Single Cell Transcriptomics Identifies Mesenchymal Stem Cell Lineage Trajectories During Maxillary Expansion

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Single Cell Transcriptomics Identifies Mesenchymal Stem Cell Lineage Trajectories During Maxillary Expansion

Abstract
Skeletal transverse harmony between maxilla and mandible is essential for balanced vertical and sagittal dental and skeletal relationship, as well as facial esthetics and periodontal health. However, patients, especially adult ones, responded to maxillary expansion differently. It remains unclear why some cases fail while others succeed despite usage of miniscrews for anchorage reinforcement. A deep understanding of the biological basis underlying maxillary expansion is the fundament to answer this question. Unfortunately, the scientific evidence behind the maxillary expansion is largely missing. Cell population within mid-palatal suture and cellular and molecular mechanisms in response to mechanical expansion forces are potential factors other than the differences in calcification patterns of the mid-palatal suture, craniofacial architecture, and patient's age. Mesenchymal stem cell (MSC) refers to a group of heterogeneous multipotent stem cells that can self-renew and further differentiate into several cell types, including osteoblasts, chondrocytes, and adipocytes, which plays an important role in bone remodeling. We show here that suture-MSCs were labeled by red fluoresces by crossing Gli1-CreERT2 mice with Cre reporter tdTomato mice. Maxillary expansion seems to promote the proliferation of the Gli1⁺ suture-MSCs. Single cell transcriptomics identified MSC lineage trajectories shift in response to maxillary expansion. Interestingly, we found the immune cells, like neutrophils, also under active immunomodulation. In summary, our data proved the presence of MSCs within mid-palatal suture and MSC lineage trajectories and their unique immune microenvironment have been revealed by single cell transcriptomics. This work may provide a more precise immune-MSC modulatory target for maxillary expansion.

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Single Cell Transcriptomics Identifies Mesenchymal Stem Cell Lineage Trajectories

During Maxillary Expansion

Xuefeng Zhao

2021

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To my lovely son, Shicheng (James) Zhao.
ABSTRACT

Skeletal transverse harmony between maxilla and mandible is essential for balanced vertical and sagittal dental and skeletal relationship, as well as facial esthetics and periodontal health. However, patients, especially adult ones, responded to maxillary expansion differently. It remains unclear why some cases fail while others succeed despite usage of miniscrews for anchorage reinforcement. A deep understanding of the biological basis underlying maxillary expansion is the fundament to answer this question. Unfortunately, the scientific evidence behind the maxillary expansion is largely missing. Cell population within mid-palatal suture and cellular and molecular mechanisms in response to mechanical expansion forces are potential factors other than the differences in calcification patterns of the mid-palatal suture, craniofacial architecture, and patient’s age. Mesenchymal stem cell (MSC) refers to a group of heterogeneous multipotent stem cells that can self-renew and further differentiate into several cell types, including osteoblasts, chondrocytes, and adipocytes, which plays an important role in bone remodeling. We show here that suture-MSCs were labeled by red fluoresces by crossing Gli1-CreERT2 mice with Cre reporter tdTomato mice. Maxillary expansion seems to promote the proliferation of the Gli1+ suture-MSCs. Single cell transcriptomics identified MSC lineage trajectories shift in response to maxillary expansion. Interestingly, we found the immune cells, like neutrophils, also under active immunomodulation. In summary, our data proved the presence of MSCs within mid-palatal suture and MSC lineage trajectories and their unique immune microenvironment have been revealed by single cell transcriptomics. This work may provide a more precise immune-MSC modulatory target for maxillary expansion.
Table of Contents

ACKNOWLEDGEMENT ........................................................................................................... 2

ABSTRACT ............................................................................................................................ 3

INTRODUCTION .................................................................................................................... 5

MATERIALS AND METHODS ............................................................................................... 7

RESULTS .............................................................................................................................. 13

DISCUSSION ........................................................................................................................ 25

BIBLIOGRAPHY .................................................................................................................. 28
INTRODUCTION

