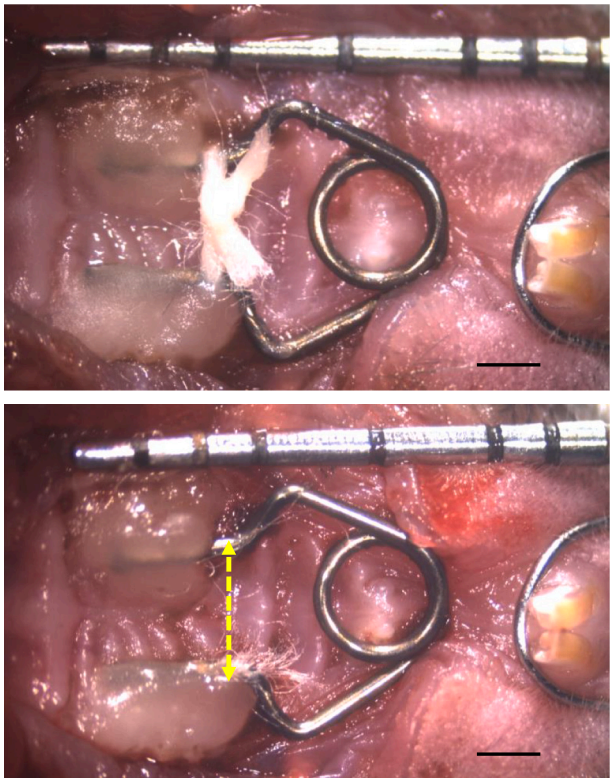

Fig. 1. Mouse maxillary expansion model. An 0.012-in. stainless steel orthodontic wire forming an opening loop was bonded to the 1st and 2nd maxillary molars bilaterally using light-cured adhesive. The initial width of the expander is set to 2.5 mm, and it is activated to 2 mm to exert a force of 25 g. Bar, 1 mm.

For bone volume fraction analysis, DICOM images were transferred to Dragonfly (Québec, Canada). Employing a protocol similar to a previous rat study [24], a region of interest (ROI) measuring 0.5 mm (width) x 0.1 mm (height) was drawn in the coronal plane, encompassing the midpalatal suture and bilateral bones at the level of the maxillary first molars bilaterally. This ROI spanned from the mesial margin of the palatal root to the distal margin of the distobuccal root, with an average total volume of 0.05 mm^3. Bone volume and bone volume fraction were then quantified within this ROI.

2.3. Histological analysis and TRAP stain

Specimens underwent decalcification in 14 % EDTA for 2.5 weeks, followed by paraffin embedding and sectioning at a thickness of 5 μm. TRAP staining, coupled with hematoxylin counterstaining, was performed as per the manufacturer's instructions (Sigma-Aldrich, Saint Louis, MO). Images of the TRAP-stained sections were captured using a Nikon Eclipse 90i microscope (Nikon, Melville, NY) under 20× and 40× objectives and analyzed using NIS Elements-AR software (Nikon). Osteoclasts, identified as TRAP-positive multinucleated cells, were quantified along the midpalatal suture across bony surfaces, periosteal membrane, and bone marrow area.

2.4. Immunofluorescence and immunohistochemistry

The sample sections underwent preparation for immunofluorescence and immunohistochemistry analysis. Antigen retrieval was conducted in 10 mM citric acid (pH 6.0) at 120 °C. Subsequently, sections were incubated overnight at 4 °C with primary antibodies targeting RUNX2 (Mouse, Santa Cruz, sc-390351) or Gli1 (Mouse, Santa Cruz, sc-515751) along with the corresponding isotype-matched negative control IgG. To

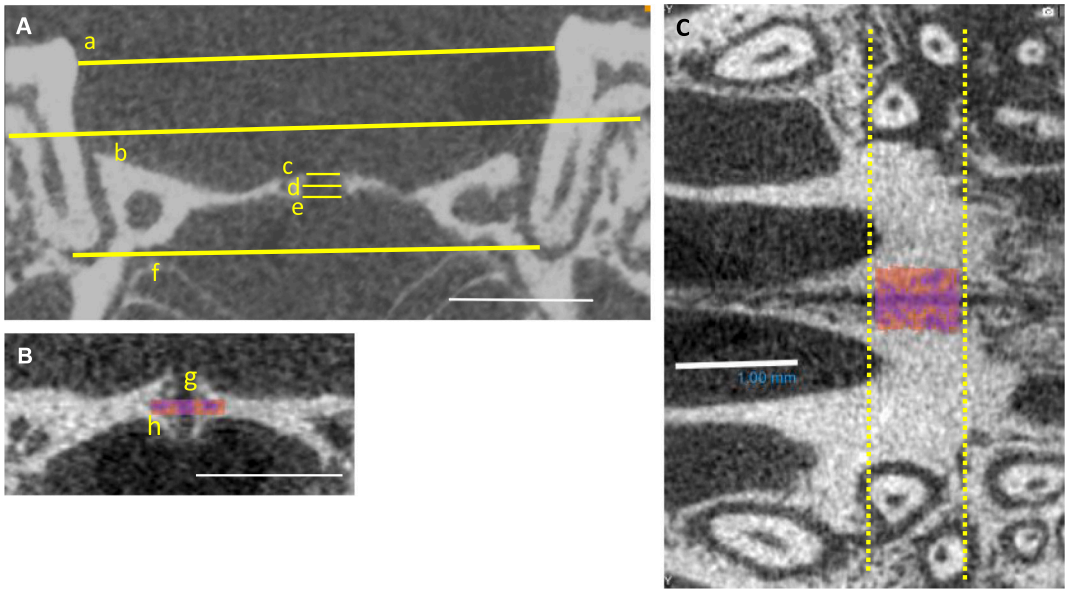

Fig. 2. MicroCT analysis. (A) Osirix analysis on a young D3 sample using a coronal slice at the palatal roots of the maxillary 1st molars. Top to bottom: a) intermolar width (crown), b) intermolar width (furcation), suture width: c) oral third,d) middle third,e) nasal third, f) intermolar width (root apices). Bar, 1 mm. (B) Bone volume fraction analysis. A region of interest (ROI) measuring 0.5 mm (width) x 0.1 mm (height) was drawn in the coronal plane, encompassing the midpalatal suture and bilateral bones at the level of the maxillary first molars bilaterally. The purple area represents g) the suture gap, and the brown represents h) the bony edges of the mid-palatal suture. Bar, 1 mm. (C) The ROI spanned from the mesial margin of the palatal root to the distal margin of the distobuccal root, with an average total volume of 0.05 mm^3. Bar, 1 mm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

visualize the staining, Alexa FluorTM 647-conjugated goat anti-mouse IgG (Invitrogen, A-21235) and DAPI-containing mounting media (Abcam, Cambridge, MA) were utilized. For the alkaline phosphatase (ALP) immunohistochemistry stain, sections were blocked using 2.5 % normal horse serum blocking solution for 20 min at room temperature, followed by incubation with primary ALP antibody (Goat, RnD, AF2910) overnight at 4 °C. Primary antibodies were detected using ImmPRESS® HRP-Anti-Goat IgG Polymer Detection Kit (Vector Laboratories, MP-7405) according to the manufacturer's instructions.

In order to further assess how aging regulates bone remodeling through its effects on MSC proliferation or apoptosis, we carried out a double immunofluorescence stain using the antibodies against Gli1 and Ki67 (Rabbit, Abcam, ab15580) or Gli1 antibody with TUNEL assay (Promega DeadEnd™ Fluorometric TUNEL System, PR-G3250). Apoptosis was quantified by TUNEL assay according to the manufacturer's recommendations.

Images were taken at 4×, 20×, and 40× magnifications using a fluorescence microscope (ECLIPSE 90i; Nikon), ensuring consistent exposure times for both experimental and negative control groups. Capture settings were adjusted to prevent immunofluorescence detection with the control IgG in all experiments. Image analysis was performed under a 40× objective using NIS Elements AR image analysis software. The percentage of positive cells for each specific antibody was calculated by dividing the number of immunopositive cells by the area of the midpalatal suture. ALP expression was quantified by measuring the pixel area and expressed as a percentage of the total midpalatal suture area using NIS Elements AR image analysis software as previously described [25].

2.5. Statistics

Statistical analysis was performed using ANOVA with Dunnett's post hoc test or non-parametric Kruskal–Wallis tests with Dunn's post hoc test to evaluate differences across multiple time points compared to the control (D0) in the young and middle-aged mouse groups, respectively. For comparisons between young and middle-aged groups at each time point, a two-tailed Student's *t*-test or the non-parametric Wilcoxon matched-pairs signed-rank test was employed. Results were presented as mean ± SEM, with statistical significance at $P < 0.05$.

3. Results

3.1. Suture gap, intermolar widths and bone volume fraction

In our study both young and middle-aged mice presented open, straight midpalatal suture lines (Fig. 3A). In response to tensile force, the width of the midpalatal suture notably expanded from day 0 to day 14 in both young and middle-aged mice ($P < 0.05$) (Table 1 and Figs. 3A-3G). Initially, in young mice, the suture width at the oral third level measured 0.09 ± 0.01 mm, increasing to 0.19 ± 0.04 mm by day 14 ($P < 0.05$). Similarly, in middle-aged mice, the suture width was 0.06 ± 0.01 mm on day 0, reaching 0.25 ± 0.03 mm by day 14 ($P < 0.05$). Notably, on day 7 ($P < 0.05$, oral third) and day 14 ($P < 0.05$, middle and nasal thirds), the suture width in middle-aged mice significantly exceeded that in young mice (Table 1). However, differences in suture widths between young and middle-aged mice were statistically insignificant on days 0 and 3 ($P > 0.05$). Intermolar widths at the crown level significantly increased from day 0 to day 14 in both young and middle-aged mice ($P < 0.05$), whereas insignificant changes were observed at the root level ($P > 0.05$). Intermolar widths at the furcation, which is considered as the center of resistance during tooth movement, significantly increased from day 0 to day 14 in both young and middle-aged mice ($P < 0.05$). The intermolar width at the furcation level was greater in young mice compared to middle-aged mice (day 7, $P < 0.05$) (day 14, $P > 0.05$) (Figs. 3H-3I and Table 2).

