

# Growth Plate Borderline Chondrocytes Behave as Transient Mesenchymal Precursor Cells

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## ABSTRACT

The growth plate provides a substantial source of mesenchymal cells in the endosteal marrow space during endochondral ossification. The current model postulates that a group of chondrocytes in the hypertrophic zone can escape from apoptosis and transform into cells that eventually become osteoblasts in an area beneath the growth plate. The growth plate is composed of cells with various morphologies; particularly at the periphery of the growth plate immediately adjacent to the perichondrium are “borderline” chondrocytes, which align perpendicularly to other chondrocytes. However, in vivo cell fates of these special chondrocytes have not been revealed. Here we show that borderline chondrocytes in growth plates behave as transient mesenchymal precursor cells for osteoblasts and marrow stromal cells. A single-cell RNA-seq analysis revealed subpopulations of *Col2a1-creER*-marked neonatal chondrocytes and their cell type-specific markers. A tamoxifen pulse to *Pthrp-creER* mice in the neonatal stage (before the resting zone was formed) preferentially marked borderline chondrocytes. Following the chase, these cells marched into the nascent marrow space, expanded in the metaphyseal marrow, and became *Col(2.3 kb)-GFP<sup>+</sup>* osteoblasts and *Cxcl12-GFP<sup>high</sup>* reticular stromal “CAR” cells. Interestingly, these borderline chondrocyte-derived marrow cells were short-lived, as they were significantly reduced during adulthood. These findings demonstrate based on in vivo lineage-tracing experiments that borderline chondrocytes in the peripheral growth plate are a particularly important route for producing osteoblasts and marrow stromal cells in growing murine endochondral bones. A special microenvironment neighboring the osteogenic perichondrium might endow these chondrocytes with an enhanced potential to differentiate into marrow mesenchymal cells. © 2019 American Society for Bone and Mineral Research.

**KEY WORDS:** DEVELOPMENTAL MODELING; GENETIC ANIMAL MODELS; GROWTH PLATE; OSTEOBLASTS; STROMAL/STEM CELLS

## Introduction

Growth of bones and their marrow space requires a constant supply of mesenchymal cells to build the new stromal compartment. The growth plate can provide a substantial source of mesenchymal cells in endochondral ossification, which has been supported by a series of murine lineage-tracing studies using *cre/creER* transgenes active in chondrocytes and their close relatives, such as *Sox9-creER*, *type II collagen (Col2)-creER*, *aggrecan (Acan)-creER*, and *type X collagen (Col10)-cre/creER*.<sup>(1–3)</sup> Our lineage-tracing study using a *Pthrp-creER* line demonstrated that PTHrP<sup>+</sup> resting chondrocytes can establish columnar chondrocytes, some of which can further differentiate into osteoblasts and marrow stromal cells through chondrocyte-to-osteoblast transformation.<sup>(4)</sup> However, these columnar chondrocytes of the central growth plate can contribute only to a minor fraction of marrow mesenchymal cells in adulthood; therefore, chondrocytes in other locations may contribute more significantly in earlier stages. “Borderline” chondrocytes at the periphery of the growth plate in a special microenvironment immediately adjacent to the perichondrium<sup>(5)</sup> can produce osteoblast-like cells in cultured

conditions. In this study, we set out to reveal cellular heterogeneity of neonatal chondrocytes by a single-cell RNA-seq analysis and in vivo cell fates of borderline chondrocytes by taking advantage of a *Pthrp-creER* line.

## Materials and Methods

### Mice

*Pthrp-creER*,<sup>(4)</sup> *Cxcl12<sup>GFP/+</sup>*<sup>(6)</sup> mice have been described elsewhere. *Col1a1(2.3 kb)-GFP* (JAX013134), *Col2a1-creER* (JAX006774), *Rosa26-CAG-loxP-stop-loxP-tdTomato* (Ai14: *R26R-tdTomato*, JAX007914) mice were acquired from the Jackson Laboratory (Bar Harbor, ME, USA). FVB/N mice were acquired from the Charles River Laboratories (Wilmington, MA, USA). 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 7681 (Ono) and the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. All mice were housed in a specific pathogen-free condition, and analyzed in a mixed background.