Skeletal transverse harmony between maxilla and mandible is essential for balanced vertical and sagittal dental and skeletal relationship, as well as facial esthetics and periodontal health. Successful management of skeletal transverse discrepancy requires accurate diagnosis and proper palatal expansion technique selection. The tooth and/or tissue borne rapid palatal expander (RPE) have been widely used in orthodontic clinic to correct pre-adolescent and adolescent patients’ maxillary transverse deficiencies and associated dentofacial deformities. More recently, adult patient pool for non-surgical expansion has been largely expanded since miniscrew-assisted rapid palatal expansion (MARPE) introduced. However, patients, especially adult ones, responded to expansion differently. It remains unclear why some cases fail while others succeed despite usage of miniscrews for anchorage reinforcement, but it is proposed that differences in calcification patterns of the mid-palatal suture, craniofacial architecture, and age are contributing factors.

A deep understanding of the biological basis underlying maxillary expansion is the fundament to answer this question. Unfortunately, the scientific evidence behind the maxillary expansion is largely missing. Cell populations within mid-palatal suture and cellular and molecular mechanisms in response to mechanical expansion forces have not been fully understood. Recent study demonstrated that transverse force stimulates two distinct phases in the mid-palatal suture. An early catabolic phase, characterized by inflammation, osteoclast recruitment, and activity, results in bone resorption and sutural widening. Then osteoclasts activate osteoblasts resulting in an anabolic phase, during which the integrity of the skeleton is reestablished. The morphology and histology of the mouse palate and the cellular changes that occur following mid-palatal suture expansion were investigated using a palatal expansion mouse model. The cell density of palatal expansion mice compared with same age non-operated control mice was significantly lower.
Mesenchymal stem cell (MSC) refers to a group of heterogeneous multipotent stem cells that can self-renew and further differentiate into several cell types, including osteoblasts, chondrocytes, and adipocytes, which plays an important role in bone remodeling\textsuperscript{11,12}. Mechanical interactions mediated by adhesion to the extracellular matrix (ECM) and cell–cell junctions play a key role in transmitting forces, to and between cells, that regulate intracellular signaling pathways as well as direct stem cell potency maintenance and differentiation\textsuperscript{13}. Abdullah Ekizer \textit{et al} demonstrated that bone marrow MSC transplantation into orthodontically expanded maxillae in rats could enhance bone formation\textsuperscript{14}. Furthermore, Samaneh Mojarrad \textit{et al} successfully isolated cells from mid-palatal suture of mouse and proved MSC characteristics of these cells by their surface markers and abilities of multi-lineage differentiation\textsuperscript{15}. Using a Flexecell Strain system, they showed cyclic mechanical tensile strain could promote osteogenic differentiation of MSCs. This raises the possibility that the distribution and trajectories of suture-MSCs may actively affect maxillary expansion. Indeed, the precise role of MSCs during maxillary expansion is at the present unknown.

We show here that suture-MSCs were labeled by red fluoresces by crossing Gli1-Cre\textsuperscript{ERT2} mice\textsuperscript{16} with Cre reporter tdTomato mice\textsuperscript{17}. Maxillary expansion, done by a single helix expander, seems to promote the proliferation of the Gli1\textsuperscript{+} suture-MSCs. Single cell transcriptomics identified MSC lineage trajectories shift in response to maxillary expansion. Interestingly, we found the immune cells, like neutrophils, also under active immunomodulation. In summary, our data proved the presence of MSCs within mid-palatal suture and MSC lineage trajectories and their unique immune microenvironment have been revealed by single cell transcriptomics. This work may provide a more precise immune-MSC modulatory target for maxillary expansion.
MATERIALS AND METHODS