In addition, we examined the bone volume fraction (BV/TV) at the level of the maxillary first molars bilaterally (Figs. 3J-3K). Middle-aged mice exhibited significantly smaller bone marrow cavity spaces compared to young mice, leading to a higher baseline bone volume fraction (BV/TV) at both day 0 and day 3 (Figs. 3J-3K and Table 2) ($P < 0.05$). Both groups demonstrated a significant decrease in BV/TV over the entire experimental period ($P < 0.05$ for young mice and $P < 0.0001$

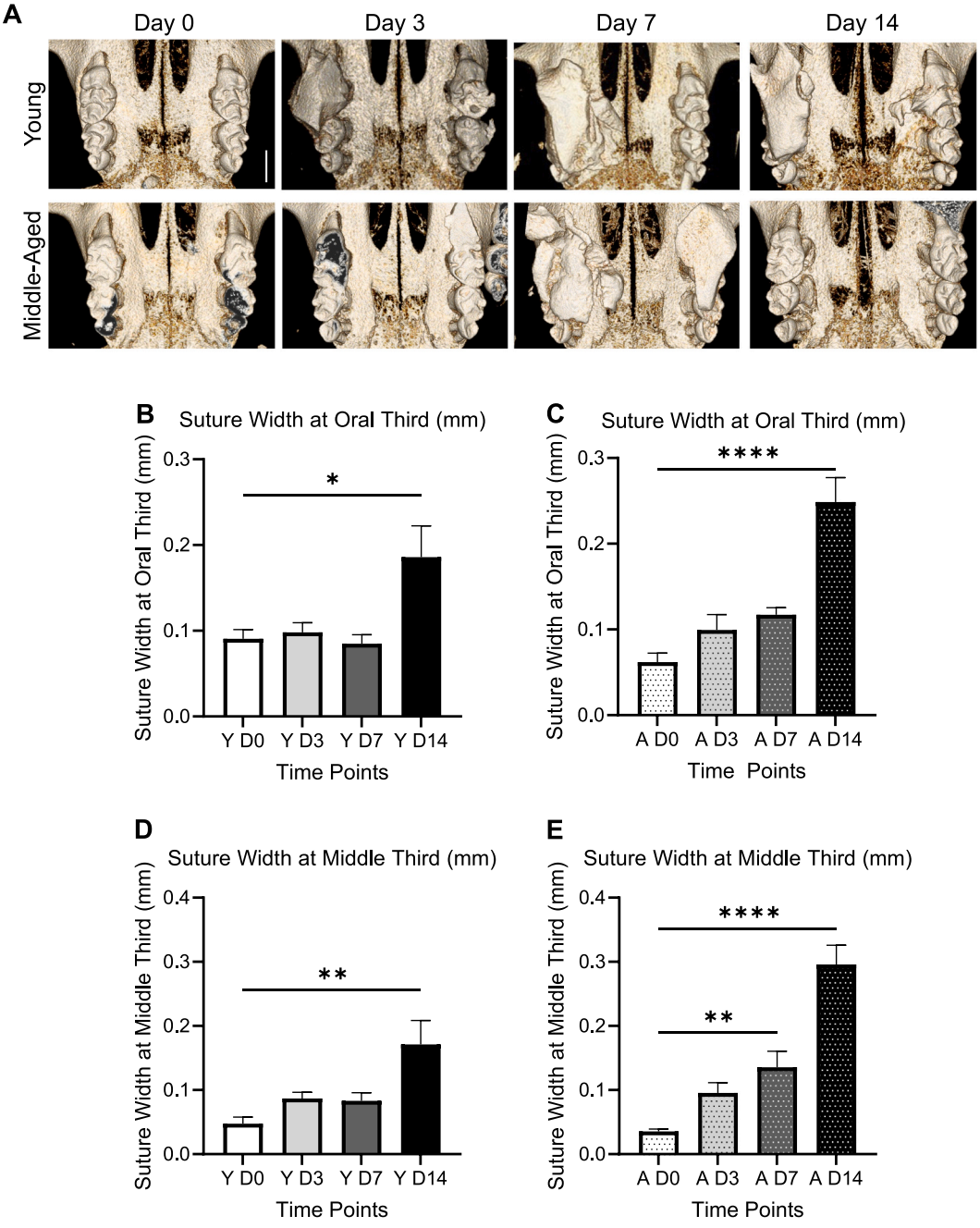

Fig. 3. MicroCT measurements. (A) 3D constructed microCT images. Bar, 1 mm. (B—C) Suture width at oral third, (D-E) Suture width at middle third, (F-G) Suture width at nasal third, (H—I) Intermolar width at furcation, (J-K) Bone volume fraction. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. Y: young mice and A: middle-aged mice.

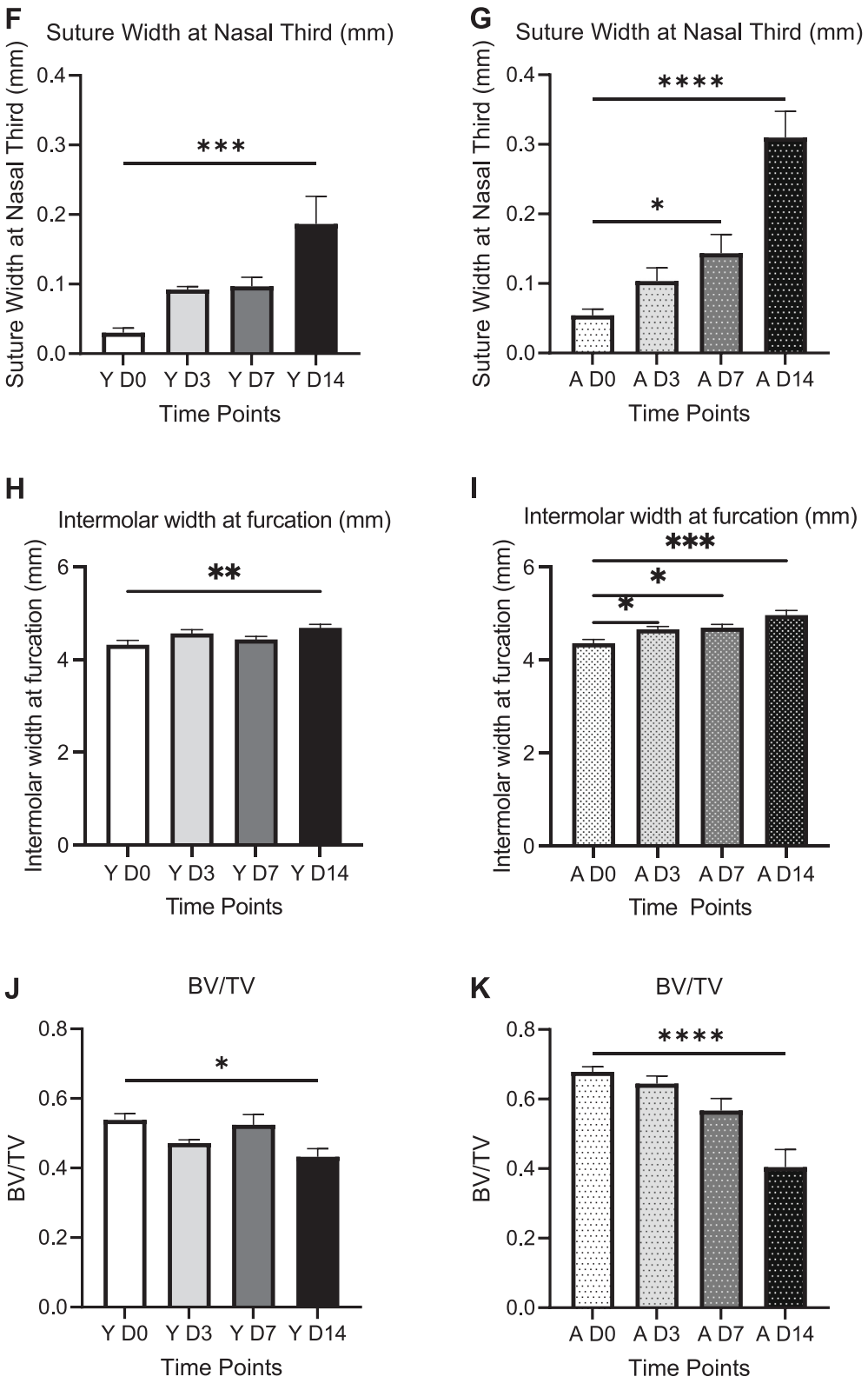

Fig. 3. (*continued*).

Table 1
Suture width (microCT).