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For neonatal experiments, tamoxifen (Sigma [St. Louis, MO, USA] T5648) or 4-hydroxytamoxifen (4-OHT, Sigma H6278) was dissolved in sunflower seed oil (Sigma S5007) and injected intraperitoneally (0.125 to 0.25 mg) shortly after birth. For prenatal experiments, tamoxifen and progesterone (Sigma P3972) were dissolved and injected intraperitoneally (4 mg each) at an indicated embryonic day. Mice were used for analysis regardless of the sex. No statistical method was used to predetermine sample size. Sample size was determined on the basis of previous literature and our previous experience to give sufficient standard deviations of the mean so as not to miss a biologically important difference between groups. The experiments were not randomized. All of the available mice of the desired genotypes were used for experiments. The investigators were not blinded during experiments and outcome assessment. One femur from each mouse was arbitrarily chosen for histological analysis. Genotypes were not particularly highlighted during quantification.

## Histology

Frozen sections were prepared using a cryostat (Leica [Buffalo Grove, IL, USA] CM1850) and adhered on positively charged glass slides (Fisherbrand ColorFrost Plus; ThermoFisher Scientific, Waltham, MA, USA). Sections were stained with anti-active Caspase-3 polyclonal (1:250; Promega [San Luis Obispo, CA, USA] PR-G7481), anti-Runx2 polyclonal (1:100; Novus Biologicals [Centennial, CO, USA] NBP1-89104) or anti-Fra1 polyclonal (1:100; Invitrogen [Carlsbad, CA, USA] PA5-40361) antibodies overnight at 4°C, followed by staining with appropriate Alexa Fluor 488/647-conjugated secondary antibodies for 3 hours at 4°C and DAPI (4',6'-diamidino-2-phenylindole, 5 µg/mL, Invitrogen D1306). In situ hybridization was performed with RNAscope 2.5 HD Reagent Kit Brown (Advanced Cell Diagnostics [Newark, CA, USA] 322300) using the following probes: *Col2a1* (407221), *Acan* (439101), and *Col10a1* (426181) according to the manufacturer's protocol. Sections were analyzed using an automated inverted fluorescence microscope with a structured illumination system (Zeiss [Thornwood, NY, USA] Axio Observer Z1 with ApoTome.2 system). 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 ×10. Serial sections spanning more than 1 mm thickness (up to 20 sections) of distal femurs were used for cell quantification.

## Single-cell RNA-seq analysis

Femur growth plate cells were dissociated enzymatically (Sigma/Roche, Liberase TM) and loaded onto a five-laser BD FACSAriaIII high-speed cell sorter with a 100-µm nozzle. Approximately 18,000 tdTomato<sup>+</sup> cells were loaded onto 10X Genomics Chromium 3' Single Cell Gene Expression Solution v3 (10X Genomics Inc., Pleasanton, CA, USA) microfluidics chip. The 10X Cell Ranger output matrix file was analyzed by Seurat R package<sup>(7)</sup> with cell cycle regression. For alignment purposes, we used a custom genome fasta and index file by including the sequences of *tdTomato-WPRE* (woodchuck hepatitis virus post-transcriptional regulatory element, a 3'UTR of the tdTomato sequence) to the mouse genome (mm10). The single-cell data set discussed in this publication are accessible in the NCBI's Gene Expression Omnibus under accession number GSE125464.