Mice

All animal studies followed University of Pennsylvania Institutional Animal Care and Use Committee (IACUC)-approved protocol # 806682, in compliance with the Guide for the Care and Use of Laboratory Animals. Gli1<sup>tm3(cre/ERT2)Alj</sup>/J (refer to as Gli1-Cre<sup>ERT2</sup> mice), Cre reporter mice B6.Cg-Gt(ROSA)26Sortm9(CAG-tdTomato)Hze/J (refer to as tdTomato mice) and wild type C57BL/6J mice, which used in single-cell RNA sequencing analyses, were purchased from The Jackson Laboratory (Bar Harbor, ME). To demonstrate sites of Cre recombination, the Gli1-Cre<sup>ERT2</sup> mice were bred with tdTomato mice. Both male and female mutant mice which carried as least one allele of mutant gene were used in the studies, if not specified. To induce the expression of red fluorescent protein variant (tdTomato), all mutant mice were orally administrated with 100 μl of 25 mg/ml tamoxifen (Fisher scientific, MA) for 3 consecutive days starting from 6 weeks after birth. For Gli1 lineage tracing, mice were sacrificed at designated date.

Genotyping

To verify the genotype of the mice obtained from breading, genotyping was performed according to the standard protocols from The Jackson Laboratory. Briefly, tips of tail tissue were harvested from 21-day old pups, then incubated in lysis buffer with Proteinase K (Bioline, TN) overnight at 55 °C. Then the sample were heated one hour at 95 °C to denature Proteinase K, followed by 10 min centrifuge at 10,000 rpm to extract the DNA.

Polymerase Chain Reaction (PCR). PCR reaction mix consists of DNA, which was extracted as described above, master mix (Fisher scientific, MA), primers and double distilled water (ddH₂O). After an initial denaturation for 5 min at 94 °C, amplification cycles consisted of denaturation at 94 °C for 1 min, annealing at 65 °C for both Gli1 and tdTomato, and 1 min
extension at 68 °C for 10 cycles with 0.5 °C per cycle decrease of annealing temperature, followed by a final extension for 10 min at 72 °C. Then 28 amplification cycles of 94 °C-60 °C- 72 °C cycle. The expected products size for Gli1 are 100 and 136 bp for mutant and wild type, respectively; products size for tdTomato are 196 and 297 bp for mutant and wild type, respectively. Primers: Gli1 transgene forward GCG GTC TGG CAG TAA AAA CTA TC, Gli1 transgene reverse GTG AAA CAG CAT TGC TGT CAC TT, Gli1 wild type forward GGG ATC TGT GCC TGA AAC TG, Gli1 wild type reverse CTT GTG GTG GAG TCA TTG GA; tdTomato mutant forward CTG TTC CTG TAC GGC ATG G, tdTomato mutant reverse GGC ATT AAA GCA GCG TAT CC, tdTomato wild type forward AAG GGA GCT GCA GTG GAG TA, tdTomato wild type reverse CCG AAA ATC TGT GCC TGA GTG GAG TA.

**Palatal expansion**

Single helix type expander was employed for palatal expansion as described previously. Briefly, 012-in Australian spooled stainless-steel wire (A.J. Wilcock) was used to bend helix in around 3 mm wide and 6 mm long. The expander was activated intraorally for one molar width and bonded to first and second maxillary molars using a light-cured adhesive (3 M Unitek, Monrovia, CA, USA) under anesthesia (0.1mg/g ketamine). Expansion group of mice were supplied with dough diet (Bio-Serv, NJ). Both control and experimental mice were subjected to daily check during 10-day expansion period to make sure the expander was intact and expansion mice were healthy and alive. Expansion mice with severe weight loss and/or severe infection were euthanized according to the protocol.

**Tissue collection and processing**

Bone tissue samples were collected and fixed in 10% paraformaldehyde (PFA) fixative solution (Thermo Fisher Scientific, MA) at room temperature overnight and then stored in PBS for further
processing. Fixed skeletal samples were either decalcified in 10% buffered EDTA for optimal cutting temperature (O.C.T.) frozen embedding or micro-computed tomography (μCT) analyses. H&E staining sections were obtained from OCT-embedded frozen sections for further histological assessment.

**Fluorescent imaging**

Fluorescent images were used to analyze the tdTomato fluorescence in tissue harvested from genetic reporter mice. The Olympus IX71 inverted fluorescence tissue microscope (Olympus, Japan) was used for acquiring images consisting of 2048 x 2048 pixels in 16 bits.