Mice		Suture width (mm)					
Duration	Group	Oral third	*P* value	Middle third	P value	Nasal third	P value
Day 0	Y	0.09 ± 0.01		0.05 ± 0.01		0.03 ± 0.01	
	A	0.06 ± 0.01		0.04 ± 0.00		0.05 ± 0.01	
Day 3	Y	0.10 ± 0.01		0.09 ± 0.01		0.09 ± 0.01	
	A	0.10 ± 0.02		0.10 ± 0.02		0.10 ± 0.02	
Day 7	Y	0.08 ± 0.01	*	0.08 ± 0.01		0.10 ± 0.01	
	A	0.14 ± 0.02		0.14 ± 0.03		0.14 ± 0.03	
Day 14	Y	0.19 ± 0.04		0.17 ± 0.04	*	0.19 ± 0.04	*
	A	0.25 ± 0.03		0.30 ± 0.03		0.31 ± 0.04	

Y: 6 week-old mice and A: 12 month-old mice.
For comparisons between young and middle-aged groups at each time point, a two-tailed Student's *t*-test or the non-parametric Wilcoxon matched-pairs signed-rank test was employed.
* $P < 0.05$.

for middle-aged mice). The middle-aged mice presented a more dramatic decrease in BV/TV than the young mice from day 0 to 14.

3.2. Osteoclast formation

Osteoclast formation was examined along the bone surface in i) the midpalatal suture, ii) periosteal membrane and iii) bone marrow cavity in the TRAP-stained sections (Figs. 4A-4C and Table 3). Interestingly, there was rare osteoclast formation along the bony surfaces in the midpalatal suture. In young mice osteoclasts were predominantly located in the bone marrow cavity and along the periosteum of the outer bony margins, while osteoclasts in middle-aged mice were primarily located along the periosteum of the outer bony margins. In the bone marrow area, osteoclastogenesis peaked on day 3 in young mice (Figs. 4B), while the osteoclast formation in the middle-aged group reached a maximum on day 7 and decreased thereafter (Figs. 4C). Along the periosteal membrane, osteoclast formation in young mice peaked on day 3 and decreased thereafter while middle-aged mice showed a peak on day 14. On day 3, young mice exhibited a significantly higher number of osteoclasts in the bone marrow cavity and along the periosteal membrane compared to middle-aged mice ($P < 0.05$) (Table 3).

3.3. Expression of RUNX2 and ALP

To investigate the osteogenesis process during maxillary expansion, we assessed the expression of RUNX2, early osteoblast marker, in the midpalatal suture area (Figs. 5A-5C). Young mice exhibited a significant increase in the percentage of RUNX2-immunopositive cells in the midpalatal suture on day 14 compared to day 0 ($P < 0.05$, Fig. 5B). In contrast, middle-aged mice showed a slight increase up to day 7, followed by a decrease thereafter (Fig. 5C). On day 14, the percentage of RUNX2-immunopositive cells in young mice was significantly higher than that in middle-aged mice ($P < 0.05$, Table 4).

In addition, we assessed the expression of alkaline phosphatase, which is highly expressed in the mineralized tissue matrix and positively related to bone formation [25,26], in the midpalatal sutures (Figs. 5D-5E). In young mice, ALP expression continuously increased in the midpalatal suture area through day 14, whereas middle-aged mice showed a slight increase on day 3, followed by a decline up to day 14. On day 14 the ALP expression was significantly higher in the young mice compared to the middle-aged mice ($P < 0.05$) (Table 4).

3.4. MSC proliferation and apoptosis

Cell proliferation, differentiation, and apoptosis are critical during tissue remodeling [27]. The young mice demonstrated that the percentage of Gli1-immunopositive cells significantly increased on day 3, slightly decreased on day 7, and then increased until day 14 after maxillary expansion ($P < 0.05$) (Figs. 6A-6B). In contrast, the middle-aged mice demonstrated an initial increase of Gli1-immunopositive cells on day 3 and decreased thereafter (Fig. 6C and Table 5).

The young mice demonstrated that the percentage of Ki67-immunopositive cells peaked on day 3 and gradually decreased, whereas the middle-aged mice showed a slight increase in Ki67-immunopositive cells on day 3 (Figs. 6D-6E). On day 3 the percentage of Ki67-immunopositive cells in young mice is significantly higher compared to middle-aged mice ($P < 0.05$) (Table 5).

The percentage of Gli1+Ki67+ double immunopositive cells showed a similar pattern with the expression of Ki67 (Figs. 6F-6G). The young mice had a significant increase on day 3 and decreased thereafter. Middle-aged mice showed a slight increase in the percentage of Gli1+Ki67+ immunopositive cells on day 3, which remained at a similar level through day 7 before declining thereafter. The percentage of Gli1+Ki67+ immunopositive cells in young mice was significantly higher than in middle-aged mice on day 3 ($P < 0.05$) (Table 5).

In contrast, the percentage of TUNEL-positive cells increased on day 3 and decreased thereafter in both young and middle-aged mice (Figs. 7A-7C and Table 6). The middle-aged mice group showed much higher TUNEL expression on day 0 ($P = 0.08$) and day 3 ($P = 0.07$), compared to young mice. Similarly, the percentage of Gli1+TUNEL+ immunopositive cells peaked on day 3 and decreased thereafter in young

Table 2
Intermolar width and bone volume fraction (microCT).

Mice		Intermolar Width (mm)						BV/TV	
Duration	Group	Crown level	P value	Furcation level	P value	Root level	P value		P value
Day 0	Y	2.74 ± 0.02	****	4.32 ± 0.09		3.05 ± 0.06		0.54 ± 0.02	***
	A	3.01 ± 0.01		4.36 ± 0.08		3.09 ± 0.06		0.68 ± 0.01	
Day 3	Y	3.14 ± 0.10		4.57 ± 0.08		3.18 ± 0.10		0.47 ± 0.01	****
	A	3.17 ± 0.04		4.66 ± 0.06		3.18 ± 0.06		0.64 ± 0.02	
Day 7	Y	3.04 ± 0.07		4.44 ± 0.07	*	2.99 ± 0.10		0.52 ± 0.03	
	A	3.18 ± 0.08		4.70 ± 0.07		3.12 ± 0.12		0.57 ± 0.03	
Day 14	Y	3.17 ± 0.06	**	4.69 ± 0.08		3.22 ± 0.07		0.43 ± 0.02	
	A	3.53 ± 0.06		4.97 ± 0.10		3.37 ± 0.07		0.40 ± 0.05	

Y: 6 week-old mice and A: 12 month-old mice.
For comparisons between young and middle-aged groups at each time point, a two-tailed Student's t-test or the non-parametric Wilcoxon matched-pairs signed-rank test was employed.
*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001

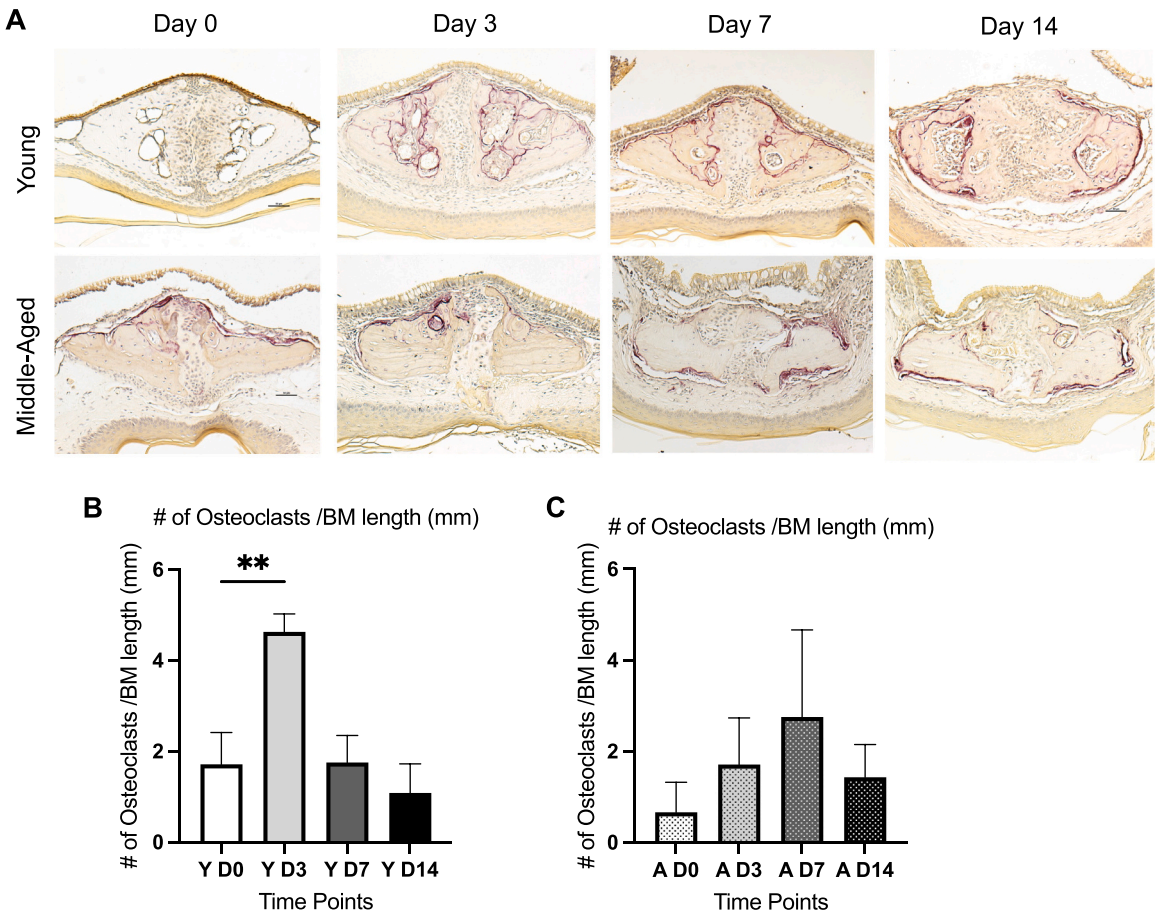

Fig. 4. Osteoclast formation. (A) TRAP-stained images (x20). Bar, 50 μm. (B—C) The number of osteoclasts in the bone marrow area. $^{**}P < 0.01$. Y: young mice and A: middle-aged mice.