## Results

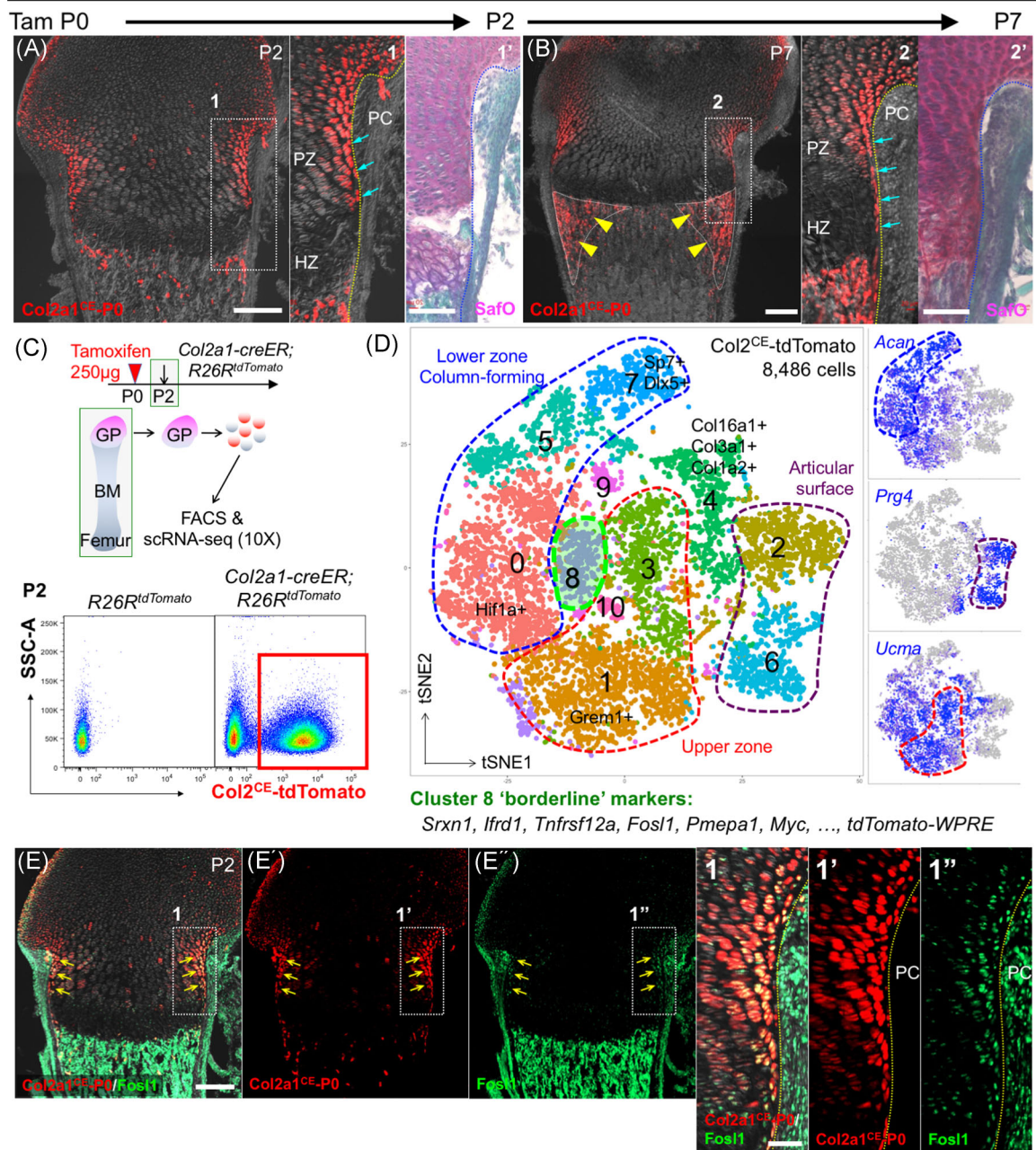
### Single-cell RNA-seq characterization of neonatal growth plate chondrocytes

