A subset of chondrogenic cells provides early mesenchymal progenitors in growing bones

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The hallmark of endochondral bone development is the presence of cartilaginous templates, in which osteoblasts and stromal cells are generated to form mineralized matrix and support bone marrow haematopoiesis. However, the ultimate source of these mesenchymal cells and the relationship between bone progenitors in fetal life and those in later life are unknown. Fate-mapping studies revealed that cells expressing cre-recombinases driven by the collagen II (Col2) promoter/enhancer and their descendants contributed to, in addition to chondrocytes, early perichondral precursors before Runx2 expression and, subsequently, to a majority of osteoblasts, Ccl2 (chemokine (C–X–C motif) ligand 12)-abundant stromal cells and bone marrow stromal/mesenchymal progenitor cells in postnatal life. Lineage-tracing experiments using a tamoxifen-inducible creER system further revealed that early postnatal cells marked by Col2–creER, as well as Sox9–creER and aggrecan (Acan)–creER, progressively contributed to multiple mesenchymal lineages and continued to provide descendants for over a year. These cells are distinct from adult mesenchymal progenitors and thus provide opportunities for regulating the explosive growth that occurs uniquely in growing mammals.

Most bones are formed through endochondral bone development, in which mesenchymal condensations first define the domain for the future bones, and then develop into the growth cartilage wherein chondrocytes appear and proliferate. Chondrocytes in the growth plate continue to proliferate well into adulthood in mice. In the centre of the developing cartilage mould, chondrocytes stop proliferating and become hypertrophic chondrocytes. These cells signal to induce the migration of mesenchymal cells into the marrow space; these cells then differentiate into osteoblasts that then form bone on top of the cartilaginous matrix. Perichondrial precursors expressing osterix (Osx) invade into the cartilage template along with blood vessels and eventually become both osteoblasts and stromal cells in the marrow space. However, mesenchymal cells constituting earlier cells of the osteoblast lineage than Osx+ precursors in vivo have not been fully characterized. The transcription factor Sox9 is expressed in mesenchymal condensations, and osteochondroprogenitors are derived from Sox9+ cells, as Sox9–cre marks all chondrocytes and osteoblasts. Sox9 binds to genes encoding major cartilaginous matrix proteins such as aggrecan (Acan) and type II collagen-α1 (Col2a1) and regulates their expression. How these early osteochondroprogenitors and their descendants relate to mesenchymal precursors in adult bone is unknown. In adult endochondral bones, the source of osteoblasts and stromal cells has been proposed to be mesenchymal stem cells (MSCs) or bone marrow stromal/mesenchymal progenitor cells (BMSCs), which are traditionally defined as cells capable of forming colonies in vitro (CFU-Fs: colony-forming unit fibroblasts) that can undergo multilineage differentiation in vitro and on transplantation. CFU-Fs are enriched among various adult marrow populations such as nestin (Nes)–GFP+ cells, platelet-derived growth factor receptor-α (PDGFRα)+ Sca1+CD45−Ter119− (PaS) cells and leptin receptor (LepR)/LepR+cre+ cells in mice and CD146+ pericytes in humans. In this study, we sought to identify cells that robustly supply osteoblasts and stromal cells in the metaphyses of growing bones and suggest the possibility that these cells might be the source of MSCs/BMSCs in adult bone marrow.

RESULTS

Col2–creERCol2–creER are expressed in early cells of the osteoblast lineage in fetal mice

