Populations of Mesenchymal Precursors

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Appendix

Appendix Materials & Methods

Msx2-creER bacterial artificial chromosome (BAC) transgenic mice.

Msx2-creER-SV40pA transgenic mice were generated by pronuclear injection of a modified bacterial artificial chromosome (BAC) clone RP23-438B5 (Children's Hospital Oakland Research Institute, Oakland, CA) containing 139kb upstream and 32kb downstream genomic sequences of the *Msx2* gene. The targeting vector for recombineering was generated by the following steps: first, 5' and 3' homology arms were PCR-amplified from RP23-438B5 using a set of mutant primers generating restriction enzyme sites (5'-Fw: GAGCTCCTGTAATTAACTCC; SacI, 5'-Rv: GAATTCGGAGAAGCCAAGAC; EcoRI, 3'-Fw: ACCGGTCATGTCCGACAAGAAG; AgeI, 3'-Rv: AGCTAGGGATTCTGGGATGC), and were subcloned into pGEM-T vector (Promega). Second, 5' and 3' homology arms were subcloned into a 7.5kb SacI/EcoRI fragment and an 8.6kb AgeI/KpnI fragment of a *pCS.frt.Neo* plasmid, respectively, to generate a *pCS.5.frt.Neo.3* plasmid. Third, a 2.2kb EcoRI fragment of a p*CreER^{T2}-SV40pA* plasmid was subcloned into an EcoRI-linearized *pCS.5.frt.Neo.3* plasmid to generate a *pCS.5.CEsA.frt.Neo.3* plasmid. A 4.3kb SacI/KpnI fragment of a *pCS.5.frt.Neo.3* plasmid was gel-purified prior to electroporation into *E.coli* strain SW105 containing RP23-438B5. Recombinants were selected on bacterial plates containing kanamycin and PCR-screened

for homologous recombination events. The *Neo*^{R} cassette was removed from correctly targeted BAC clones by L-arabinose induction of *flpe* recombinase, and *lox* sites (*loxP* and *lox411*) in the BAC plasmid were replaced with *Amp*^{R} and *Neo*^{R} cassettes by recombineering. The modified BAC clone was verified by sequencing high-fidelity long-range PCR products and standard southern blot analysis, and purified by NucleoBond BAC 100 (Clontech) prior to injection. The concentration of the BAC construct was verified on gel. The BAC clone was pronuclearly injected into hybrid SJL/C57BL6 mouse fertilized eggs at a concentration of 1ng/μl. F0 mouse was backcrossed with C57/BL6 mice at least for four generations before analysis.

Mice.

Osteocalcin (Oc)-GFP (JAX13134) and *Osx-creER*^{T2} (Maes et al, 2011) mice have been described elsewhere. *Rosa26-CAG-loxP-stop-loxP*-tdTomato (Ai14: *R26R*-tdTomato, JAX7914) mice were acquired from the Jackson laboratory. All procedures were conducted in compliance with the Guidelines for the Care and Use of Laboratory Animals approved by the University of Michigan's Institutional Animal Care and Use Committee (IACUC), protocol PRO00005703 (Ono). All mice were housed in a specific pathogen-free condition in a standard Allentown cage, and analyzed in a mixed background. Mice were identified by micro-tattooing or ear tags. Tail biopsies of mice were lysed by proteinase K (14-22μg/ml) in DirectPCR lysis reagent (Viagen Biotech), or a HotShot protocol (incubating the tail sample at 95^{o}C for 30 min in an alkaline lysis reagent followed by neutralization), and subsequently used for PCR-based genotyping (GoTaq Green Master Mix, Promega, and Nexus X2, Eppendorf). Perinatal mice were also genotyped fluorescently (BLS miner's lamp) whenever possible. Mice were euthanized by over-dosage of carbon dioxide or decapitation under inhalation anesthesia in a drop jar (Fluriso, Isoflurane USP,

VetOne). For all experiments, male breeder mice (*Msx2-creER* or *Osx-creER*; *R26R*$^{tdTomato/tdTomato}$) were mated to female CD1 mice (>8 weeks old, Charles River Laboratories 022) and the vaginal plug was checked in the morning. Pregnant mice received 4 mg tamoxifen (Sigma T5648) and 4 mg progesterone (Sigma P3972) intraperitoneally at an indicated embryonic day in a home cage. Progesterone was added in the mix to prevent the adverse effect of tamoxifen, which may cause spontaneous abortion due to its anti-estrogenic activity. Embryos or pups were used for analysis regardless of the sex. At least three independent biological samples of the indicated genotype were examined for each data shown in the figures.

Figure 1C, D: Msx2-creER; R26R$^{tdTomato/tdTomato}$ male mice were mated to female CD-1 mice. Pregnant CD-1 mice received 4mg of tamoxifen and progesterone intraperitoneally at E10.5. Embryos at E11.5 were used for analysis regardless of the sex. *n*=3 mice.

Figure 1E: Msx2-creER; R26R$^{tdTomato/tdTomato}$ male mice were mated to female CD-1 mice. Pregnant CD-1 mice received 4mg of tamoxifen and progesterone intraperitoneally at E13.5. Embryos at E14.5 were used for analysis regardless of the sex. *n*=3 mice.

Figure 2A: Msx2-creER; R26R$^{tdTomato/tdTomato}$ male mice were mated to female CD-1 mice. Pregnant CD-1 mice received 4mg of tamoxifen and progesterone intraperitoneally at E9.5. Embryos at E15.5 were used for analysis regardless of the sex. *n*=3 mice.

Figure 2B, D, E: Msx2-creER; R26R$^{tdTomato/tdTomato}$ male mice were mated to female CD-1 mice. Pregnant CD-1 mice received 4mg of tamoxifen and progesterone intraperitoneally at E10.5. Embryos at E15.5 were used for analysis regardless of the sex. *n*=3 mice.

Figure 2C: Msx2-creER; R26R$^{tdTomato/tdTomato}$ male mice were mated to female CD-1 mice. Pregnant CD-1 mice received 4mg of tamoxifen and progesterone intraperitoneally at E11.5. Embryos at E15.5 were used for analysis regardless of the sex. *n*=3 mice.

Figure 2F, I, J: Osx-creER; R26R$^{tdTomato/tdTomato}$ male mice were mated to female CD-1 mice. Pregnant CD-1 mice received 4mg of tamoxifen and progesterone intraperitoneally at E12.5. Embryos at E15.5 were used for analysis regardless of the sex. *n*=3 mice.