**Micro-computed tomography (μCT)**

Micro-computed tomography (μCT) analysis was performed according to the standard guidelines using a SCANCO Medical μCT 45 system (SCANCO Medical, Wayne, PA) with an isotropic voxel size of 8 μm for tissue imaging. The threshold was set as 220 for 3D image reconstruction.

**Mid-palatal cells isolation and suture mesenchymal stem cells (MSCs) culture**

Mid-palatal cells isolation and suture-MSCs culture were performed as previous described. After palatal mucosa tissue flap, the exposed palatal suture was excised along with 0.5 mm of adjacent structures on both sides. The suture tissue was minced into small pieces using a scalpel blade #15 and subjected to enzymatic digestion using an enzyme solution consisting of 3 mg/ml collagenase type I and 4 mg/ml dispase 1:1 ratio for 1h at 37°C. Next, 3 ml of dexamethasone (-) culture medium (see receipt below) was added to stop digestion, then was filtered through 70 micro millimeter strainer and centrifuged at 1500 rpm for 5 minutes. Suspended cells were seeded in 100 mm culture dishes (Genesee), and incubated in an atmosphere of 5% CO₂ at 37 °C of temperature. After 24 hours, non-adherent cells were removed, and adherent cells were cultured
for an additional 7 days with additional 10 ml dexamethasone (-) culture medium. Supplied dexamethasone at day 10 for further culture. These adherent single colonies were passaged at passage one (P1) with frequent medium changes to eliminate potential hematopoietic cell contamination. Receipt for culture medium: alpha minimum essential medium (α-MEM, Invitrogen) supplemented with 20% fetal bovine serum (FBS), 2 mM L-glutamine (Invitrogen), 55 μM 2-mercaptoethanol (Invitrogen), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen).

**Cell viability test**

Cell viability test was performed on the cells isolated from mid-palatal suture using LIVE/DEAD™ Viability/Cytotoxicity Kit (Invitrogen, CA) according to manufactory’s instruction.

**Flow cytometry analysis**

Flow cytometry analysis was used to determine the expression of hematopoietic or erythroid specific markers of the isolated cells. Cells isolated from mid-palatal suture were dissociated into single cells by treating the cells with 3 ml 0.05% trypsin/ 0.02% EDTA for 10 min at 37°C and vigorous pipetting. Cells were then centrifuged at 1500 rpm for 6 min at 4°C. A total of 0.2-0.3 x10^6 cells were resuspended in 100 μl of wash buffer per FACS tube/antibody. The following antibodies were used: anti-CD45 (hematopoietic marker, Thermo Fisher Scientific, MA), anti-TER119 (erythroid marker, BD Bioscience, NJ) and isotype-matched control (negative control) at a concentration of 1μg of primary antibody or isotype-matched antibody for 30-45 minutes on ice. Cells were then washed twice in 1 ml wash buffer, centrifuged at 1500 rpm for 6 min at 4°C, and resuspend in 500 μl of 2 % PFA (Wash Buffer: FACS Fixative = 1:1). All samples were analyzed with FACSCalibur and CellQuest software (BD Bioscience, NJ).
Single-cell RNA sequencing

The mid-palatal bones of three 8-week-old C57BL/6J wild type mice from each control and expansion group were combined to extract single-cell suspensions. Mid-palatal suture cells were harvested as described above. Cells were suspended in 2% FBS/PBS with 25 mM HEPES buffer. The centrifuged cells were resuspended in 0.4% BSA PBS solution. FACS analysis was performed using a FACS Aria III at low pressure (100 p.s.i.) using a nozzle of 100 μm and FACSDiva software (BD Biosciences). Living cells gated on the FSC/SSC and debris and dead cells were sorted with the FACSDiva8 software (BD Biosciences) on the basis of the particle size. Placenta blue stain was used to calculate the number of cells and cell viability on a hemocytometer as well to double confirm cell number and viability. Then cells were re-suspended in 0.04% BSA/PBS (culture grade, no Mg\(^{2+}\) or Ca\(^{2+}\)) for sequencing. Nearly 20,000 cells were loaded in aim of acquiring one single library of 10,000 cell for each age group by Chromium controller (V3 chemistry version, 10X Genomics Inc, San Francisco, USA), barcoded and purified as described by the manufacturer, and sequenced using a 2 * 150 pair-end configuration on an Illumina HiSeq platform at a sequencing depth of ~400 million reads.