We chose to explore the hypothesis that cells defined by activities of the Sox9, aggrecan (Acan) and type II collagen (Col2) gene promoter/enhancers might encompass mesenchymal precursors of osteoblasts and stromal cells. Previous studies indicate that osteochondroprogenitors are marked by cre recombinases driven by...
the Col2a1 promoter10–12. First, we mapped cell fates using a Col2–cre; R26R–tdTomato reporter13,14 in combination with Col2(2.3kb)–GFP (ref. 15) as a readout of osteoblastic cells. In this system, cells expressing Col2–cre and their descendants become red, and if they concurrently express Col1–GFP, they become yellow. In addition, a thymidine analogue, EdU, was administered shortly before analysis to evaluate cell proliferation. At embryonic day 12.5 (E12.5), red cells were observed in the growth cartilage (Fig. 1a, asterisks) and perichondrium (Fig. 1a, sharpars), where many of these cells were proliferating (see also Supplementary Fig. 1a for an earlier day). At E14.5, almost all Col1–GFP+ cells appearing in the osteogenic perichondrium were yellow (Fig. 1b, arrowheads); thus, these perichondrial preosteoblasts were derived from Col2–cre+ cells. Active cell proliferation was observed in the perichondrium, but not in its adjacent hypertrophic chondrocytes (Fig. 1b). At E15.5, Col2–cre-derived cells proliferated within the marrow space, as the nascent primary ossification centre was occupied by red cells (Fig. 1c, asterisks).

Second, we mapped cell fates using an Osx–cre::GFP; R26R–tdTomato reporter16. In this system, cells expressing Osx–cre::GFP become green in the nucleus, and these cells and their descendants become red. At E12.5, Osx+ yellow cells (expressing Osx–cre::GFP and tdTomato) were observed in the growth cartilage and perichondrium, in a domain more restricted than that of Col2–cre-targeted cells (Fig. 1d, arrows). At E14.5, Osx+ yellow cells dominated the inner part of the perichondrium in a domain broader than Col1+ cells seen in Fig. 1b (Fig. 1e, arrows), with some of them in proliferation (Supplementary Fig. 1f,g). Osx+ prehypertrophic chondrocytes appearing green were not proliferating (Fig. 1e, arrowheads). At E15.5, mesenchymal cells appearing in the primary ossification centre were largely yellow (Fig. 1f, asterisks), and therefore expressing Osx. These comparative fate-mapping analyses suggest that one fate of Col2+ cells may be to become Osx+ cells in the perichondrium and the marrow space.

Runx2 is a crucial transcription factor in osteoblastic differentiation genetically upstream of Osx (ref. 17). To understand whether Col2+ cells require Runx2 expression, as for example, nestin–GFP+ perichondrial cells do18, we analysed mice carrying Runx2-null alleles18 in addition to the Col2–cre; R26R–tdTomato; Col1–GFP transgenes. In these mice, no Col1–GFP+ cell appeared in the perichondrium at E14.5. However, despite the absence of Runx2, Col2–cre-derived red cells were still present in the perichondrium (Fig. 1g, arrows) and its underlying growth cartilage (Fig. 1g right panel, arrowheads). Therefore, Runx2 is dispensable for generating these Col2–cre-derived progenitor cells, although it is required for their further differentiation into osteoblasts. This places Col2+ cells in the perichondrium upstream of Runx2 expression in the lineage.

We next examined the distribution of Col2–cre–targeted red cells at postnatal day 3 (P3), when bone marrow haematopoiesis had been established. Col2–cre–targeted red cells contributed not only to chondrocytes and perichondrial cells in the growth cartilage, but also to Col1–GFP+ osteoblasts, osteocytes and Cxcl12+GFP+ stromal cells20,21 in the marrow space (Fig. 1h,i, see also Supplementary Fig. 1b,c). Osx–cre–targeted red cells contributed to all of these cell types22,23 (Fig. 1j,k, see also Supplementary Fig. 1d,e). Flow cytometry analysis of dissociated bone cells revealed that Osx–cre–targeted cells contributed to essentially all osteoblasts (95.5 ± 0.7% of Col1–GFPhigh and 97.8 ± 1.3% of Oc–GFPhigh; ref. 15) and Cxcl12-expressing stromal cells (92.1 ± 6.9% of Cxcl12–GFPhigh, n = 3 per group, data are presented as mean ± s.d.; ref. 24) (Fig. 1m). Col2–cre–targeted cells also contributed to a great majority of osteoblasts (80.0 ± 2.8% of Col1–GFP+ and 78.1 ± 5.9% of Oc–GFP+) and Cxcl12-expressing stromal cells (89.6 ± 5.2% of Cxcl12–GFPhigh, n = 3 per group, data are presented as mean ± s.d.) (Fig. 1l). Therefore, most osteoblasts and Cxcl12-expressing stromal cells in endochondral bones are derived from mesenchymal progenitors/precursors that express Col2–cre and Osx–cre at some point in their development.