*Col2a1-creER* marks a group of early skeletal progenitors in growing bones during the perinatal stage.<sup>(2)</sup> A tamoxifen pulse to *Col2a1-creER; R26R<sup>tdTomato</sup>* mice at postnatal day 0 (P0) predominantly marked chondrocytes at the periphery of the growth plate, including those termed as borderline chondrocytes located immediately adjacent to the perichondrium, shortly after tamoxifen injection at P2 (Fig. 1A). After a week of chase at P7, these chondrocytes continued to occupy the periphery of the growth plate in a borderline-enriched manner (Fig. 1B, arrows), and their descendants contributed to well-defined reverse triangular areas adjacent to the metaphyseal endosteal surface (Fig. 1B, arrowheads). Therefore, *Col2a1-creER* can mark a group of chondrocytes including precursors for borderline chondrocytes in the neonatal growth plate, which further contribute to mesenchymal cells in the marrow space. We utilized a single-cell RNA-seq analysis to define the characteristics of borderline chondrocytes. Flow cytometry analysis of cells dissociated from *Col2a1-creER; R26R<sup>tdTomato</sup>* femur growth plates at P2 (pulsed at P0) revealed that tdTomato<sup>+</sup> cells accounted for approximately two-thirds of harvested cells (Fig. 1C). We subsequently profiled 8486 *Col2a1<sup>CE</sup>-tdTomato<sup>+</sup>* single cells isolated by fluorescence-activated cell sorting (FACS) using the 10X Genomics Chromium platform. An initial *t*-distributed stochastic neighbor embedding (*t*-SNE) analysis revealed 11 clusters (Fig. 1D). Of the nine clusters of interest, we discovered three clusters of column-forming chondrocytes in the lower zone growth plate abundant in *Acan* (Clusters 0, 5, 7), two clusters of chondrocytes in the upper zone growth plate abundant in *Ucma* (Clusters 1, 3),<sup>(8,9)</sup> and two clusters of cells in the articular surface abundant in *Prg4* (Clusters 2, 6)<sup>(10)</sup> (Fig. 1D, Supplemental Fig. S1A). Interestingly, chondrocytes in Cluster 8 were abundant in *tdTomato-WPRE* and exhibited a borderline state between the upper and lower zone growth plate; their cell type-specific markers included *Srxn1*, *Ifrd1*, *Tnfrsf12a*, *Fos1*, *Pmepa1*, and *Myc* (Supplemental Fig. S1B). These cells expressed genes encoding secreted factors *Pthlh* (*Pthlh* is also known as *Pthrp*) and *Cxcl14* but not *Ihh*, *Grem1*, or *Cxcl12* (Supplemental Fig. S1A, C). Histological validation of an identified marker revealed that FOSL1 (also known as Fra1) was indeed expressed at a particularly high level by tdTomato<sup>bright</sup> chondrocytes at the periphery of the growth plate (Fig. 1E). Moreover, these cells did not express hypertrophic markers *Col10a1* or *Sp7* (Supplemental Fig. S1D). Therefore, these analyses reveal fundamental transcriptional heterogeneity and cellular diversity of neonatal growth plate chondrocytes.

### Pthrp-creER specifically marks borderline chondrocytes in the fetal and neonatal stage

*PTHrP* is initially expressed in the periarticular region of the fetal and neonatal growth plate and later in the resting zone of the postnatal growth plate.<sup>(4)</sup>

First, we set out to determine cell fates of fetal PTHrP<sup>+</sup> cells by in vivo lineage-tracing experiments. We injected tamoxifen to *Pthrp-creER; R26R<sup>tdTomato</sup>* mice carrying *Cxcl12<sup>GFP/+</sup>* reporter at embryonic day 12.5 (E12.5) and analyzed these mice at postnatal day 9 (P9) (Supplemental Fig. S2A). The descendant of