Figure 2G: Osx-creER; R26R$^{tdTomato/tdTomato}$ male mice were mated to female CD-1 mice. Pregnant CD-1 mice received 4mg of tamoxifen and progesterone intraperitoneally at E13.5. Embryos at E15.5 were used for analysis regardless of the sex. *n*=3 mice.

Figure 2H: Osx-creER; R26R$^{tdTomato/tdTomato}$ male mice were mated to female CD-1 mice. Pregnant CD-1 mice received 4mg of tamoxifen and progesterone intraperitoneally at E14.5. Embryos at E15.5 were used for analysis regardless of the sex. *n*=3 mice.

Figure 3A, B: Msx2-creER; R26R$^{tdTomato/tdTomato}$ male mice were mated to female CD-1 mice. Pregnant CD-1 mice received 4mg of tamoxifen and progesterone intraperitoneally at E10.5. Mice at P0 were used for analysis regardless of the sex. *n*=3 mice.

Figure 3C: Msx2-creER; R26R$^{tdTomato/tdTomato}$ male mice were mated to female CD-1 mice. Pregnant CD-1 mice received 4mg of tamoxifen and progesterone intraperitoneally at E10.5. Mice at P28 were used for analysis regardless of the sex. *n*=3 mice.

Figure 3D, E: Osx-creER; R26R$^{tdTomato/tdTomato}$ male mice were mated to female CD-1 mice. Pregnant CD-1 mice received 4mg of tamoxifen and progesterone intraperitoneally at E13.5. Mice at P0 were used for analysis regardless of the sex. *n*=3 mice.

Figure 3F: Osx-creER; R26R$^{tdTomato/tdTomato}$ male mice were mated to female CD-1 mice. Pregnant CD-1 mice received 4mg of tamoxifen and progesterone intraperitoneally at E13.5. Mice at P28 were used for analysis regardless of the sex. *n*=3 mice.

Appendix Figure 1B, C: Msx2-creER; R26R$^{tdTomato/tdTomato}$ male mice were mated to female CD-1 mice. Pups received 0.1mg of tamoxifen intraperitoneally at P2. Mice at P4 were used for analysis regardless of the sex. *n*=3 mice.

Appendix Figure 1D, E: Msx2-creER; R26R$^{tdTomato/tdTomato}$ male mice were mated to female CD-1 mice. Pregnant CD-1 mice received 4mg of tamoxifen and progesterone intraperitoneally at E10.5. Embryos at E11.5 were used for analysis regardless of the sex. *n*=3 mice.

Appendix Figure 2A, C: Msx2-creER; R26R$^{tdTomato/tdTomato}$ male mice were mated to female CD-1 mice. Pregnant CD-1 mice received 4mg of tamoxifen and progesterone intraperitoneally at E9.5. Embryos at E10.5 were used for analysis regardless of the sex. *n*=3 mice.

Appendix Figure 2B, D: Msx2-creER; R26R$^{tdTomato/tdTomato}$ male mice were mated to female CD-1 mice. Pregnant CD-1 mice received 4mg of tamoxifen and progesterone intraperitoneally at E10.5. Embryos at E11.5 were used for analysis regardless of the sex. *n*=3 mice.

Appendix Figure 3A, B: Msx2-creER; R26R$^{tdTomato/tdTomato}$ male mice were mated to female CD-1 mice. Pregnant CD-1 mice received 4mg of tamoxifen and progesterone intraperitoneally at E11.5. Embryos at E12.5 were used for analysis regardless of the sex. *n*=3 mice.

Appendix Figure 3C: Msx2-creER; R26R$^{tdTomato/tdTomato}$ male mice were mated to female CD-1 mice. Pregnant CD-1 mice received 4mg of tamoxifen and progesterone intraperitoneally at E17.5. Embryos at E18.5 were used for analysis regardless of the sex. *n*=3 mice.

Appendix Figure 3D: Msx2-creER; R26R$^{tdTomato/tdTomato}$ male mice were mated to female CD-1 mice. Pups received 0.1mg of tamoxifen intraperitoneally at P1. Mice at P4 were used for analysis regardless of the sex. *n*=3 mice.

Appendix Figure 4B, C, D, E: Msx2-creER; R26R$^{tdTomato/tdTomato}$ male mice were mated to female CD-1 mice. Pregnant CD-1 mice received 4mg of tamoxifen and progesterone intraperitoneally at E9.5. Embryos at E15.5 were used for analysis regardless of the sex. *n*=3 mice.

Appendix Figure 4F: Msx2-creER; R26R$^{tdTomato/tdTomato}$ male mice were mated to female CD-1 mice. Pregnant CD-1 mice received 4mg of tamoxifen and progesterone intraperitoneally at E10.5. Embryos at E15.5 were used for analysis regardless of the sex. *n*=3 mice.

Appendix Figure 4G: Msx2-creER; R26R$^{tdTomato/tdTomato}$ male mice were mated to female CD-1 mice. Pregnant CD-1 mice received 4mg of tamoxifen and progesterone intraperitoneally at E11.5. Embryos at E15.5 were used for analysis regardless of the sex. *n*=3 mice.

Appendix Figure 5A: Osx-creER; R26R$^{tdTomato/tdTomato}$ male mice were mated to female CD-1 mice. Pregnant CD-1 mice received 4mg of tamoxifen and progesterone intraperitoneally at E10.5. Embryos at E11.5 were used for analysis regardless of the sex. *n*=3 mice.

Appendix Figure 5B: Osx-creER; R26R$^{tdTomato/tdTomato}$ male mice were mated to female CD-1 mice. Pregnant CD-1 mice received 4mg of tamoxifen and progesterone intraperitoneally at E12.5. Embryos at E13.5 were used for analysis regardless of the sex. *n*=3 mice.

Appendix Figure 5C: Osx-creER; R26R$^{tdTomato/tdTomato}$ male mice were mated to female CD-1 mice. Pregnant CD-1 mice received 4mg of tamoxifen and progesterone intraperitoneally at E12.5. Embryos at E15.5 were used for analysis regardless of the sex. *n*=3 mice.

Appendix Figure 5D, E: Osx-creER; R26R$^{tdTomato/tdTomato}$ male mice were mated to female CD-1 mice. Pregnant CD-1 mice received 4mg of tamoxifen and progesterone intraperitoneally at E14.5. Embryos at E15.5 were used for analysis regardless of the sex. *n*=3 mice.

Appendix Figure 6A, C: Msx2-creER; R26R$^{tdTomato/tdTomato}$ male mice were mated to female CD-1 mice. Pregnant CD-1 mice received 4mg of tamoxifen and progesterone intraperitoneally at E10.5. Mice at P21 were used for analysis regardless of the sex. *n*=3 mice.