Preprocessing of scRNA-seq data

After obtaining the initial sequencing data, we compared them to the mouse genome mm10, and folded the UMI with Cellranger (version 3.1, 10x Genomics) software to obtain a single-cell gene expression matrix. Then we imported the expression matrix into the Seurat package (v 4.0) for further analysis. Genes expressed in less than three cells are deleted, mitochondrial genes >25%, and cells with genes <300 are filtered out. The FindVariableGenes function in the Seurat package is used to select variable genes, and then principal component analysis (PCA) was performed, and UMAP dimensionality reduction and visualization were performed based on PCA results.
According to the specific genes of different subgroups, we annotated the cell types of different subclusters.

**Single-cell trajectories analysis**

We also performed the trajectory analysis using Slingshot. Briefly, UMAP was used as dimensional reduction after the PCA were calculated for individual or integrated data- sets. Then Seurat objects were transformed into SingleCellExperiment objects. Slingshot trajectory analysis was conducted using the Seurat clustering information and with dimensionality reduction produced by UMAP.

**Statistical analysis**

Statistical analyses were from at least triplicate experiments. Unpaired t-test or ANOVA were used to evaluate significance in two samples groups. All analyses were carried out using Prism 6 statistical analysis program (GraphPad, La Jolla, CA). Statistical significance was reported as follows: NS for not significant or $P > 0.05$; * for $P < 0.05$; ** for $P < 0.01$; and *** for $P < 0.001$). All data are reported as mean ± standard deviation (s.d.)
RESULTS

Gli1 lineage tracing

It has been demonstrated that Gli1\(^+\) cells within the suture mesenchyme were identified as the main stem cell population for mesenchyme of most craniofacial sutures such as the inter-palatal, maxilla-palatal, maxilla-premaxilla, and inter-maxilla sutures\(^{16}\). To trace the Gli1\(^+\) mesenchymal stem cells (MSCs) in mid-palatal suture, Gli1-Cre\(^{ERT2}\) mice were crossed with Cre reporter tdTomato mice (Fig 1A). The expression of the red fluorescent protein variant (tdTomato) was induced upon tamoxifen injection in a 3-consecutive-day fashion for 6-week-old double mutant mice. Tissue was harvested at day 1, 7, 21 and day 35 (Fig 1B). There were minimal Gli1\(^+\) MSCs reside in mid-palatal suture at day 1. Then cells derived from Gli1\(^+\) MSCs were expanded within mid-palatal suture starting from day 7 as demonstrated by the red fluorescence (Fig 1C). These data indicate MSCs are presented in mid-palatal suture area, which proliferate and differentiate into sub cell populations along with the development of mid-palatal suture.
Figure 1: Gli1-Cre<sup>ERT2</sup>/tdTomato mice generation and Gli1<sup>+</sup> lineage tracing. (A) Schematic representation of the transgenic mouse model to trace Gli1<sup>+</sup> lineage. (B) Schematic representation of the tamoxifen treatment and time points for tissue harvest of reporter mice. (C) Fluorescence image of mid-palatal suture area of reporter mice. Red fluorescence indicates Gli1<sup>+</sup> cells and cells derived from Gli1<sup>+</sup> cells.