**Fig. 1.** Single-cell RNA-seq characterization of neonatal growth plate chondrocytes. (A, B) Cell-fate analysis of *Col2a1-creER*<sup>+</sup> neonatal growth plate chondrocytes (P0-pulsed) at P2 (A) and P7 (B). PC = perichondrium; PZ = proliferating zone; HZ = hypertrophic zone. Right panels: Safranin O and FastGreen staining of identical sections. Arrows = tdTomato<sup>+</sup> borderline chondrocytes; arrowheads = tdTomato<sup>+</sup> cells in peripheral metaphyseal marrow space. Scale bars = 200 µm (left panels), 100 µm (right panels). *n* = 3 mice for each experiment. (C, D) Single-cell RNA-seq analysis of *Col2a1-creER*<sup>+</sup> neonatal growth plate chondrocytes. (D) t-SNE-based visualization of major classes of FACS-sorted *Col2a1*<sup>CE</sup>-tdTomato<sup>+</sup> single cells at P2 (Cluster 0–10). Clusters 0, 5, 7: lower zone column-forming chondrocytes; Clusters 1, 3: upper zone chondrocytes; Clusters 2, 6: articular surface chondrocytes; Cluster 4: collagen-rich articular cells; Cluster 8: borderline chondrocytes; Clusters 9, 10: cells in cell cycle. Dots = individual cells; color = cell type. Green contour = borderline chondrocytes. Right panels: plots of *Acan*, *Prg4*, and *Ucma*. Blue = high expression; grey = no expression. *n* = 8486 cells. (E) In situ validation of the identified marker, *Fos1*. *Col2a1-creER*; *R26R*<sup>tdTomato</sup> distal femurs at P2 (P0-pulsed). (E', E''): tdTomato, *Fos1*-Alexa488 single channel, (1–1''): magnified views of the dotted area. Scale bars = 200 µm (E–E''), 50 µm (1–1''). *n* = 3 mice.

PTHrP<sup>+</sup> periarticular cells at E12.5 (PTHrP<sup>CE</sup>-E12.5 cells) became a small number of cells on the metaphyseal endosteal region (Supplemental Fig. S2B). Second, we pulsed these mice at later fetal stages at E14.5 or E17.5. Both PTHrP<sup>CE</sup>-E14.5 and

PTHrP<sup>CE</sup>-E17.5 cells became cells on the metaphyseal endosteal region, *Cxcl12*-GFP<sup>high</sup> stromal cells in the metaphyseal marrow space, and cells on the articular surface (Supplemental Fig. S2C, D). Therefore, *Pthrp-creER* marks precursors for metaphyseal

marrow mesenchymal cells during the fetal stage, while also marking precursors for articular chondrocytes in later fetal stages. These precursors might reside in the perichondrium, therefore representing a discrete population from borderline chondrocytes.

Second, we pulsed *Pthrp-creER*; *R26R<sup>tdTomato</sup>* mice carrying *Col1(2.3 kb)-GFP* reporter at birth (P0) and analyzed these mice at 48 hours later at P2. A small number of chondrocytes at the periphery of the growth plate, including "borderline chondrocytes" adjoining the perichondrium, was marked by tdTomato (PTHrP<sup>CE</sup>-P0 cells, 25.6 ± 5.8 tdTomato<sup>+</sup> cells per growth plate) (Fig. 2A). These PTHrP<sup>CE</sup>-P0 cells were localized within the cartilage, expressing *type II collagen (Col2a1)* (18/18 tdTomato<sup>+</sup> cells examined, 100%) and *aggrecan (Acan)* (21/23 tdTomato<sup>+</sup> cells examined, 91%); some of them also expressed *type X collagen (Col10a1)* mRNAs (4/25 tdTomato<sup>+</sup> cells examined, 16%) (Fig. 2B–D). PTHrP<sup>CE</sup>-P0 cells underwent apoptosis less frequently; only a small fraction of these tdTomato<sup>+</sup> cells were positive for activated Caspase 3 (4.4 ± 8.8% of PTHrP<sup>CE</sup>-P0 cells, in contrast to 27.0 ± 8.2% of hypertrophic chondrocytes) (Fig. 2E, Supplemental Fig. S3A). These tdTomato<sup>+</sup> cells were embedded in the cartilaginous matrix, with a fraction of them expressing transcription factors RUNX2 (13.8 ± 10.5%) (Fig. 2F, Supplemental Fig. S3B). Following the chase, PTHrP<sup>CE</sup>-P0 cells started to move into the metaphyseal marrow space (Supplemental Fig. S3C, Fig. 2G). To determine the cell cycle status of tdTomato<sup>+</sup> peripheral chondrocytes, we performed an EdU label-exclusion assay. *Pthrp-creER*; *R26R-tdTomato* mice were first pulsed with tamoxifen at P0, then serially pulsed with EdU for 3 days and analyzed at P3. A great majority of tdTomato<sup>+</sup> cells were resistant to EdU incorporation (Supplemental Fig. S3D, EdU<sup>+</sup>; 4.6 ± 4.4% of tdTomato<sup>+</sup> cells compared with 97.7 ± 0.7% of cells in the adjacent perichondrium, *n*=3 mice), indicating that most of these peripheral chondrocytes were not dividing. Therefore, borderline chondrocytes can passively translocate to the marrow space without involving active cell proliferation.

