Osteoporotic changes in the periodontium impair alveolar bone healing

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Supplemental information

Methods and Materials

Animals, ovariectomy and tooth extraction surgeries

Stanford Committee on Animal Research approved protocol (#13146), which conforms to ARRIVE guidelines. Forty-one 5-week-old BALB/c female mice were purchased from Charles River Laboratories (Wilmington, MA). Eighteen *Axin2*$^{CreERT2/+}$;*R26R*$^{mTmG/+}$ mice (#018867) that were purchased from Jackson Laboratories were used. All mice were housed in a temperature-controlled environment with 12-h light/dark cycles and were fed an ad libitum diet. Middle-aged (>12-month-old) mice were aged in-house. To induce Cre expression in *Axin2*$^{CreERT2/+}$;*R26R*$^{mTmG/+}$ mice, tamoxifen (4 mg/25 g body weight) was delivered intraperitoneally for 3 consecutive days.

In the young mice group, animals were either examined at 8 weeks of age (the intact group), or the mice underwent a maxillary first molar tooth (mxM1) extraction at 8 weeks of age and then were followed until post-extraction day (PED) 1, 3, or 7.

In the OVX group, 6-week-old mice underwent an OVX surgery (described below). Eight weeks after OVX surgery, this group of mice underwent an mxM1 extraction and were then followed until PED 1, 3, or 7.

Aging is associated with cellular cementum accrual at the root tips (Supplemental Fig. 1), which complicates complete tooth removal. Therefore, to induce an osteoporotic phenotype an OVX

was performed, following established protocols (Kalu and Chen 1999). After surgery mice were randomly assigned to experimental groups, outlined above.

Bilateral mxM1 extractions were performed using micro-forceps. After extraction, bleeding was controlled by local compression. Mice recovered in a controlled, heated environment, then were fed a soft food diet. No adverse events (e.g., uncontrolled pain, infection, prolonged inflammation) were encountered.

Micro-computed tomography (µCT)

Scanning and analyses followed published guidelines (Bouxsein et al. 2010). Three-dimensional µCT imaging was performed at intact and PED7 samples. In brief, samples were fixed in 4% PFA at 4°C overnight and washed in PBS. During scanning, the samples were kept in 70% ethanol solution. µCT scanning was performed on VivaCT40 data-acquisition system (Scanco, Brüttisellen, Switzerland) at 10.5µm voxel size (70kV, 115µA and 300ms integration time). Bone morphometry and measuring width of periodontal ligament (PDL) were performed using CTAn software (SkyScan, Belgium). Multiplanar reconstruction and volume rendering were carried out using Avizo (FEI, Hillsboro, OR), Dataviewer (SkyScan) software and ImageJ (NIH, Bethesda, MD) software. To evaluate average PDL width, three transverse images taken from different positions along the long axis of the root were chosen (e.g., from a crestal region, a middle region and an apical region). Buccal, palatal, mesial and distal PDL widths for each of the three roots were measured using CTAn software (SkyScan) and these values were averaged. The values of each of the three regions were averaged, and eventually average PDL width was calculated.

Tissue preparation and histology

After harvesting, samples were fixed in 4% paraformaldehyde (PFA), decalcified in 10% EDTA solution at 25°C for a minimum of two weeks under constant microwave irradiation. The samples were then dehydrated using an ascending graded ethanol series, and embedded into paraffin blocks for sectioning. Sections were generated at an 8-µm thickness by a Leica microtome (Leica Instruments GmbH, Hubloch, Germany). Before histological staining, all

sections were de-paraffinized in Citrisolv (#1601, Decon Labs Inc. PA), and hydrated via a descending graded ethanol series. After staining, sections were dehydrated in a graded series of ethanol and Citrisolv, and subsequently cover-slipped with Permount (#SP15, Fisher Scientific) mounting media.

For Aniline blue staining, slides were treated with a saturated solution of picric acid, followed by a 5% Phosphotungstic acid solution and 1% Aniline blue staining. For Picrosirius Red staining, slides were stained with picrosirius solution (0.5 g Sirius red (#35780, Pfaltz & Bauer, Inc., CT) dissolved in 500 mL saturated picric acid solution), and then viewed under polarized light. For Pentachrome staining, after dehydration, slides were stained with 1% Alcian Blue (#A5268, Sigma), Verhoeffs Hematoxylin (#S71299, Fisher Scientific), Sodium Thiosulfate (#14518, Alfa Aesar, MA), Crocein-Scarlet-Acid Fuchsin solution (#22914, Chem Impex International, IL; #F8129, Sigma), 5% Phosphotungstic Acid (#P4006, Sigma) and Saffron (#3801, Harlecon), with washing steps between each stain using ethanol, acetic acid and distilled water. With this staining, mature bone was stained yellow, osteoid and mineralized cartilage were stained green, nuclei were stained black and cytoplasm was stained red.