Mid-palatal expansion promotes proliferation of suture-MSCs

To study the cellular behavior of suture-MSCs in response to expansion force, Gli1-Cre<sup>ERT2</sup>/tdTomato double mutant mice were subjected to 10-day palatal expansion using single helix expanders. Mutant mice without expanders were included as control group. All mice were orally administrated tamoxifen to induce fluorescence expression and subjected to daily check (Fig 2A). The weight of expansion mice was decreased significantly upon initiation of expansion then regained slowly (Fig 2B), with few of them showing barbering phenotype. μCT analyses and 3D
reconstruction data reveal significant mid-palatal suture opening, 0.31 mm on average, in comparison to the control mice (Fig 2 C and D). It’s worth mentioning that since the mice were only 6-week-old when the experiments started, the mid-palatal sutures were not fused yet.

Fig 2A
Figure 2: Mid-palatal expansion on Gli1-Cre$^{ERT2}$/tdTomato mice. (A) Schematic representation of the tamoxifen treatment and expansion of reporter mice. Representative images of the expansion and control mice. (B) The weight of expansion mice was decreased significantly upon initiation of expansion then regained slowly (n=6 for both control and expansion group). (C) μCT examination of skulls showing expanded mid-palatal suture. (D) 0.31 mm average suture opening in expansion mice (n=3 for both control and expansion group).

Histological analysis of skulls demonstrated decreased interdigitation in the mid-palatal suture in expansion mice (Fig 3A). Interestingly, the number of Gli1$^+$ MSCs was increased significantly in expansion mice showing by the fluoresces imaging (Fig 3B).
Figure 3: Mid-palatal expansion promotes proliferation of suture-MSCs. (A) H&E sections present decreased interdigitation in the mid-palatal suture of expansion mice. (B) The number of Gli1+ MSCs, labeled with red fluorescens, was increased significantly in expansion mice.

Characterization of mid-palatal single-cell atlas

Single-cell RNA sequencing (scRNA-seq) makes it be possible to analyze tissue heterogeneity at the level of individual cell and explore the contribution of different cell subtypes to physiological function and pathogenesis\textsuperscript{19}. The quality of cells in terms of number and viability is essential for scRNA-seq. To confirm it, we harvested cells from mid-palatal bones followed previous described protocols\textsuperscript{15}. Cells cultured \textit{in vitro} presented colonies formation and typical MSC-like morphology
(Fig 4A). On the other hand, live/dead staining and FACS sorting were performed immediately on the cells isolated from mid-palatal suture to check cell viability, which showing the majority of the cells are alive (Fig 4 B and C). Not surprisingly, flow cytometry analysis demonstrated that most of the cells are of hematopoietic origin by targeting CD45 and TER119, markers for hematopoietic and erythrocatic cells, respectively (Fig 4D).

**Figure 4**: Quality of cells isolated from mid-palatal suture. (A) Representative images of MSCs isolated from mid-palatal suture showing typical MSC-like morphology. (B) Live/dead staining. Green color indicates live cell and red color indicates dead cells. (C) FACS sorting results. (D) FACS analysis targeting CD45 and TER 119.

To characterize mid-palatal single-cell atlas, the mid-palatal bones of three 8-week-old C57BL/6J wild type mice from each control and expansion group were combined to extract single-cell suspensions and subjected to droplet-based scRNA-seq (Fig 5A). A total number of 7,118 cells were obtained. Then the UMAP method was applied to reduce the dimensionality. Cells were divided into 12 subgroups based on classic cell surface markers (neutrophil—S100a9, T cell—
Ccl5, macrophage—Csf1r, erythrocyte—Hbb-bt, B cell—Hist1h1b, pro-B cell—Tmcc2, mesenchymal cell—Col1a1, neural cells—Gfyl, epithelium cell—Reg3g and endothelium cell—Plvap) (Fig 5 B, C and D). Clearly, immune cells, like neutrophile, B cell, T cell and macrophage, and erythroid cells constitute the majority of the mid-palatal cells. MSCs only account for 2.16% of whole cell population. Be merging cell populations from control and expansion mice, comparative analysis reveals significant cell population shift among most of the cell populations, including neutrophile, B cell, T cell and MSC in response to expansion (Fig 5E, 6A).