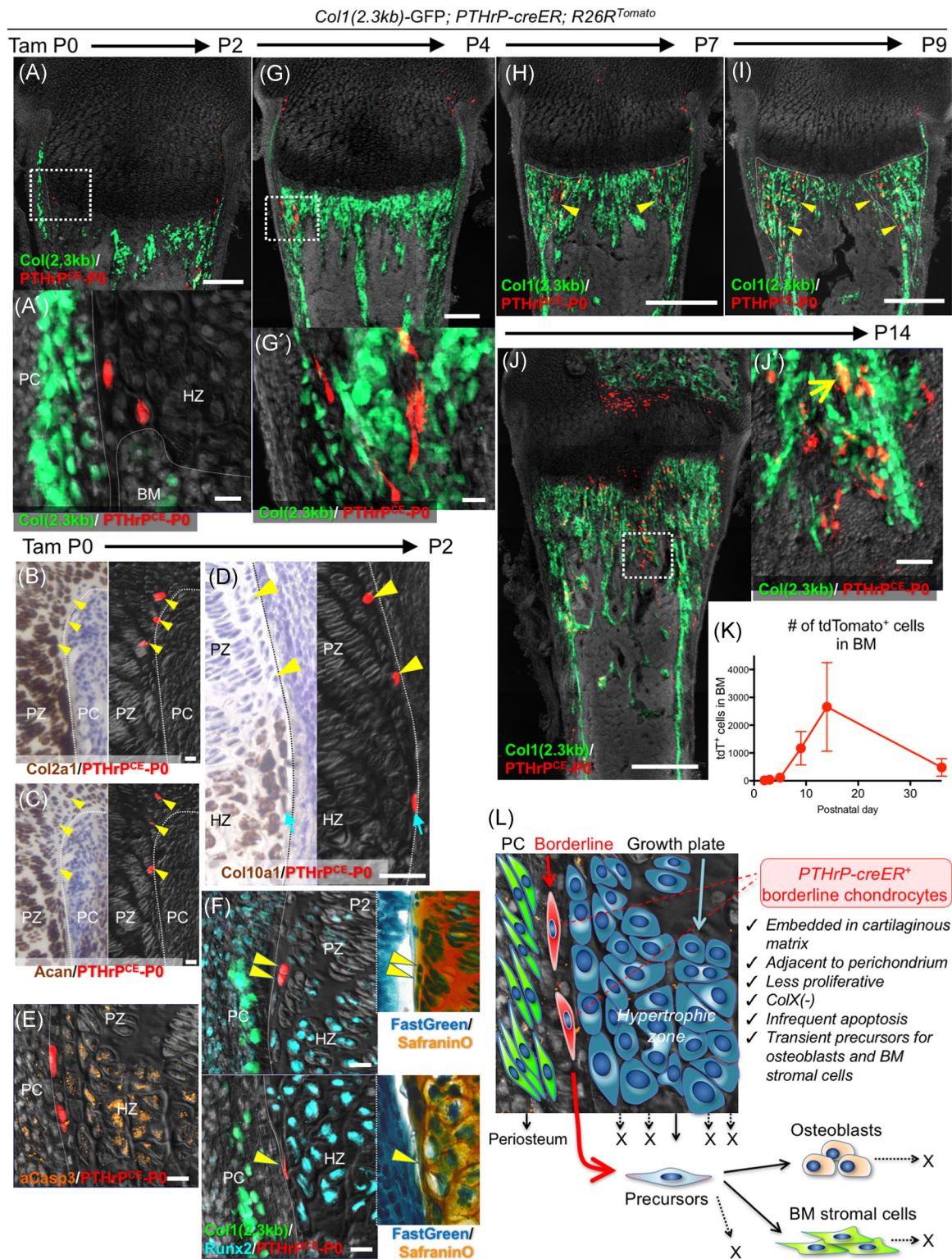
### Neonatal borderline chondrocytes behave as transient mesenchymal precursor cells