Immunohistochemistry

Immunostaining was performed as described (Yuan et al. 2018). In brief, tissue sections were permeabilized with 0.5% TritonX-100. After antigen retrieval, slides were blocked with 5% goat serum (Vector S-1000) for 1h at room temperature and incubated with primary antibodies overnight at 4°C. After washing with PBS, slides were incubated with Cyanine5 conjugated goat anti-rabbit secondary antibody (Invitrogen, A10523) for 30min, then mounted with DAPI mounting medium (Vector Laboratories). Primary antibodies were anti-Periostin (ab14041, Abcam), anti-Osterix (ab22552, Abcam), anti-Runx2 (ab23981, Abcam) and anti-Ki67 (ab16667, Abcam) and carried out as described.

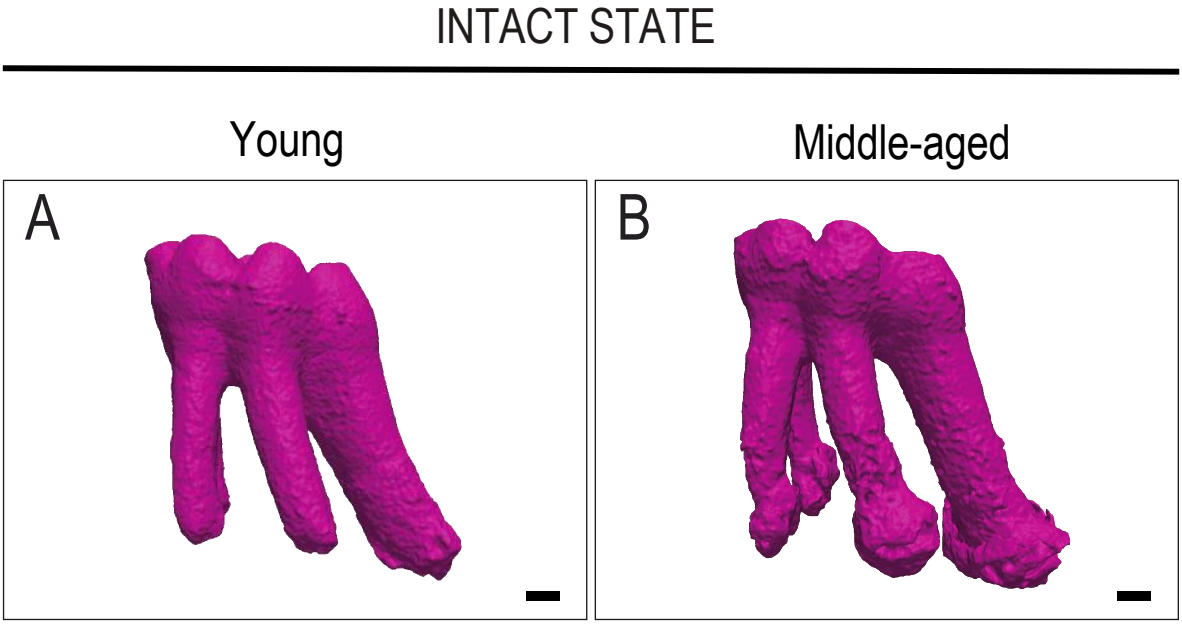
Histomorphometric analyses

Histomorphometric measurements were performed using ImageJ software (NIH, Bethesda, MD). To quantify the number of immunopositive cells, tissue sections containing the PDL and tooth extraction socket were photographed using a Leica digital image system. The number of $Ki67^{+ve}$, $Osterix^{+ve}$, $Runx2^{+ve}$ and GFP^{+ve} cells within the PDL area and tooth extraction socket, and the number of $DAPI^{+ve}$ cells within $Periostin^{+ve}$ area were manually counted to find the number of cells. The percentage of $Ki67^{+ve}$, $Osterix^{+ve}$, $Runx2^{+ve}$ and GFP^{+ve} cells were calculated by dividing the number of positively labelled cells to number of total cells defined by DAPI stain in the PDL area and tooth extraction socket.

Statistical Analyses

Results are presented in the form of mean ± standard deviation, with N equal to the number of samples analyzed. All statistical analyses were performed using the GraphPad 5.0 Software, (San Diego, CA). Histomorphometric results were based on the Student's *t*-tests and one-way ANOVA. Significance was attained at $p<0.05$ (*), at $p<0.01$ (**), at $p<0.001$ (***).

Supplemental Figure



Supplemental Figure. Three-dimensional volume renderings of mxM1 in (A) the young and (B) the middle-aged rodents. Scale bars=100μm