**MSC lineage trajectories shift in response to expansion**

Next, we investigate the lineage trajectories of MSCs in response to maxillary expansion, which exhibit significant shift from comparative analysis (Fig 6A). MSCs were divided into three distinct subclusters (Fig 6 B and C). Clusters 0 expressed markers of adipocyte and osteoblast progenitors, such as Col1a1 and Sp7. Cluster 1 expressed markers of chondrocyte progenitors, such as Sox9. And cluster 2 expressed markers of chondrocyte, such as Col2a1. Detailed gene expression analyses demonstrated MSC and osteogenesis markers (MSC-Ly6a, osteogenesis-Col1a1 and Sp7) elevated while chondrogenesis markers (Col2a1 and Sox9) decreased (Fig 6D) in expansion mice in comparison with control group.
**Figure 5**: Characterization of the single-cell atlas of mouse mid-palatal suture. (A) Schematic representation of the workflow of scRNA-seq. (B) Cells identified by scRNA-seq were visualized with UMAP. Different cell populations were defined and distinguished by color. Each point represented an independent cell. (C) Specific expression of marker genes in different cell types. (D) The expression levels of marker genes were projected onto UMAP atlas. (E) Merged data from expansion and control mice.

**Figure 6**: Characterization of the single-cell atlas of MSC subcluster. (A) Merged MSC subcluster from expansion and control mice. (B) Cells identified by scRNA-seq were visualized with UMAP. Different MSC subpopulations were defined and distinguished by color. Each point represented
an independent cell. (C) Specific expression of marker genes in different cell types. (D) The expression levels of marker genes were projected onto UMAP atlas.

**Neutrophile lineage trajectories shift in response to expansion**

Neutrophil is the predominant cell population (27.07%) in mid-palatal suture. It’s important to study its role in regulating bone remodeling during maxillary expansion. To answer this question, we started from the lineage trajectories analysis. Four neutrophil populations were defined by distinct signature genes based on a recent study of comprehensive transcriptional landscape of neutrophil maturation (Fig 7 A, B and C)\textsuperscript{20}. Maxillary expansion reprograms the genetic architecture of neutrophil populations, alters dynamic transitions toward matured neutrophils without affecting overall heterogeneity (Fig 7D). Furthermore, the biological functions of different subclusters were analyzed by gene ontology (GO) enrichment analysis (Fig 7E). We found that neutrophil degeneration was enriched in cluster 0 which coincident with decrease primes neutrophils. Interleukin 1 (IL-1) beta production and IL-1 pathway was highly activated in cluster 1. Cluster 2 was related to immune and inflammatory response. The biological functions of the cluster 3 were enriched in DNA packaging and chromosome condensation.
Fig 7A

0: Neutrophil progenitors
1: Early Neutrophil
2: Mature Neutrophil 1
3: Mature Neutrophil 2

Fig 7B

Fig 7C
Figure 7: Characterization of the single-cell atlas of neutrophil subcluster. (A) Cells identified by scRNA-seq were visualized with UMAP. Different neutrophil subpopulations were defined and distinguished by color. Each point represented an independent cell. (B) Specific expression of marker genes in different cell types. (C) The expression levels of marker genes were projected onto UMAP atlas. (D) Merged data from expansion and control mice. (E) GO enrichment analysis of the biological functions of different subpopulations.
DISCUSSION

Gli1+ cells are known as the main stem cell population for mesenchyme of most craniofacial sutures\textsuperscript{16}. Recent study demonstrated that the Gli1+ cells are a predominant source for osteoblasts throughout the life of a mouse\textsuperscript{21}, thus, suggesting that Gli1 can be identified as a molecular marker for MSCs or osteogenic progenitors. We have generated a conditional and tamoxifen-inducible transgenic mouse model for Gli1+ MSC \textit{in vivo} labeling. Using this mouse model, we conducted Gli1 lineage tracing within mid-palatal suture. Our data proved MSCs are presented in mid-palatal suture area, which proliferate and differentiate into sub cell populations along with the development of mid-palatal suture. Furthermore, the proliferation of suture-MSCs was elevated in response to mid-palatal expansion. Together, the data suggested that suture-MSCs mediated bone remodeling plays a key role not only during normal growth and development of mid-palatal suture but also in response to maxillary expansion.