We interrogated the fates of PTHrP<sup>CE</sup>-P0 cells at sequential time points after the pulse. After 5 days of chase, PTHrP<sup>CE</sup>-P0 cells further moved into the endosteal region, some of which became Cxcl12-GFP<sup>high</sup> stromal cells in the adjacent marrow space (Supplemental Fig. S4A). After a week of chase at P7, PTHrP<sup>CE</sup>-P0 cells became Col1(2.3 kb)-GFP<sup>+</sup> osteoblasts on the metaphyseal trabecular bone surface and reticular stromal cells in the inter-trabecular marrow space (Fig. 2H). After 9 days of chase at P9, PTHrP<sup>CE</sup>-P0 cells occupied a well-defined reverse triangular area of the spongiosa at the corner of the hypertrophic zone and the endosteal surface, demonstrating that borderline chondrocytes can particularly contribute to marrow mesenchymal cells of the peripheral areas (Fig. 2I). After 2 weeks of chase at P14, PTHrP<sup>CE</sup>-P0 cells continued to become Col1(2.3 kb)-GFP<sup>+</sup> osteoblasts and Cxcl12-GFP<sup>high</sup> stromal cells in the metaphyseal marrow space (Fig. 2J, Supplemental Fig. S4B). Quantification of Col1(2.3 kb)-GFP<sup>+</sup>tdTomato<sup>+</sup> and Cxcl12-GFP<sup>high</sup>tdTomato<sup>+</sup> cells after being pulsed at P0 or P6 demonstrated that PTHrP<sup>CE</sup>-P0 cells, but not PTHrP<sup>CE</sup>-P6 cells, became osteoblasts and marrow stromal cells at this stage, indicating that these tdTomato<sup>+</sup> cells were derived from neonatal borderline chondrocytes (Supplemental

Fig. S4C). In addition, columnar chondrocytes derived from PTHrP<sup>CE</sup>-P0 cell resting chondrocytes had not yet reached the entire length of the growth plate at this stage. We further chased PTHrP<sup>CE</sup>-P0 cells for a longer period into adulthood. At 5 weeks of chase at P36, PTHrP<sup>CE</sup>-P0 cells sporadically remained as Col1(2.3 kb)-GFP<sup>+</sup> osteoblasts and Cxcl12-GFP<sup>high</sup> stromal cells in the metaphyseal marrow space, without any expansion of tdTomato<sup>+</sup> areas (Supplemental Fig. S4D, E), although we could no longer discriminate the origin of these cells at this stage because columnar chondrocytes originating from these resting chondrocytes had already reached the primary spongiosa. After 9.5 weeks of chase at P67, a vast majority of PTHrP<sup>CE</sup>-P0 cells disappeared from the marrow space, with only a small number of tdTomato<sup>+</sup> cells remaining on trabecular bone surfaces (Supplemental Fig. S4F). Quantification of tdTomato<sup>+</sup> cells in the marrow space revealed that PTHrP<sup>CE</sup>-P0 cells transiently increased their number in the marrow space at P14, then drastically decreased their number toward P36 (Fig. 2K). Taken together, these data suggest that PTHrP<sup>+</sup> borderline chondrocytes behave as mesenchymal precursor cells for osteoblasts and marrow stromal cells that transiently expand in the marrow space during the early phase of postnatal bone growth.