Appendix Figure 6B, D: Osx-creER; R26R$^{tdTomato/tdTomato}$ male mice were mated to female CD-1 mice. Pregnant CD-1 mice received 4mg of tamoxifen and progesterone intraperitoneally at E13.5. Mice at P21 were used for analysis regardless of the sex. *n*=3 mice.

Tamoxifen.

Tamoxifen (Sigma T5648) and progesterone (Sigma P3972) were mixed with 100% ethanol until completely dissolved. Subsequently, a proper volume of sunflower seed oil (Sigma S5007) was added to the tamoxifen-ethanol mixture and rigorously mixed. The tamoxifen-progesterone-ethanol-oil mixture was incubated at 60^{o}C in a chemical hood until the ethanol evaporated completely. The tamoxifen-progesterone-oil mixture was stored at room temperature until use.

Histology and immunohistochemistry.

Samples were dissected under a stereomicroscope (Nikon SMZ-800) to remove soft tissues, and fixed in 4% paraformaldehyde for a proper period, typically ranging from 3 hours to overnight at 4^{o}C, then decalcified in 15% EDTA for a proper period, typically ranging from 3 hours to 14

days. Decalcified samples were cryoprotected in 30% sucrose/PBS solutions and then in 30% sucrose/PBS:OCT (1:1) solutions, each at least overnight at 4°C. Samples were embedded in an OCT compound (Tissue-Tek, Sakura) under a stereomicroscope and transferred on a sheet of dry ice to solidify the compound. Embedded samples were cryosectioned at 15-25µm using a cryostat (Leica CM1850) and adhered to positively charged glass slides (Fisherbrand ColorFrost Plus). Cryosections were stored at -20°C in freezers until use. Sections were postfixed in 4% paraformaldehyde for 15 min. For immunostaining, sections were permeabilized with 0.25% TritonX/TBS for 30 min, blocked with 3% BSA/TBST for 30 min and incubated with rabbit anti-Sox9 polyclonal antibody (1:1,000, EMD-Millipore, AB5535), goat anti-osteopontin (OPN) polyclonal antibody (1:500, R&D, AF808), rabbit anti-periostin (POSTN) polyclonal antibody (1:2,000, EMD-Millipore, ABT280), rabbit anti-type I collagen (COL1) polyclonal antibody (1:500, Cederlane, CL50151AP), sheep anti-dentin matrix protein 1 (DMP1) polyclonal antibody (1:100, R&D, AF4386), goat anti-osteocalcin (OCN) polyclonal antibody (1:200, AbD Serotec/Bio-rad, 7060-1815) and/or rat anti-E-cadherin (E-cad) monoclonal antibody (1:200, Abcam, ab11512) overnight at 4°C, and subsequently with Alexa Fluor 488-conjugated donkey anti-rabbit IgG (A21206), Alexa Fluor 647-conjugated donkey anti-goat IgG (A21082) or goat anti-rat IgG (A21094) (1:400, Invitrogen) for 3 hours at 4°C. Sections were further incubated with DAPI (4',6-diamidino-2-phenylindole, 5µg/ml, Invitrogen D1306) to stain nuclei prior to imaging. Stained samples were mounted in TBS with No.1.5 coverslips (Fisher).

Imaging.

Images for fixed sections were captured by an automated inverted fluorescence microscope with a structured illumination system (Zeiss Axio Observer Z1 with ApoTome.2 system) and Zen 2

(blue edition) software. The filter settings used were: FL Filter Set 34 (Ex. 390/22, Em. 460/50 nm), Set 38 HE (Ex. 470/40, Em. 525/50 nm), Set 43 HE (Ex. 550/25, Em. 605/70 nm), Set 50 (Ex. 640/30, Em. 690/50 nm) and Set 63 HE (Ex. 572/25, Em. 629/62 nm). The objectives used were: Fluar 2.5x/0.12, EC Plan-Neofluar 5x/0.16, Plan-Apochromat 10x/0.45, EC Plan-Neofluar 20x/0.50, EC Plan-Neofluar 40x/0.75, Plan-Apochromat 63x/1.40. Images were typically tile-scanned with a motorized stage, Z-stacked and reconstructed by a maximum intensity projection (MIP) function. Differential interference contrast (DIC) was used for objectives higher than 10x. Representative images of at least three independent biological samples are shown in the figures.

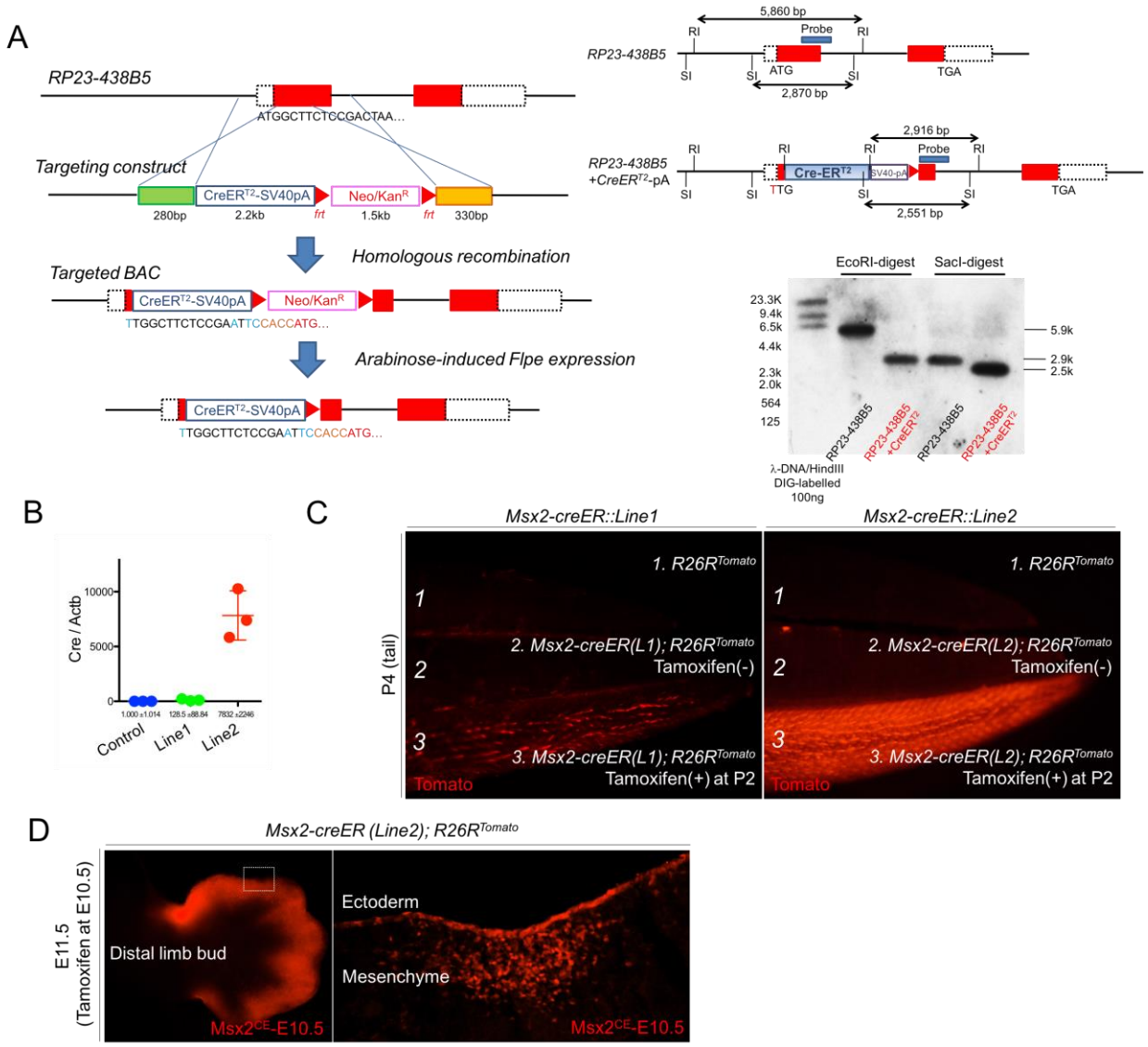
Quantitative Real-time PCR Analysis.