Table 3
Osteoclast formation (TRAP stain).

Mice		Osteoclast number / mm					
Duration	Group	Midpalatal suture	P value	Periosteal membrane	P value	Bone marrow cavity	P value
Day 0	Y	0.21 ± 0.21		1.98 ± 1.14		1.71 ± 0.70	
	A	0 ± 0		2.89 ± 1.19		0.66 ± 0.66	
Day 3	Y	0.22 ± 0.22		5.50 ± 0.93	*	4.63 ± 0.40	*
	A	0.20 ± 0.20		2.84 ± 0.71		1.71 ± 1.02	
Day 7	Y	1.09 ± 1.09		4.51 ± 0.85		1.75 ± 0.59	
	A	0.30 ± 0.30		4.72 ± 0.82		2.75 ± 0.91	
Day 14	Y	0 ± 0		3.05 ± 1.14		1.08 ± 0.64	
	A	0 ± 0		5.62 ± 0.65		1.43 ± 0.72	

Y: 6 week-old mice and A: 12 month-old mice.
For comparisons between young and middle-aged groups at each time point, a two-tailed Student's t-test or the non-parametric Wilcoxon matched-pairs signed-rank test was employed.
* $P < 0.05$.

and middle-aged mice (Figs. 7D-7E). The middle-aged mice presented a higher expression of Gli1+Ki67+ immunopositive cells than young mice on day 0 and 3 ($P < 0.05$).

4. Discussion

The role of MSCs in the craniofacial suture is indispensable during mechanical force-induced bone remodeling [28–30]. The number of MSCs in our study significantly increased after maxillary expansion, consistent with the previous studies. Tensile force stimulates the osteogenic differentiation of suture MSCs. Increased MSC proliferation and differentiation into osteoblasts significantly contribute to new bone formation, commencing as early as day 1. Our study supports that aging negatively impacts bone remodeling during maxillary expansion. This is evidenced by reduced MSC proliferation and increased MSC apoptosis in middle-aged mice. Our findings align with previous studies indicating age-related impairment of MSC function across various tissues [17,31–35]. Compared to younger cells, aged human adipose-derived MSCs exhibit lower proliferation rates, reduced chondrogenic and osteogenic potential, and increased senescent characteristics [17]. In the human PDL, aging reduces the number of PDL stem cells, characterized by expression of STRO-1 and CD146, and alters MSC proliferation and differentiation, diminishing tissue homeostasis and the repair process [33,36]. Age-related bone loss partly results from decreased MSC

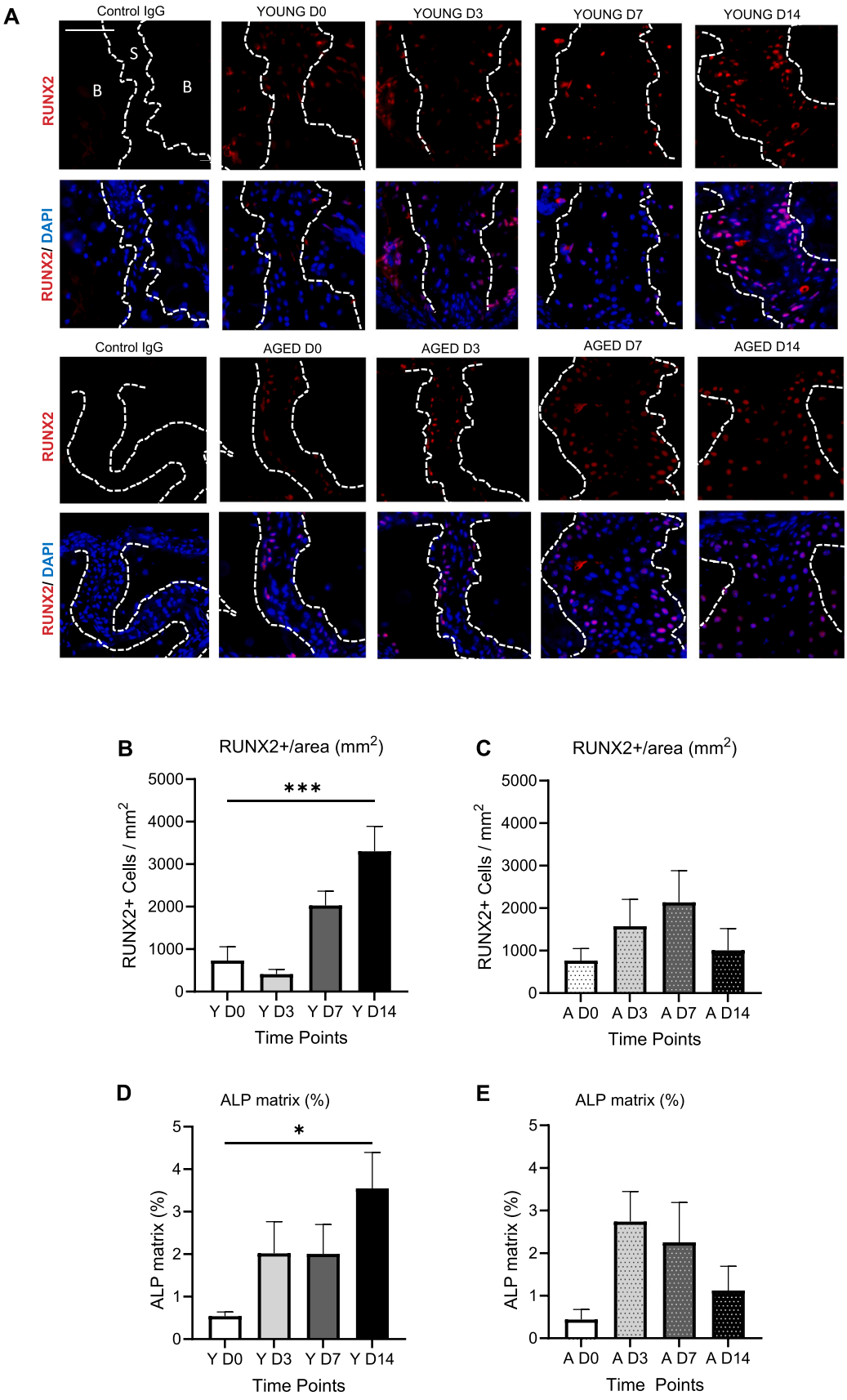

Fig. 5. Expression of Osteogenic Markers. (A) Representative images of RUNX2 immunofluorescence stain in the midpalatal suture. B: bone and S: midpalatal suture. 40×. Bar, 50 μm. (B—C) The percentage of RUNX2^{+} cells per area. (D-E) The percentage of ALP matrix. *$P < 0.05$, ***$P < 0.001$. Y: young mice and A: middle-aged mice.

Table 4
Expression of Osteogenic Markers.

Mice		Osteogenesis			
Duration	Group	RUNX2+/area (mm^2)	P value	ALP matrix (%)	P value
Day 0	Y	731.3 ± 326		0.54 ± 0.10	
	A	763.6 ± 284.9		0.44 ± 0.24	
Day 3	Y	409.5 ± 113.2		2.02 ± 0.75	
	A	1573 ± 634.2		2.74 ± 0.71	
Day 7	Y	2028 ± 337.1		2.00 ± 0.69	
	A	2135 ± 745.1		2.25 ± 0.94	
Day 14	Y	3301 ± 586.3	*	3.55 ± 0.84	*
	A	1006 ± 511.3		1.12 ± 0.57	

Y: 6 week-old mice and A: 12 month-old mice.
For comparisons between young and middle-aged groups at each time point, a two-tailed Student's t-test or the non-parametric Wilcoxon matched-pairs signed-rank test was employed.
* $P < 0.05$.

proliferation and function, alongside an increased commitment of MSCs to adipogenic lineages using mouse bone marrow-derived MSCs [18,37]. This shift in lineage commitment, coupled with decreased self-renewal capacity, contributes to bone loss during aging. Moreover, transcriptional analysis at the single-cell level confirms that MSCs from aged donors reduce wound healing capacities compared to MSCs from young donors [22]. Interestingly, the functions of aged PDL stem cells are reversible and influenced by the extrinsic microenvironment [32,33]. Young PDL cell–conditioned medium improves the proliferation and differentiation of PDL stem cells from aged donors and enhances their regenerative capabilities. Conversely, young PDL stem cells induced by aged PDL cell–conditioned medium lead to connective tissue formation, demonstrating the potential reversal of aging on stem cells [22,33]. On the contrary, some studies on the effects of aging on MSCs have yielded contradictory results. A study on human bone marrow-derived MSCs reveals no age-related differences in the gene expression associated with adipogenesis, osteogenesis, and bone remodeling, suggesting that MSCs from older donors may retain functional parity with those from younger donors [21]. Additionally, no age-related changes in MSC numbers are reported based on in vitro assays [19,38,39].