Craniofacial bones hold a distinct niche from long bone considering their different developmental origin and postnatal remodeling pattern\textsuperscript{22}. However, a systematic explanation of mid-palatal suture at single-cell level is still lacking. Taking advantage of the cutting-edge technology of single cell sequencing, we were able to map cell population at single-cell resolution within mid-palatal suture with or without maxillary expansion. To our best knowledge, this is the first scRNA-seq study of mid-palatal suture. Suture-MSC lineage trajectories has been established. We found that MSC-marker Ly6a markable elevated in expansion group, which is consistent with expanded Gli1+ cells demonstrated by lineage tracing. Osteogenesis markers, like Col1a1 and Sp7, significantly increased while chondrogenesis markers, like Col2a1 and Sox9, decreased in expansion mice in comparison with control group in response to maxillary expansion. This finding is consistent with previous \textit{in vitro} study\textsuperscript{15}, in which the cyclic mechanical tensile strain could
promote osteogenic differentiation of suture-MSCs, as well as MSCs derived from alveolar bone\textsuperscript{23,24}.

Not surprisingly, MSCs only accounts for 2.16\% of whole cell population reside in mid-palatal suture, which is slightly higher than in mandibular alveolar bone\textsuperscript{25}. The majority cells are immune cells including neutrophiles, B cells, T cells and macrophages, which reflects the nature of anatomical architecture surrounding the mid-palatal suture, including periodontal tissue and abundant blood supplies. Immune cell is an emerging regulatory factor on differentiation of bone stromal cells during bone remodeling\textsuperscript{26,27}. Recently, a study of single-cell atlas has revealed the unique immune microenvironment for the mandibular alveolar bone\textsuperscript{25}. With higher expression level of Oncostatin M (Osm) compare to long bone, monocytes/macrophages derived from alveolar bone promotes osteogenic differentiation and inhibits adipogenic differentiation of MSCs.

It is believed that the maxillary expansion is an inflammation process\textsuperscript{9}. The inflammation-induced osteoclastic bone resorption is critical for suture opening in response to orthopedic tensile force. In our study, comparative analysis reveals significant cell population shift among immune cells in response to expansion. As the most abundant cell population (27.07\%) within mid-palatal suture, neutrophils’ genetic architecture has been reprogrammed dramatically upon expansion. There is a dynamic transition toward matured neutrophils without affecting overall heterogeneity. GO enrichment analysis reveals that neutrophil degeneration was enriched in cluster 0 which coincident with decrease primes neutrophils. Interleukin 1 beta (IL-1β) production and IL-1 pathway were highly activated in cluster 1. Cluster 2 was related to immune and inflammatory response.

The cross-regulation between bone cells and their progenitors, MSCs, and immune system plays an important role in regulating bone remodeling. Many immune cytokines, such as
Interleukins (i.e., IL-6, -11, -17, and -23), Tumor Necrosis Factor (TNF)-α, Receptor-Activator of Nuclear factor Kappa B (RANK), and its Ligand (RANKL), Nuclear Factor of Activated T-cell, cytoplasmatic-1 (NFATc1), and others have all been found to be crucial in osteoclast and osteoblast biology\textsuperscript{28,29}. IL-1 family members promote the activity of cells of the innate immune system, such as neutrophils, eosinophils, basophils, mast cells and natural killer cells. They have important functions in activating and reinforcing the function of polarized T cells. Diseases driven by innate immune cells, such as atherosclerosis and the response to tissue injury, maxillary expansion in this case, also seem to have a large contribution from IL-1 family members\textsuperscript{30}. Future study is needed to gain deep understanding of possible regulatory effects and mechanisms of IL-1 signaling on MSCs.

In summary, our data proved the presence of MSCs within mid-palatal suture and MSC lineage trajectories and their unique immune microenvironment have been revealed by single cell transcriptomics. This work may provide a more precise immune-MSC modulatory target for maxillary expansion.
BIBLIOGRAPHY