A tail biopsy (~5mm) was homogenized in TRIzol reagent (Invitrogen), and total RNA was isolated using NucleoSpin RNA XS kit (Macherey-Nagel) without a carrier RNA. First-strand cDNA was synthesized using QuantiTect Reverse Transcription kit (Qiagen). Quantitative real-time PCR analysis was carried out using QuantiFast SYBR Green PCR kit (Qiagen) and StepOne Plus Real-time PCR systems (Applied Biosystems). The PCR conditions were 95 °C for 10 sec and 60°C for 30 sec for 50 cycles. The primer sequences are as follows: *Actb*, 5'-GGCTGTATTCCCCTCCATCG-3' (forward) and 5'-CCAGTTGGTAACAATGCCATGT-3' (reverse); *Cre*, 5'-CGTACTGACGGTGGGAGAAT-3' (forward) and 5'-CCCGGCAAAACAGGTAGTTA-3' (reverse).

Statistical analysis.

Results are presented as mean values ± S.D. Statistical evaluation was conducted using the unpaired *t*-test. A *P* value of <0.05 was considered significant. No statistical method was used to

pre-determine sample size. Sample size was determined on the basis of previous literature and our prior experience that give sufficient S.D. of the mean so as not to miss a biologically important difference between groups. The experiments were not randomized. All the available mice of the desired genotypes were used for experiments. The investigators were not blinded to during experiments and outcome assessment. Genotypes were not particularly highlighted during quantification.



Appendix Figure 1. Generation and characterization of *Msx2-creER* BAC transgenic mice.

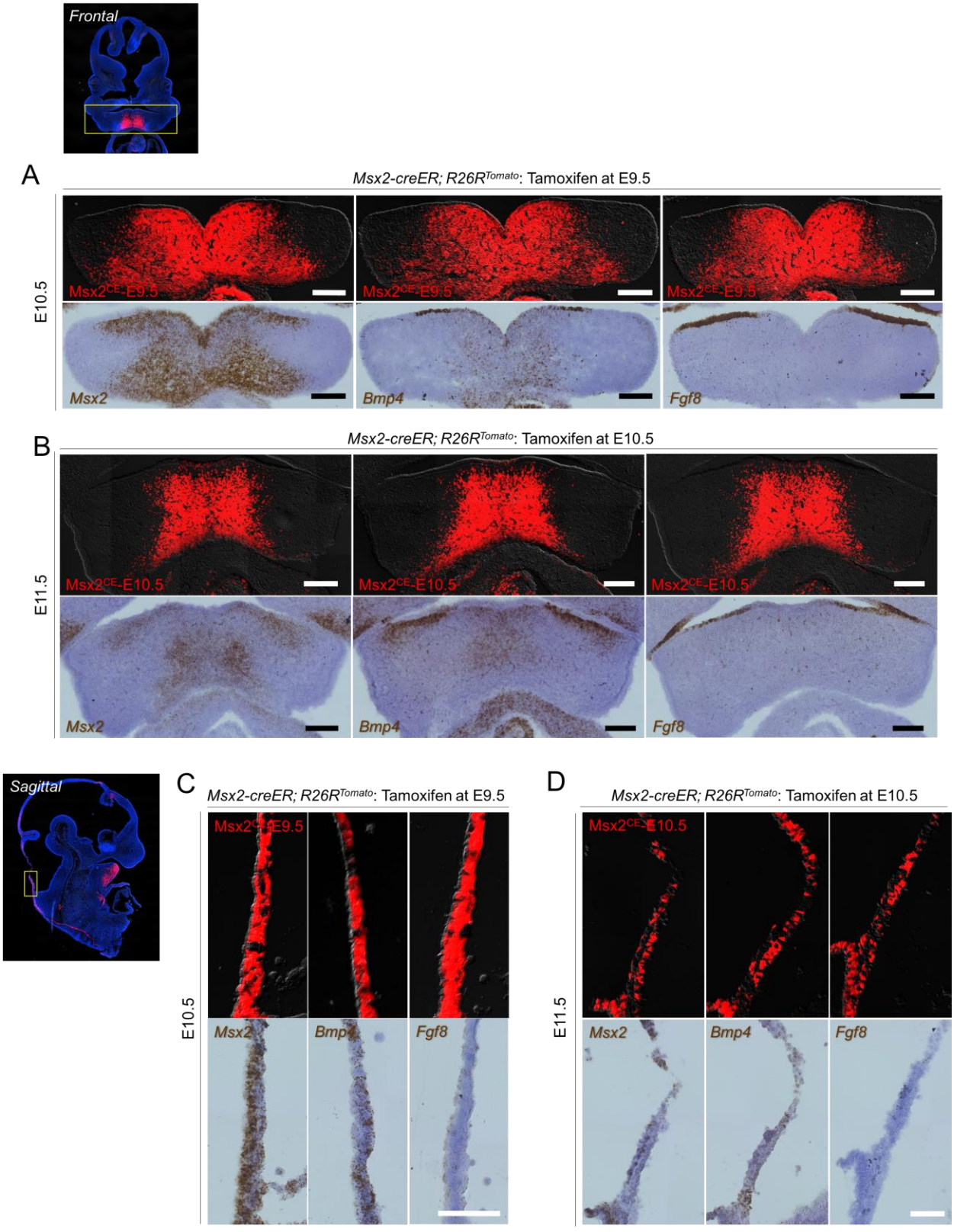
(A) Recombineering strategy to generate the *Msx2-creER-SV40pA* bacterial artificial chromosome (BAC) transgene. *Kozak-Msx2-creER-SV40pA-frt-Neo^{R}-frt* cassette containing homology arms (5': 280bp, 3': 330bp) was recombineered into BAC clone RP23-438B5 containing 139kb upstream and 32kb downstream genomic sequences of the *Msx2* gene through homologous recombination. *Neo^{R}* and backbone *lox* sites were removed prior to pronuclear injection. Right panel: southern blot analysis to verify the modified BAC clone. DIG-labeled 3'