The murine model is widely used to study human craniofacial suture biology. In our study both young and middle-aged mice presented open, straight midpalatal suture lines, allowing for successful expansion of the midpalatal sutures even in 12-month-old mice. Interestingly, young and middle-aged mice exhibit similar suture widths prior to maxillary expansion. However, significant differences emerge with middle-aged mice displaying larger suture widths, particularly noticeable at the oral third on day 7 and at the middle and nasal third on day 14. To investigate the underlying mechanisms, we evaluated i) intermolar distance at the root furcation, which serves as a fulcrum for assessing bone separation by mechanical force, ii) osteoclast formation in the midpalatal suture, a key component of bone remodeling and iii) new bone formation in the midpalatal suture, another essential aspect of bone remodeling. First, an insignificant difference in bone separation at the intermolar distance at the root furcation was observed between young and middle-aged mice on day 14 (young: 4.69 ± 0.08 mm vs. middle-aged: 4.97 ± 0.10 mm). Second, osteoclast formation in the midpalatal suture was rare, with no significant difference between young and middle-aged mice. After maxillary expansion, osteoclasts emerge on day 3, peak on day 5, and decrease by day 7, primarily at the outer bony margins and within the bone marrow cavity, not in the midpalatal suture [10,23,40]. Third, after maxillary expansion, younger mice exhibited more robust new bone formation in the midpalatal suture, which leads to the smaller suture gap caused by expansion, as evidenced by significantly increased expression of RUNX2 and alkaline phosphatase.

In addition, the middle-aged mice had significantly smaller bone marrow cavities compared to young mice, resulting in a higher baseline bone volume fraction (BV/TV) at day 0 and day 3. From day 0 to day 14, BV/TV significantly decreased in young and middle-aged mice. Bone marrow cavity size is closely linked to bone remodeling activity, functioning as the primary source of osteoblasts and osteoclasts [41]. Consequently, a larger bone marrow cavity size in young mice generally indicates a greater capacity for bone remodeling. Moreover, the cellular activity within the bone marrow changes with age as senescence reduces its ability to produce osteoblast precursors [41]. Expectedly, after maxillary expansion, younger mice demonstrated more robust osteoclast formation in the bone marrow and along the periosteal membrane and new bone formation in the midpalatal suture. Therefore, these responses led to a narrower suture width and a smaller reduction in BV/TV in young mice compared to the middle-aged mice.

Cell proliferation, differentiation and apoptosis are the key events during tissue remodeling. MSCs respond to mechanical loading through mechanosensing and mechanotransduction, affecting their

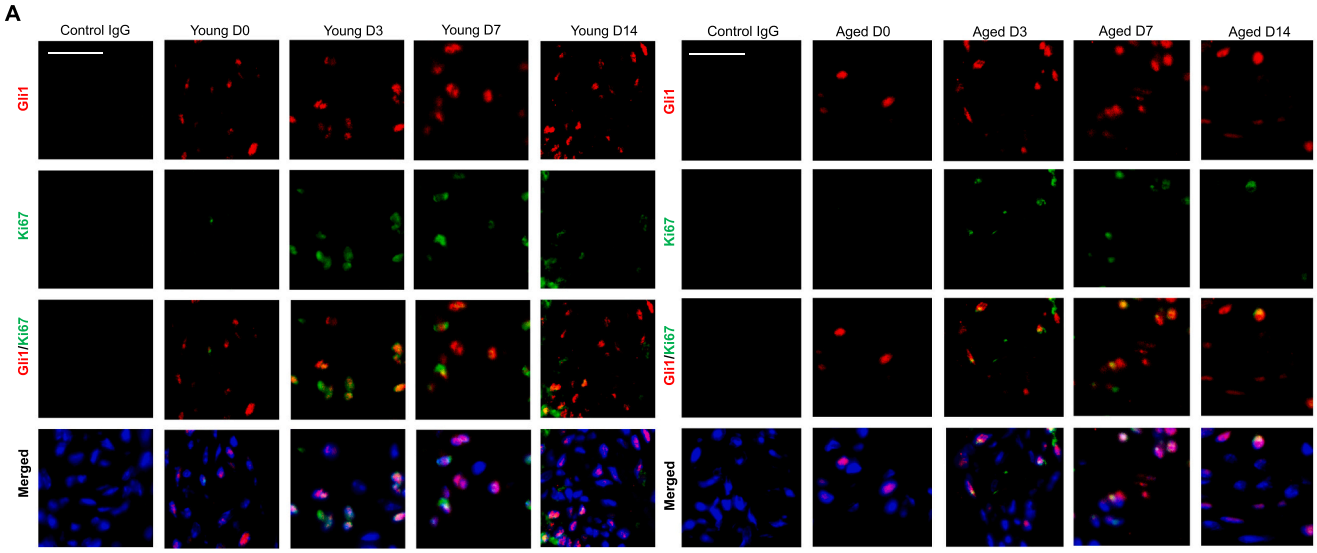

Fig. 6. Gli1/Ki67 Immunofluorescence stain. (A) Representative images in the midpalatal suture. 40×. Bar, 25 μm. (B—C) The percentage of Gli1-immunopositive cells. (D-E) The percentage of Ki67-immunopositive cells. (F-G) The percentage of Gli1/Ki67-immunopositive cells. *$P < 0.05$, **$P < 0.01$. Y: young mice and A: middle-aged mice.

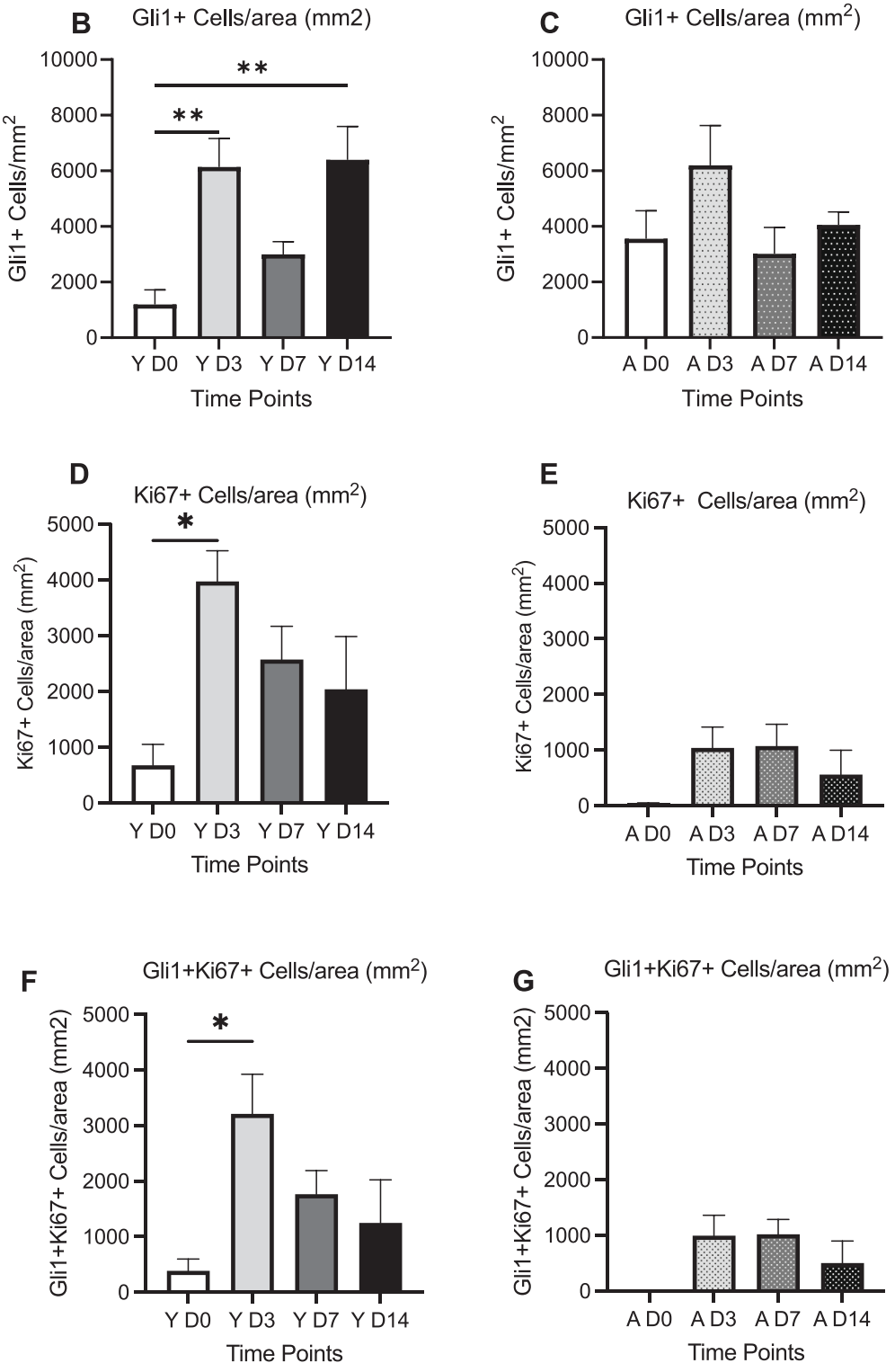

Fig. 6. (*continued*).

proliferation, differentiation and self-renewal [42]. The proliferation of Gli1+ stem cells in response to mechanical loading involves several key signaling pathways and molecules, including Hedgehog, Wnt/β-catenin, TGF-β and Yes-associated protein (YAP)/Transcriptional co-activator with PDZ-binding motif (TAZ). The activation of Gli1+ stem cells is primarily regulated by the Hedgehog signaling pathway [30]. Genetic disruption of Hedgehog signaling impairs proliferation and osteoblast differentiation of mesenchymal progenitors [43]. Conversely, activation of Hedgehog signaling significantly improved bone formation in postnatal mice [44,45]. Uniaxial mechanical stretch stimulates proliferation and osteogenic differentiation of bone marrow MSCs by activating the Wnt/b-catenin signaling [46]. Wnt ligands facilitate the nuclear translocation of β-catenin, which drives the proliferation and differentiation of Gli1+ stem cells. Upon suture expansion, the co-localization of Gli1 and β-catenin genes is observed using RNAscope [28]. Disrupting Wnt signaling impairs the differentiation potential of Gli1-lineage cells and

Table 5
Gli1/Ki67 Immunofluorescence stain.