## Discussion

Our lineage-tracing study provides evidence that borderline chondrocytes in the fetal and neonatal growth plate can generate osteoblasts and marrow stromal cells in vivo in a mouse model. Further, our single-cell RNA-seq analysis reveals cellular identity of these special chondrocytes. Chondrocytes at the periphery of the growth plate appear to represent an important pathway to generate marrow mesenchymal cells particularly in rapidly growing endochondral bones (Fig. 2L); these cells go through a unique state expressing *Col10a1* without hypertrophy on their way to becoming mesenchymal cells in the marrow space. In combination with our previous finding that hypertrophic chondrocytes in the central growth plate can produce marrow mesenchymal cells predominantly in the adult stage,<sup>(4)</sup> we speculate that different routes of chondrocytes are utilized to generate osteoblasts and marrow stromal cells in a stage-specific manner. Additionally, our data indicate that *Pthrp-creER<sup>+</sup>* borderline chondrocytes represent a non-self-renewing subset of *Col2a1-creER<sup>+</sup>* chondrocytes; we observed that borderline chondrocyte-derived marrow mesenchymal cells could only transiently persist within the marrow space. More immature Col2a1<sup>+</sup>PTHrP<sup>high</sup> chondrocytes within the growth plate such as Grem1<sup>+</sup> cells in the upper zone,<sup>(11)</sup> rather than immature perichondrial cells, are likely to serve as precursors for PTHrP<sup>+</sup> borderline chondrocytes that continue to feed into the nascent marrow space. Additionally, *Pthrp-creER* can mark perichondrial precursor cells during the fetal stage. Marked by the same *Pthrp-creER* transgene at different time points, the characteristics of PTHrP<sup>+</sup> borderline chondrocytes appear to be fundamentally different from those of PTHrP<sup>+</sup> resting chondrocytes that can self-renew long term within the resting zone. We speculate that differentiation of borderline chondrocytes occurs more effectively than transdifferentiation of hypertrophic chondrocytes in generating mesenchymal cells of the marrow space. How PTHrP<sup>+</sup> chondrocytes at the periphery and the center are functionally different needs to be addressed through further studies.



**Fig. 2.** *Pthrp-creER<sup>+</sup>* borderline chondrocytes in the neonatal stage behave as transient mesenchymal precursor cells. Cell-fate analysis of *Pthrp-creER<sup>+</sup>* borderline chondrocytes (P0-pulsed) at P2 (A–F), P4 (G), P7 (H), P9 (I), and P14 (J). (A, F–J) *Col1(2.3 kb)-GFP; Pthrp-creER; R26R<sup>tdTomato</sup>* distal femurs; (B–E) *Pthrp-creER; R26R<sup>tdTomato</sup>* distal femurs. (A', G', J) Magnified views of the dotted areas. In B–D, left panels (bright field) and right panels (dark field) represent the identical sections. Right panels in F: Safranin O and FastGreen staining of left panels. Arrowheads in B–F: tdTomato<sup>+</sup> borderline chondrocytes denoting identical cells. PC = perichondrium; PZ = proliferating zone; HZ = hypertrophic zone. Arrowheads in H, I: tdTomato<sup>+</sup> cells in peripheral metaphyseal marrow space; arrow in J: GFP<sup>+</sup>tdTomato<sup>+</sup> osteoblasts. Scale bars = 200 μm (A, G), 500 μm (H–J), 20 μm (A–F), 50 μm (J'). n = 3 mice for each experiment. (K) Quantification of tdTomato<sup>+</sup> marrow cells at each time point. n = 6 (P2), n = 4 (P4), n = 3 (P5, P9, P14, P36) mice; data are presented as mean ± SD. (L) Concluding diagram. Borderline chondrocytes in growth plates can translocate into marrow space and behave as transient precursors for osteoblasts and stromal cells by escaping hypertrophy and apoptosis.

## Disclosures

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All authors state that they have no conflicts of interest.

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Authors' roles: Study design and conduct; data collection; analysis and interpretation: KM, MN, YM, and NO. Drafting manuscript: KM and NO. Revising manuscript content: WO. Approving final version of manuscript: KM, MN, YM, WO, and NO. NO takes responsibility for the integrity of the data analysis.

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