probe was used to detect EcoRI BAC fragments (unmodified: 5.9kb, recombineered: 2.9kb) or SacI BAC fragments (unmodified: 2.9kb, recombineered: 2.5kb).

(B) Quantitative PCR (qPCR) analysis of *cre* transgene expression in tail biopsies at P4. Blue: control, green: Line 1, red: Line 2. Data were normalized to *β-actin* (*Actb*). *n*=3. All data represented as mean ± S.D.

(C) Whole-mount epifluorescence image of tails at P4. Left panel: *Msx2-creER* (Line1), right panel: *Msx2-creER* (Line2). (1): *R26R*^{Tomato} control, (2): *Msx2-creER*; *R26R*^{Tomato} mice without tamoxifen injection, (3): *Msx2-creER*; *R26R*^{Tomato} mice with tamoxifen injection at P2 (48 hours prior to analysis). Red: tdTomato.

(D) Distal limb bud of *Msx2-creER* (Line2); *R26R*^{Tomato} mice at E11.5 (tamoxifen at E10.5). Left panel: whole mount, right panel: section of the dotted area. Red: tdTomato.

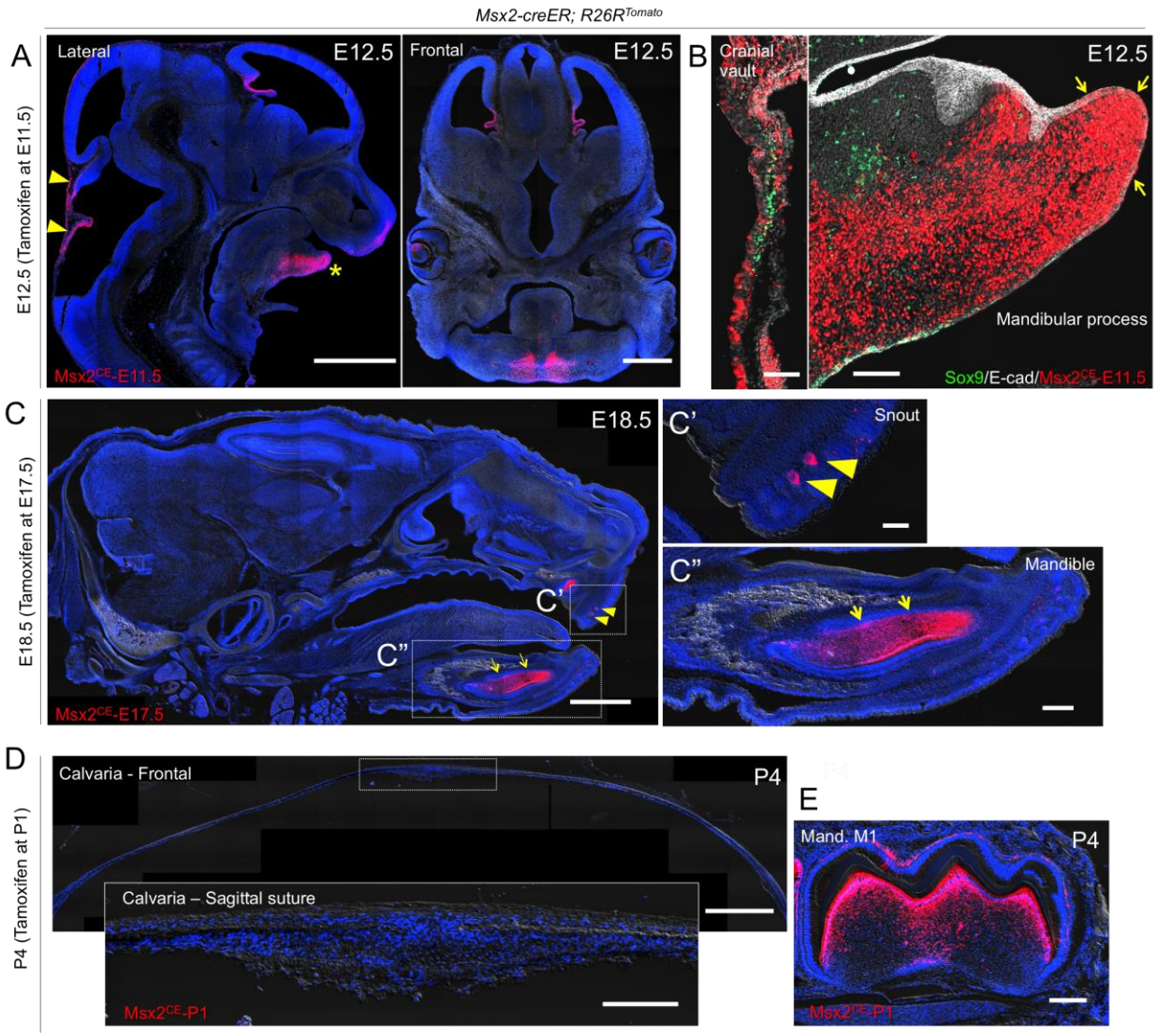

Frontal
A
Msx2-creER; R26R^Tomato: Tamoxifen at E9.5
E10.5
Msx2^CE-E9.5
Msx2^CE-E9.5
Msx2^CE-E9.5
Msx2
Bmp4
Fgf8
B
Msx2-creER; R26R^Tomato: Tamoxifen at E10.5
E11.5
Msx2^CE-E10.5
Msx2^CE-E10.5
Msx2^CE-E10.5
Msx2
Bmp4
Fgf8
Sagittal
C
Msx2-creER; R26R^Tomato: Tamoxifen at E9.5
E10.5
Msx2^CE-E9.5
Msx2
Bmp4
Fgf8
D
Msx2-creER; R26R^Tomato: Tamoxifen at E10.5
E11.5
Msx2^CE-E10.5
Msx2
Bmp4
Fgf8

Appendix Figure 2. *Msx2-creER*$^{+}$ cells are preferentially located in proximity to BMP4-positive domain of the mesenchyme.