Duration	Group	Gli1+/area (mm^2)	P value	Ki67+/area (mm^2)	P value	Gli1+Ki67+/area (mm^2)	P value
Day 0	Y	1192 ± 527.8		675.5 ± 377.7		385.3 ± 211.0	
	A	3556 ± 1003		26.06 ± 26.06		0.00 ± 0.00	
Day 3	Y	6134 ± 1027		3974 ± 550.1	***	3210 ± 713.9	*
	A	6196 ± 1433		1040 ± 373.4		992.5 ± 367.1	
Day 7	Y	3000 ± 453.7		2573 ± 594.9		1766 ± 425.8	
	A	3010 ± 954.1		1073 ± 392.5		1018 ± 268.6	
Day 14	Y	6396 ± 1202		2040 ± 943.4		1247 ± 775.6	
	A	4051 ± 469.7		559.7 ± 437.0		503.4 ± 393.6	

Y: 6 week-old mice and A: 12 month-old mice.
For comparisons between young and middle-aged groups at each time point, a two-tailed Student's t-test or the non-parametric Wilcoxon matched-pairs signed-rank test was employed.
*P < 0.05, ***P < 0.001.

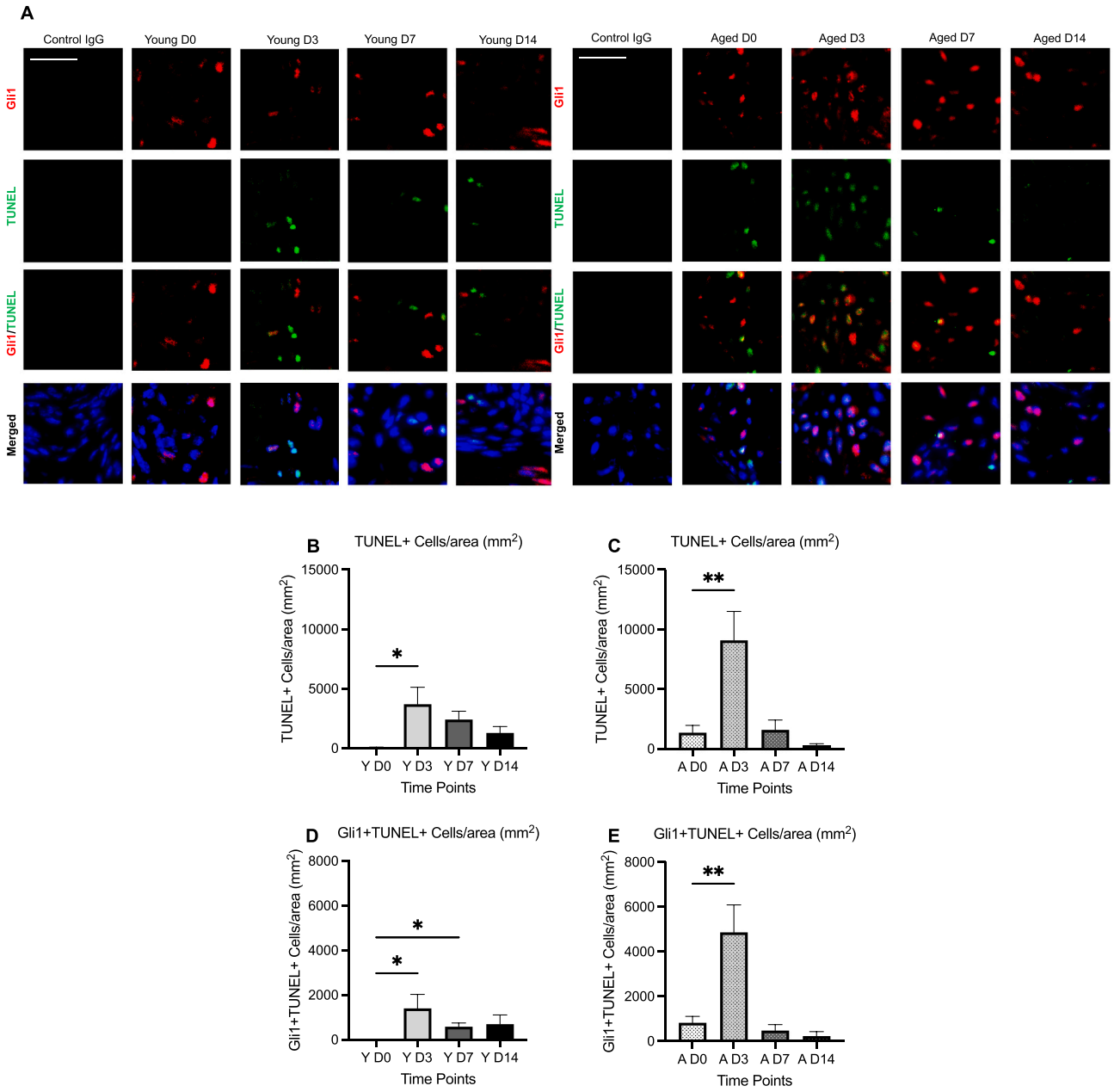

Fig. 7. Gli1/TUNEL Immunofluorescence stain. (A) Representative images in the midpalatal suture. 40×. Bar, 25 μm. (B—C) The percentage of TUNEL-positive cells. (D-E) The percentage of Gli1/TUNEL-immunopositive cells. *$P < 0.05$, **$P < 0.01$. Y: young mice and A: middle-aged mice.

Table 6
Gli1/TUNEL Immunofluorescence stain.

Duration	Group	TUNEL+/area (mm^2)	P value	Gli1+TUNEL+/area (mm^2)	P value
Day 0	Y	57.85 ± 40.71		0.00 ± 0.00	*
	A	1343 ± 611.7		815.7 ± 287.0	
Day 3	Y	3698 ± 1421		1395 ± 631.5	*
	A	9087 ± 2416		4853 ± 1229	
Day 7	Y	2401 ± 708.2		577.5 ± 166.3	
	A	1582 ± 819.3		472.0 ± 267.6	
Day 14	Y	1276 ± 542.6		686.9 ± 415.7	
	A	226.0 ± 196.6		226.0 ± 196.6	

Y: 6 week-old mice and A: 12 month-old mice.
For comparisons between young and middle-aged groups at each time point, a two-tailed Student's t-test or the non-parametric Wilcoxon matched-pairs signed-rank test was employed.
* $P < 0.05$.

inhibits bone formation following expansion. Moreover, the deletion of β-catenin shifts the fate of mesenchymal progenitors toward adipogenesis at the expense of bone formation [43]. Inhibition of Wnt/β-catenin signaling reduces the proliferation and differentiation of the Gli1+MSCs in vitro [47] and ß-catenin knockout in Gli1+MSCs significantly impairs their osteogenic differentiation in vivo, suppressing bone remodeling after expansion [28]. TGF-β enhances the activation and proliferation of Gli1+ stem cells, often in coordination with Hedgehog and Wnt signaling. Immunohistochemical analysis of Gli1+ cells revealed that disrupting TGF-β signaling led to reduced proliferation and increased apoptosis [48]. Moreover, mechanical loading can activate YAP and TAZ, which interact with Hedgehog signaling to regulate Gli1+ stem cell activity including proliferation, cell plasticity and stemness [49–52]. These pathways collectively mediate the adaptive response of Gli1+ stem cells to mechanical loading, contributing to tissue repair and regeneration.

Excessive or abnormal mechanical loading can induce MSCs apoptosis through several pathways and molecules, including the reactive oxygen species, integrin, TNF-α/NF-κB, p53 and MAPK (c-Jun N-terminal kinase and p38). Excessive mechanical stress can increase the production of reactive oxygen species, leading to oxidative stress and apoptosis in MSCs [53]. Pro-apoptotic molecules such as Bax and Bak are upregulated under high mechanical strain, leading to mitochondrial outer membrane permeabilization and apoptosis whereas anti-apoptotic molecules like Bcl-2 are downregulated in response to excessive loading [54]. Additionally, integrins, which mediate cell-matrix interactions, can transmit mechanical signals to MSCs [55]. Excessive mechanical stress disrupts integrin-mediated signaling, leading to MSC apoptosis [56]. Excessive mechanical loading also increases TNF-α levels and activates the NF-κB pathway [57]. Chronic NF-κB activation promotes pro-apoptotic signaling in MSCs. Moreover, mechanical overloading can activate p53, c-Jun N-terminal kinase and p38 MAPK, leading to cell cycle arrest and apoptosis.

Aging is closely related to MSC senescence and inflammation [58]. Aging reduces proliferation of pre-osteoblasts, inhibit differentiation to osteoblasts and diminish expression of matrix proteins. Several factors regulate the mechanisms underlying MSC senescence. For example, Insulin-like growth factor 1 (IGF-1)-binding protein 4 promotes MSC senescence and inhibits proliferation, which is reversed by an IGF1 receptor antagonist [59]. Senescent cells also exhibit elevated expression of p16 and p21, key regulators of cell cycle arrest. Local inhibition of the p21 expression using p21 siRNA accelerates wound healing in aged mice by enhancing the proliferation of wound fibroblasts [60,61]. Additionally, NF-κB plays a pivotal role in inflammation and the aging process. In MSCs from aged mice, increased NF-κB activity is linked to reduced osteogenesis with reduced osteoblast numbers and activity [9,14,62]. Modulating NF-κB activity in aged MSCs offers a potential approach to mitigating age-related bone loss in elderly populations. Furthermore, the literature indicates that cells in aged bones show reduced sensitivity to mechanical stimuli and a diminished ability to respond to strain signals during loading, primarily due to increased tissue stiffness [63]. Moreover, the reduced anabolic responses to mechanical loading in aged mice are due to an inability to sustain Wnt activity [64]. This could partly account for the lower osteoclast and less osteoblast activities observed in middle-aged mice.