In situ hybridization (RNAscope) analysis of *Msx2-creER*; *R26R*^{Tomato} mandibular and posterior cranial vault sections.

(A, B) Frontal sections of *Msx2-creER*; *R26R*^{Tomato} mandibular process at E10.5 (tamoxifen at E9.5) (A) and E11.5 (tamoxifen at E10.5) (B). Upper panels: tdTomato epifluorescence before RNAscope, lower panels: after RNAscope hybridization. Left panels: *Msx2*, center panels: *Bmp4*, right panels: *Fgf8.* Red: tdTomato, brown: DAB, blue: hematoxylin. Scale bars: 200µm.

(C, D) Sagittal sections of *Msx2-creER*; *R26R*^{Tomato} posterior cranial vault at E10.5 (tamoxifen at E9.5) (C) and E11.5 (tamoxifen at E10.5) (D). Upper panels: tdTomato epifluorescence before RNAscope, lower panels: after RNAscope hybridization. Left panels: *Msx2*, center panels: *Bmp4*, right panels: *Fgf8.* Red: tdTomato, brown: DAB, blue: hematoxylin. Scale bars: 100µm.



Appendix Figure 3. Characterization of cells marked by *Msx2-creER* during craniofacial development.

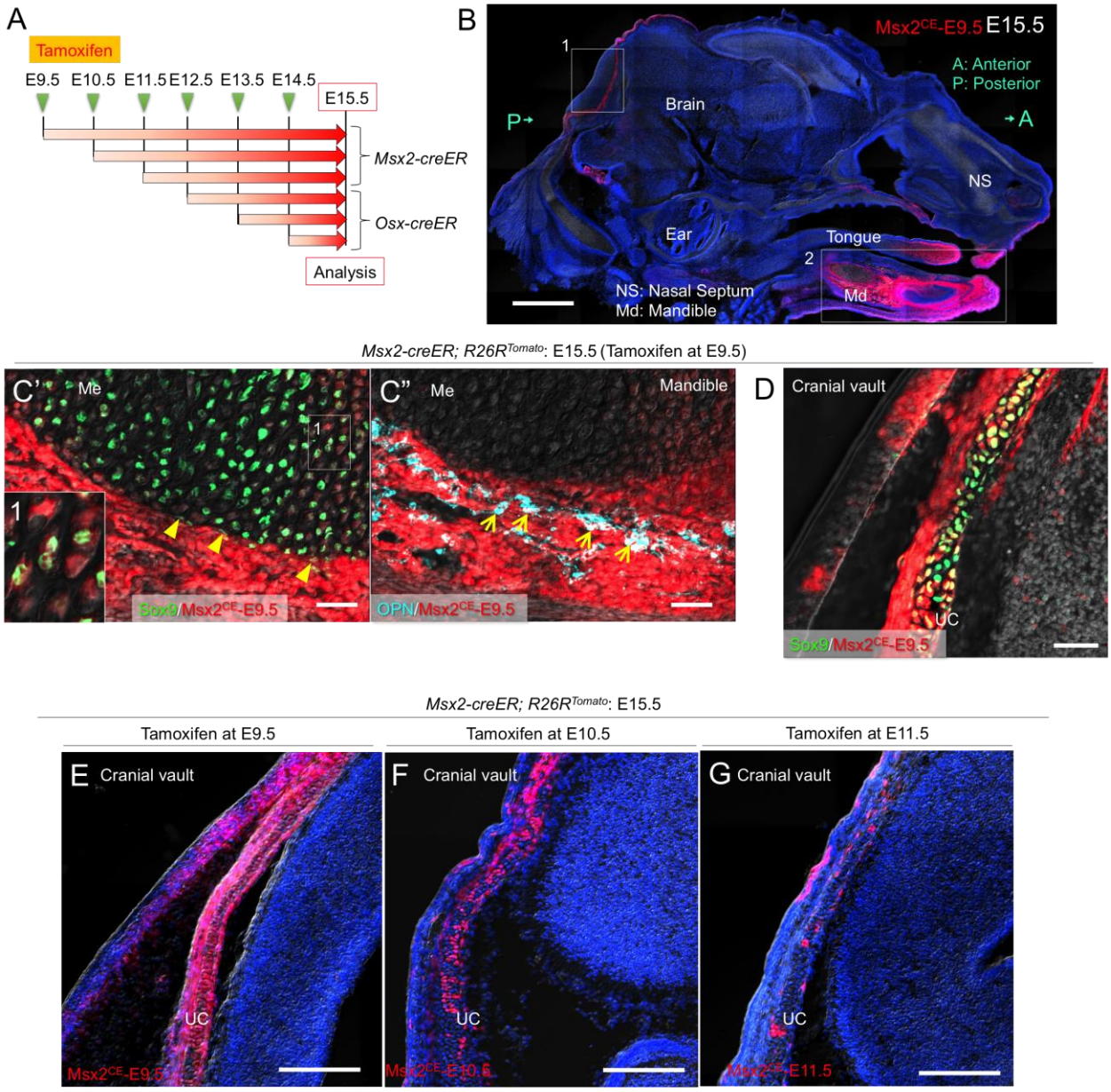
(A, B) Sagittal (A, left panel) and frontal (A, right panel) sections of *Msx2-creER*; *R26R*$^{\text{Tomato}}$ mice at E12.5 (tamoxifen at E11.5) stained for nuclei. Cranial vault (B, left panel) and mandibular process. (B, right panel) were stained for Sox9 and E-cad. Arrowheads: tdTomato^{+} cells in the posterior cranial vault, asterisk: tdTomato^{+} cells in the mandibular process, arrows: E-cad^{+}tdTomato^{+} in the apical ectodermal ridge. Green: Sox9-Alexa488, white: E-cadherin-

Alexa647, red: tdTomato, gray: DAPI and DIC. Scale bars: 1mm (A, left panel), 500µm (A, right panel), 100µm (B).

(C-C") Sagittal sections of *Msx2-creER*; *R26R*^{Tomato} mice at E18.5 (tamoxifen at E17.5) stained for nuclei. C': maxillary snout, C": mandible. Arrowheads: tdTomato^{+} cells in whisker follicles, arrows: tdTomato^{+} cells in the dental papilla of the mandibular incisor. Red: tdTomato, gray: DAPI and DIC. Scale bars: 1mm (left panel), 100µm (right upper panel), 200µm (right lower panel).