There are several limitations in this study. Firstly, it entails a short-term evaluation until day 14 after maxillary expansion. While we examined the short-term effects of aging on suture width, bone volume fraction, osteoclastogenesis, and osteogenesis as well as MSC proliferation and apoptosis within this timeframe, a longer-term evaluation using the retention models could provide a more thorough understanding of mechanically induced bone remodeling. Such insights could facilitate the development of more targeted treatments tailored to age-related patient needs. Secondly, our comparison was limited to young and middle-aged groups, which correspond roughly to individuals in their 40s. Middle-aged groups may not entirely represent the effects of aging. Conducting further studies encompassing young adults and older age groups could provide valuable insights into age-related changes in bone remodeling processes under mechanical loading.

5. Conclusion

We demonstrate that aging negatively affects mechanical force-induced bone remodeling by reducing MSC proliferation and increasing MSC apoptosis in middle-aged mice compared to young mice. In addition, in response to the mechanical force, young mice exhibited earlier and more intense osteoclast formation in the bone marrow cavities and along the periosteal membrane, as well as elevated expression of osteogenic markers in midpalatal sutures compared to middle-aged mice.

CRediT authorship contribution statement

Hyeran Helen Jeon: Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Mary Cruz Contreras Salas:** Writing – review & editing, Writing – original draft, Methodology, Funding acquisition, Formal analysis. **Kyungjoon Park:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **Lindsay Fisher:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis. **Sara Ha:** Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis. **Caroline Palmer:** Data curation, Formal analysis. **Fionna Chan:** Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis. **Dana T. Graves:** Writing – review & editing, Supervision, Methodology, Conceptualization.

Declaration of competing interest

The authors state no conflict of interest.

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Data availability

Data will be made available on request.

References

[1] J. Kurol, L. Berglund, Longitudinal study and cost-benefit analysis of the effect of early treatment of posterior cross-bites in the primary dentition, Eur. J. Orthod. 14 (3) (1992) 173–179.

[2] K.J. Lee, Y.C. Park, J.Y. Park, W.S. Hwang, Miniscrew-assisted nonsurgical palatal expansion before orthognathic surgery for a patient with severe mandibular prognathism, Am. J. Orthod. Dentofacial Orthop. 137 (6) (2010) 830–839.

[3] S. Safari, A. Mahdian, S.R. Motamedian, Applications of stem cells in orthodontics and dentofacial orthopedics: current trends and future perspectives, World J Stem Cells 10 (6) (2018) 66–77.

[4] H. Zhao, Y. Chai, Stem cells in teeth and craniofacial bones, J. Dent. Res. 94 (11) (2015) 1495–1501.

[5] S. Park, H. Zhao, M. Urata, Y. Chai, Sutures possess strong regenerative capacity for Calvarial bone injury, Stem Cells Dev. 25 (23) (2016) 1801–1807.

[6] S.S. Liu, H. Xu, J. Sun, E. Kontogiorgos, P.R. Whittington, K.G. Misner, H.M. Kyung, P.H. Buschang, L.A. Opperman, Recombinant human bone morphogenetic protein-2 stimulates bone formation during interfrontal suture expansion in rabbits, Am. J. Orthod. Dentofacial Orthop. 144 (2) (2013) 210–217.

[7] R.M. Delaine-Smith, G.C. Reilly, Mesenchymal stem cell responses to mechanical stimuli, Muscles Ligaments Tendons J 2 (3) (2012) 169–180.

[8] Y.K. Luu, J.E. Pessin, S. Judex, J. Rubin, C.T. Rubin, Mechanical signals as a non-invasive means to influence mesenchymal stem cell fate, Promoting Bone and Suppressing the Fat Phenotype, Bonekey Osteovision 6 (4) (2009) 132–149.

[9] T.H. Lin, E. Gibon, F. Loi, J. Pajarinen, L.A. Cordova, A. Nabeshima, L. Lu, Z. Yao, S.B. Goodman, Decreased osteogenesis in mesenchymal stem cells derived from the aged mouse is associated with enhanced NF-kappaB activity, J. Orthop. Res. 35 (2) (2017) 281–288.

[10] B. Hou, N. Fukai, B.R. Olsen, Mechanical force-induced midpalatal suture remodeling in mice, Bone 40 (6) (2007) 1483–1493.

[11] A. Ekizer, M.E. Yalvac, T. Uysal, M.F. Sonmez, F. Sahin, Bone marrow mesenchymal stem cells enhance bone formation in orthodontically expanded maxillae in rats, Angle Orthod. 85 (3) (2015) 394–399.

[12] H. Zhao, J. Feng, T.V. Ho, W. Grimes, M. Urata, Y. Chai, The suture provides a niche for mesenchymal stem cells of craniofacial bones, Nat. Cell Biol. 17 (4) (2015) 386–396.

[13] M. Jin, Y. An, Z. Wang, G. Wang, Z. Lin, P. Ding, E. Lu, Z. Zhao, H. Bi, Distraction force promotes the osteogenic differentiation of Gli1(+) cells in facial sutures via primary cilia-mediated hedgehog signaling pathway, Stem Cell Res Ther 15 (1) (2024) 198.

[14] M. Al-Azab, M. Safi, E. Idiiatullina, F. Al-Shaebi, M.Y. Zaky, Aging of mesenchymal stem cell: machinery, markers, and strategies of fighting, Cell. Mol. Biol. Lett. 27 (1) (2022) 69.

[15] B. Rath-Deschner, A.V.B. Nogueira, S. Beisel-Memmert, M. Nokhbehsaim, S. Eick, J.A. Cirelli, J. Deschner, A. Jager, A. Damanaki, Interaction of periodontitis and orthodontic tooth movement-an in vitro and in vivo study, Clin. Oral Investig. 26 (1) (2022) 171–181.

[16] L. Christensen, F. Luther, Adults seeking orthodontic treatment: expectations, periodontal and TMD issues, Br. Dent. J. 218 (3) (2015) 111–117.

[17] M. Maredziak, K. Marycz, K.A. Tomaszewski, K. Kornicka, B.M. Henry, The influence of aging on the regenerative potential of human adipose derived mesenchymal stem cells, Stem Cells Int. 2016 (2016) 2152435.

[18] L. Singh, T.A. Brennan, E. Russell, J.H. Kim, Q. Chen, F. Brad Johnson, R. J. Pignolo, Aging Alters Bone-Fat Reciprocity by Shifting In Vivo Mesenchymal Precursor Cell Fate towards an Adipogenic Lineage, Bone vol. 85, 2016, pp. 29–36.

[19] A. Stolzing, E. Jones, D. McGonagle, A. Scutt, Age-related changes in human bone marrow-derived mesenchymal stem cells: consequences for cell therapies, Mech. Ageing Dev. 129 (3) (2008) 163–173.

[20] M. Zaim, S. Karaman, G. Cetin, S. Isik, Donor age and long-term culture affect differentiation and proliferation of human bone marrow mesenchymal stem cells, Ann. Hematol. 91 (8) (2012) 1175–1186.

[21] P. Ganguly, J.J. El-Jawhari, A.N. Burska, F. Ponchel, P.V. Giannoudis, E.A. Jones, The analysis of in vivo aging in human bone marrow mesenchymal stromal cells using Colony-forming unit-fibroblast assay and the CD45(low)CD271(+) phenotype, Stem Cells Int. 2019 (2019) 5197983.

[22] S.M.L. Khong, M. Lee, N. Kosaric, D.M. Khong, Y. Dong, U. Hopfner, M. M. Aitzetmuller, D. Duscher, R. Schafer, G.C. Gurtner, Single-cell transcriptomics of human mesenchymal stem cells reveal age-related cellular subpopulation depletion and impaired regenerative function, Stem Cells 37 (2) (2019) 240–246.

[23] J. Li, T.T. Yu, H.C. Yan, Y.Q. Qiao, L.C. Wang, T. Zhang, Q. Li, Y.H. Zhou, D.W. Liu, T cells participate in bone remodeling during the rapid palatal expansion, FASEB J. 34 (11) (2020) 15327–15337.

[24] Y. Cheng, J. Sun, Z. Zhou, J. Pan, S. Zou, J. Chen, Effects of lactoferrin on bone resorption of midpalatal suture during rapid expansion in rats, Am. J. Orthod. Dentofacial Orthop. 154 (1) (2018) 115–127.

[25] X. Xiao, J. Chen, Q. Zhai, L. Xin, X. Zheng, S. Wang, J. Song, Suppressing STAT3 activation impairs bone formation during maxillary expansion and relapse, J. Appl. Oral Sci. 31 (2023) e20230009.

[26] J. Li, X. Wang, F. Yang, J. Yuan, Q. Cui, F. Nie, J. Zhang, Matrine enhances osteogenic differentiation of bone marrow-derived mesenchymal stem cells and promotes bone regeneration in rapid maxillary expansion, Arch. Oral Biol. 118 (2020) 104862.

[27] N. Katebi, E. Kolpakova-Hart, C.Y. Lin, B.R. Olsen, The mouse palate and its cellular responses to midpalatal suture expansion forces, Orthod. Craniofacial Res. 15 (3) (2012) 148–158.

[28] D. Jing, Z. Chen, Y. Men, Y. Yi, Y. Wang, J. Wang, J. Yi, L. Wan, B. Shen, J.Q. Feng, Z. Zhao, H. Zhao, C. Li, Response of Gli1(+) suture stem cells to mechanical force upon suture expansion, J. Bone Miner. Res. 37 (7) (2022) 1307–1320.