(D) Frontal sections of *Msx2-creER*; *R26R*^{Tomato} parietal bones at P4 (tamoxifen at P1) stained for nuclei. Inset: magnified view of the midsagittal calvarial suture. Red: tdTomato, gray: DAPI and DIC. Scale bars: 1mm (upper panel), 200µm (inset).

(E) Sagittal sections of *Msx2-creER*; *R26R*^{Tomato} molars at P4 (tamoxifen at P1) stained for nuclei. Red: tdTomato, gray: DAPI and DIC. Scale bars: 200µm.



Appendix Figure 4. *Msx2-creER* marks early mesenchymal precursors during craniofacial bone development.

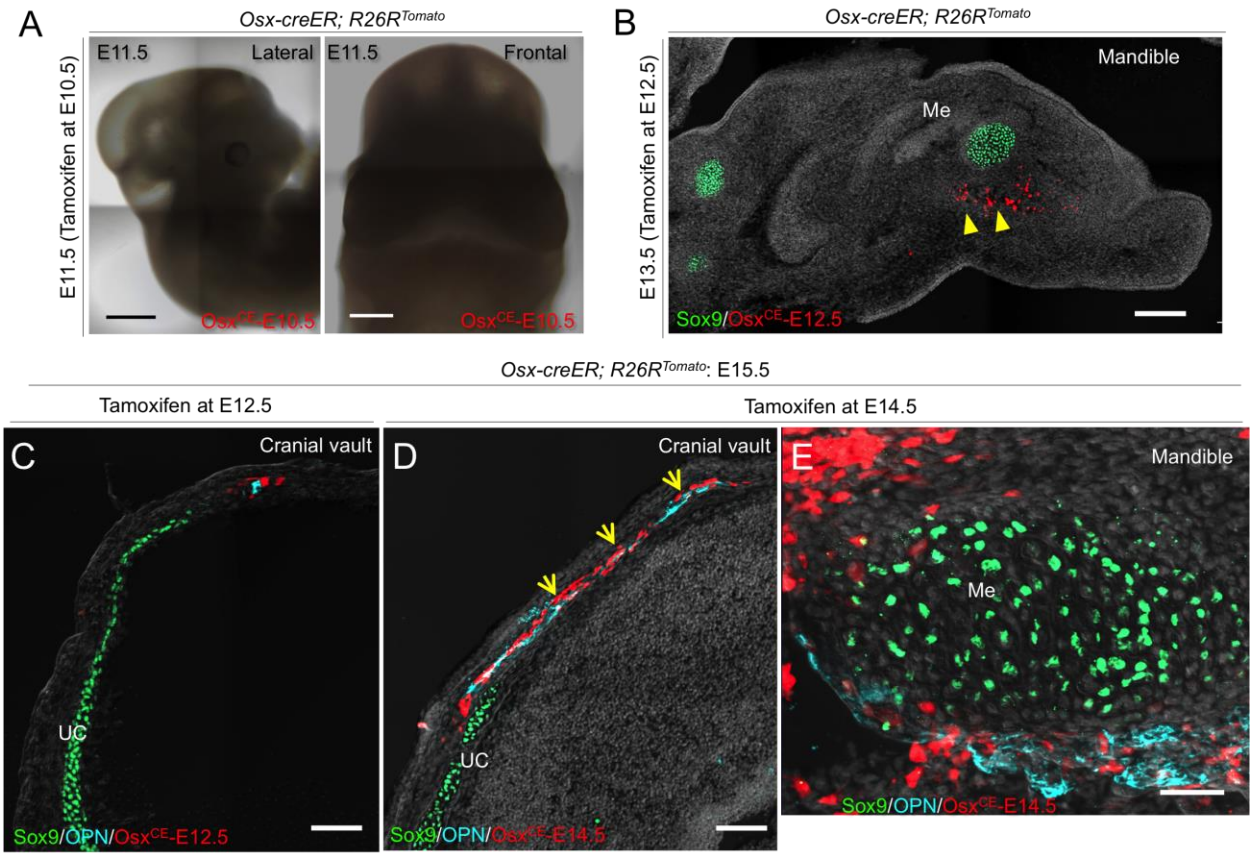
(A) The scheme of experiments in Figure 2. A single dose of tamoxifen was administered at various preceding time points, and the treated mice were analyzed at E15.5.

(B) Sagittal sections of *Msx2-creER*; *R26R*^{Tomato} mice at E15.5 (tamoxifen at E9.5) stained for nuclei. (1): cranial vault, (2): mandible. Red: tdTomato, gray: DAPI and DIC. Scale bars: 1000μm.

(C'-C") Sagittal sections of *Msx2-creER*; *R26R*^{Tomato} mandible at E15.5 (tamoxifen at E9.5) stained for Sox9, OPN and nuclei. Magnified view of the dotted area in **Fig.2A**. Inset: magnified view of Area (1). Me: Meckel's cartilage. Arrows: OPN^{+}tdTomato^{+} osteoblasts, arrowheads: Sox9^{+}tdTomato^{+} chondrocytes and perichondrial cells. Light blue: OPN-Alexa647, green: Sox9-Alexa488, red: tdTomato, gray: DAPI and DIC. Scale bars: 50μm.

(D) Sagittal sections of *Msx2-creER*; *R26R*^{Tomato} cranial vault at E15.5 (tamoxifen at E9.5) stained for Sox9 and nuclei. UC: underlying cartilage. Green: Sox9-Alexa488, red: tdTomato, gray: DAPI and DIC. Scale bars: 50μm.

(E-G) Sagittal sections of *Msx2-creER*; *R26R*^{Tomato} cranial vault at E15.5 [tamoxifen at (E): E9.5, (F): E10.5, (G): E11.5] stained for nuclei. UC: underlying cartilage. Red: tdTomato, gray: DAPI and DIC. Scale bars: 200μm.