[29] X. Huang, Z. Li, P. Liu, M. Wu, A.Q. Liu, C. Hu, X. Liu, H. Guo, X. Yang, X. Guo, B. Li, X. He, K. Xuan, Y. Jin, Gli1(+) cells residing in bone sutures respond to mechanical force via IP(3)R to mediate osteogenesis, Stem Cells Int. 2021 (2021) 8138374.

[30] L. Wu, Z. Liu, L. Xiao, M. Ai, Y. Cao, J. Mao, K. Song, The role of Gli1(+) mesenchymal stem cells in osteogenesis of craniofacial bone, Biomolecules 13 (9) (2023).

[31] R.X. Wu, C.S. Bi, Y. Yu, L.L. Zhang, F.M. Chen, Age-related decline in the matrix contents and functional properties of human periodontal ligament stem cell sheets, Acta Biomater. 22 (2015) 70–82.

[32] B.D. Sui, C.H. Hu, C.X. Zheng, Y. Jin, Microenvironmental views on mesenchymal stem cell differentiation in aging, J. Dent. Res. 95 (12) (2016) 1333–1340.

[33] W. Zheng, S. Wang, D. Ma, L. Tang, Y. Duan, Y. Jin, Loss of proliferation and differentiation capacity of aged human periodontal ligament stem cells and rejuvenation by exposure to the young extrinsic environment, Tissue Eng. Part A 15 (9) (2009) 2363–2371.

[34] G. Hajishengallis, T. Chavakis, J.D. Lambris, Current understanding of periodontal disease pathogenesis and targets for host-modulation therapy, Periodontol 84 (1) (2000) 14–34, 2020.

[35] A.S. Ahmed, M.H. Sheng, S. Wasnik, D.J. Baylink, K.W. Lau, Effect of aging on stem cells, World J Exp Med 7 (1) (2017) 1–10.

[36] P.M. Bartold, S. Shi, S. Gronthos, Stem cells and periodontal regeneration, Periodontol 2000 (40) (2006) 164–172.

[37] A. Infante, C.I. Rodriguez, Osteogenesis and aging: lessons from mesenchymal stem cells, Stem Cell Res Ther 9 (1) (2018) 244.

[38] J. Justesen, K. Stenderup, E.F. Eriksen, M. Kassem, Maintenance of osteoblastic and adipocytic differentiation potential with age and osteoporosis in human marrow stromal cell cultures, Calcif. Tissue Int. 71 (1) (2002) 36–44.

[39] K. Stenderup, J. Justesen, E.F. Eriksen, S.I. Rattan, M. Kassem, Number and proliferative capacity of osteogenic stem cells are maintained during aging and in patients with osteoporosis, J. Bone Miner. Res. 16 (6) (2001) 1120–1129.

[40] J.A. Guerrero, R.S. Silva, I.L. de Abreu Lima, B.C.D. Rodrigues, B.R. Barrioni, F. A. Amaral, A.P. Tabanez, G.P. Garlet, D.A.G. Alvarado, T.A. Silva, E.B. de Las Casas, S. Macari, Maxillary suture expansion: a mouse model to explore the molecular effects of mechanically-induced bone remodeling, J. Biomech. 108 (2020) 109880.

[41] S.C. Manolagas, R.L. Jilka, Bone marrow, cytokines, and bone remodeling, Emerging insights into the pathophysiology of osteoporosis, N Engl J Med 332 (5) (1995) 305–311.

[42] K.H. Vining, D.J. Mooney, Mechanical forces direct stem cell behaviour in development and regeneration, Nat. Rev. Mol. Cell Biol. 18 (12) (2017) 728–742.

[43] Y. Shi, G. He, W.C. Lee, J.A. McKenzie, M.J. Silva, F. Long, Gli1 identifies osteogenic progenitors for bone formation and fracture repair, Nat. Commun. 8 (1) (2017) 2043.

[44] S. Ohba, H. Kawaguchi, F. Kugimiya, T. Ogasawara, N. Kawamura, T. Saito, T. Ikeda, K. Fujii, T. Miyajima, A. Kuramochi, T. Miyashita, H. Oda, K. Nakamura, T. Takato, U.I. Chung, Patched1 haploinsufficiency increases adult bone mass and modulates Gli3 repressor activity, Dev. Cell 14 (5) (2008) 689–699.

[45] K.K. Mak, Y. Bi, C. Wan, P.T. Chuang, T. Clemens, M. Young, Y. Yang, Hedgehog signaling in mature osteoblasts regulates bone formation and resorption by controlling PTHrP and RANKL expression, Dev. Cell 14 (5) (2008) 674–688.

[46] C. Zhao, Y. Li, X. Wang, S. Zou, J. Hu, E. Luo, The effect of uniaxial mechanical stretch on Wnt/beta-catenin pathway in bone mesenchymal stem cells, J. Craniofac. Surg. 28 (1) (2017) 113–117.

[47] S. Chen, L. Lan, J. Lei, Y. He, Y. Zhang, Gli1+ osteogenic progenitors contribute to condylar development and fracture repair, Front. Cell Dev. Biol. 10 (2022) 819689.

[48] L. Song, M. Golman, A.C. Abraham, E. Zelzer, S. Thomopoulos, A role for TGFbeta signaling in Gli1+ tendon and enthesis cells, FASEB J. 38 (6) (2024) e23568.

[49] B. Sun, Y. Wen, X. Wu, Y. Zhang, X. Qiao, X. Xu, Expression pattern of YAP and TAZ during orthodontic tooth movement in rats, J. Mol. Histol. 49 (2) (2018) 123–131.

[50] L. Deng, Y. Chen, J. Guo, X. Han, Y. Guo, Roles and mechanisms of YAP/TAZ in orthodontic tooth movement, J. Cell. Physiol. 236 (11) (2021) 7792–7800.

[51] J.X. Pan, L. Xiong, K. Zhao, P. Zeng, B. Wang, F.L. Tang, D. Sun, H.H. Guo, X. Yang, S. Cui, W.F. Xia, L. Mei, W.C. Xiong, YAP promotes osteogenesis and suppresses adipogenic differentiation by regulating beta-catenin signaling, Bone Res 6 (2018) 18.

[52] T. Panciera, L. Azzolin, M. Cordenonsi, S. Piccolo, Mechanobiology of YAP and TAZ in physiology and disease, Nat. Rev. Mol. Cell Biol. 18 (12) (2017) 758–770.

[53] F. Atashi, A. Modarressi, M.S. Pepper, The role of reactive oxygen species in mesenchymal stem cell adipogenic and osteogenic differentiation: a review, Stem Cells Dev. 24 (10) (2015) 1150–1163.
[54] P. Wolf, A. Schoeniger, F. Edlich, Pro-apoptotic complexes of BAX and BAK on the outer mitochondrial membrane, Biochim. Biophys. Acta, Mol. Cell Res. 1869 (10) (2022) 119317.
[55] X. Pang, X. He, Z. Qiu, H. Zhang, R. Xie, Z. Liu, Y. Gu, N. Zhao, Q. Xiang, Y. Cui, Targeting integrin pathways: mechanisms and advances in therapy, Signal Transduct. Target. Ther. 8 (1) (2023) 1.
[56] X. Di, X. Gao, L. Peng, J. Ai, X. Jin, S. Qi, H. Li, K. Wang, D. Luo, Cellular mechanotransduction in health and diseases: from molecular mechanism to therapeutic targets, Signal Transduct. Target. Ther. 8 (1) (2023) 282.
[57] T. Fang, X. Zhou, M. Jin, J. Nie, X. Li, Molecular mechanisms of mechanical load-induced osteoarthritis, Int. Orthop. 45 (5) (2021) 1125–1136.
[58] B.C. Lee, K.R. Yu, Impact of mesenchymal stem cell senescence on inflammaging, BMB Rep. 53 (2) (2020) 65–73.
[59] H. Li, S. Yu, F. Hao, X. Sun, J. Zhao, Q. Xu, D. Duan, Insulin-like growth factor binding protein 4 inhibits proliferation of bone marrow mesenchymal stem cells and enhances growth of neurospheres derived from the stem cells, Cell Biochem. Funct. 36 (6) (2018) 331–341.
[60] J. Yan, S. Chen, Z. Yi, R. Zhao, J. Zhu, S. Ding, J. Wu, The role of p21 in cellular senescence and aging-related diseases, Mol. Cell 47 (11) (2024) 100113.
[61] D. Jiang, J.C. de Vries, J. Muschhammer, S. Schatz, H. Ye, T. Hein, M. Fidan, V. S. Romanov, Y. Rinkevich, K. Scharffetter-Kochanek, Local and transient inhibition of p21 expression ameliorates age-related delayed wound healing, Wound Repair Regen. 28 (1) (2020) 49–60.
[62] S. Pacios, W. Xiao, M. Mattos, J. Lim, R.S. Tarapore, S. Alsadun, B. Yu, C.Y. Wang, D.T. Graves, Osteoblast lineage cells play an essential role in periodontal bone loss through activation of nuclear factor-kappa B, Sci. Rep. 5 (2015) 16694.
[63] B. Javaheri, A.A. Pitsillides, Aging and Mechanoadaptive responsiveness of bone, Curr. Osteoporos. Rep. 17 (6) (2019) 560–569.
[64] N. Holguin, M.D. Brodt, M.J. Silva, Activation of Wnt signaling by mechanical loading is impaired in the bone of old mice, J. Bone Miner. Res. 31 (12) (2016) 2215–2226.