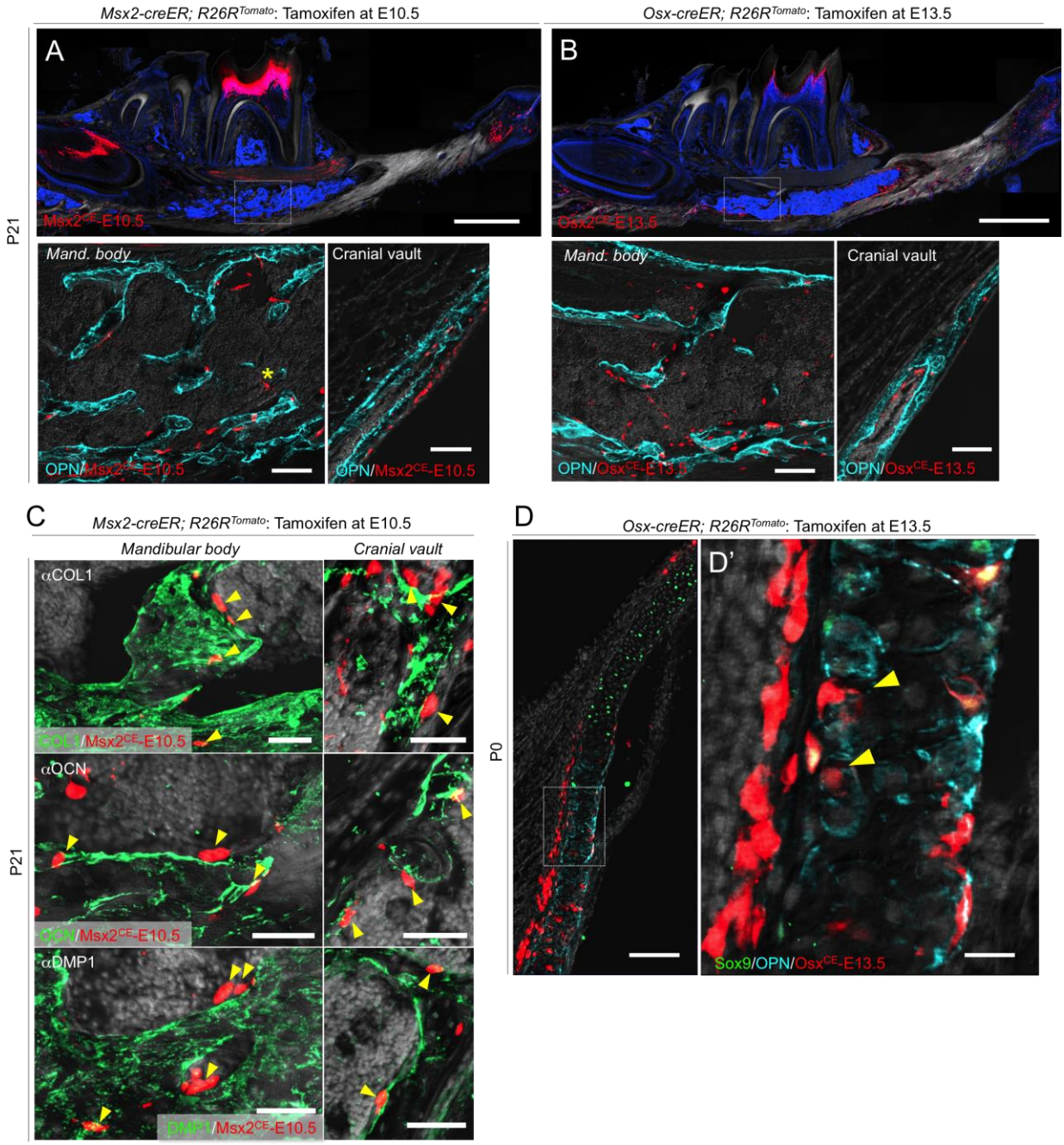
Appendix Figure 5. *Osx-creER* marks perichondrial cells of the transient cartilage in the fetal stage.

(A) Whole-mount image of *Osx-creER*; *R26R*^{Tomato} mice at E11.5 (tamoxifen at E10.5). Left: lateral view, right: front view of the head. Red: tdTomato. Scale bars: 1mm.

(B) Sagittal sections of *Osx-creER*; *R26R*^{Tomato} mandible at E13.5 (tamoxifen at E12.5) stained for Sox9 and nuclei. Me: Meckel's cartilage. Arrowheads: tdTomato^{+} perichondrial cells. Green: Sox9-Alexa488, red: tdTomato, gray: DAPI and DIC. Scale bars: 200μm.

(C, D) Sagittal sections of *Osx-creER*; *R26R*^{Tomato} cranial vault at E15.5 [tamoxifen at (C): E12.5, (D): E14.5] stained for Sox9, OPN and nuclei. UC: underlying cartilage. Arrows: superior domain of tdTomato^{+} perichondrial cells. Light blue: OPN-Alexa647, green: Sox9-Alexa488, red: tdTomato, gray: DAPI and DIC. Scale bars: 100μm.

(E) Sagittal sections of *Osx-creER*; *R26R*^{Tomato} mandible at E15.5 (tamoxifen at E14.5) stained for Sox9, OPN and nuclei. Me: Meckel's cartilage. Light blue: OPN-Alexa647, green: Sox9-Alexa488, red: tdTomato, gray: DAPI and DIC. Scale bars: 50μm.



Appendix Figure 6. Contribution of *Msx2-creER*-marked and *Osx-creER*-marked descendants to osteoblasts in postnatal development.

(A) Sagittal sections of *Msx2-creER*; *R26R*$^{\text{Tomato}}$ mandible at P21 (tamoxifen at E10.5) stained for OPN and nuclei. Lower panel: magnified view of the dotted area. Asterisk: tdTomato^{+}

reticular stromal cells in bone marrow. Light blue: OPN-Alexa647, red: tdTomato, gray: DAPI and DIC. Scale bars: 100µm.

(B) Sagittal sections of *Osx-creER*; *R26R*^{Tomato} mandible at P21 (tamoxifen at E10.5) stained for OPN and nuclei. Lower panel: magnified view of the dotted area. Light blue: OPN-Alexa647, red: tdTomato, gray: DAPI and DIC. Scale bars: 100µm.

(C) Sagittal sections of *Msx2-creER*; *R26R*^{Tomato} mandible at P21 (tamoxifen at E10.5) stained for nuclei and COL1 (upper panels), OCN (middle panels) or DMP1 (bottom panels). Arrowheads: osteoblasts/cytes on the bone surface and within the bone matrix. Green: COL1-Alexa488 (upper), OCN-Alexa488 (middle) or DMP1-Alexa488 (bottom), red: tdTomato, gray: DAPI and DIC. Scale bars: 50µm.

(D) Sagittal sections of *Osx-creER*; *R26R*^{Tomato} posterior cranial vault at P0 (tamoxifen at E13.5) stained for Sox9, OPN and nuclei, far posterior region. Inset: magnified view of the hypertrophic area. UC: underlying cartilage. Arrowheads: invading tdTomato^{+} cells. Light blue: OPN-Alexa647, green: Sox9-Alexa488, red: tdTomato, gray: DAPI and DIC. Scale bars: 100µm, 20µm (inset).