To further clarify the relationships between Col2+ cells and Osx+ cells within the osteogenic lineage, we took advantage of tamoxifen-inducible creER recombinases (Col2–creER (ref. 25) or Osx–creER (ref. 2)) and performed pulse-chase experiments with a single tamoxifen injection. An E11.5 pulse to Col2–creER mice marked perichondral cells and chondrocytes at E12.5 (Fig. 2a), and their descendants (Col2creER–E11.5) contributed to the perichondrium and the primary ossification centre at E15.5 (Fig. 2b) and yielded a number of tdTomato+ cells throughout the bone at P0 (Fig. 2c) and P21 (Supplementary Fig. 2a). In contrast, an E11.5 pulse to Osx–creER mice did not give rise to descendants at P0 (Fig. 2d), suggesting that Col2–creER+ cells appeared earlier than Osx–creER+ cells during early skeletal development. An E13.5 pulse to Col2–creER mice marked chondrocytes beneath the perichondrium, as well as perichondral cells, at E14.5 (Fig. 2e), and their descendants (Col2creER–E13.5) contributed to the primary ossification centre at E16.5 (Fig. 2f). Col2creER–E13.5 cells continued to yield tdTomato+ cells robustly in the growth cartilage, the perichondrium and the bone at P0 (Fig. 2g) and including the secondary ossification centre in the epiphysial region at P21 (Fig. 2h). Osx–creER+ cells at E13.5 proliferate in the primary ossification centre at E16.5 (ref. 2) but do not persist in the perichondrium18. Their descendants (OsxcreER–E13.5) appeared as osteoblasts and stromal cells among cells derived from the primary ossification centre, but not those of the secondary ossification centre at P0 (Fig. 2i), and then gradually disappeared from the metaphysis by P21 (Fig. 2j and Supplementary Fig. 2b). These data underscore the transient nature of embryonic Osx–creER+ cells, supporting the notion that these cells are replenished by their precursors, probably derived from Col2–creER cells during early bone development.

Col2–creER-expressing cells at an early postnatal stage generate multiple mesenchymal lineages

As cells expressing Col2–creER in fetal life seem to be early cells in the osteoblast lineage, we next investigated whether this transgene is also active in postnatal life. For this purpose, Col2–creER; R26R–tdTomato26 mice were pulsed with a low-dose tamoxifen injection at P3. A small number of tdTomato+ cells were detected at P5 and P10 in the absence of tamoxifen administration (Supplementary Fig. 2d,e). Two days after a tamoxifen pulse at P3 (Col2creER–P3), chondrocytes in the growth plate and beneath the perichondrium, as well as cells in the perichondrium and metaphyseal spongiosa, were labelled by Col2–creER (Fig. 4a). After a week, a group of Col2creER–P3 cells appeared directly under the growth plate in the primary spongiosa and beneath the unlabelled cells of the perichondrium with further contiguity to the endocortical surface (Fig. 3a, arrowheads, see...
Figure 1 Fate mapping of Col2-cre<sup>+</sup> and Osx-cre<sup>+</sup> cells during endochondral ossification. (a-c) Fate mapping of Col2-cre<sup>+</sup> cells was performed using Col1-GFP; Col2-cre; R26R<sup>Tomato</sup> mice, with EdU administration before analysis (a). E12.5; sharps: Tomato<sup>+</sup> perichondrial cells, asterisks: Tomato<sup>+</sup> chondrocytes. (b) E14.5; arrowheads: Col1<sup>+</sup>Tomato<sup>+</sup> perichondrial cells. (c) E15.5; asterisks: Tomato<sup>+</sup> cells in primary ossification centre. Green: EGFP, red: tdTomato, blue: Alexa647, grey: DAPI. Scale bars: 100 μm.

(d-f) Fate mapping of Osx-cre<sup>+</sup> cells was performed using Osx-cre::GFP; R26R<sup>Tomato</sup> mice, with EdU administration before analysis (e,f). (d) E12.5; arrows: Osx<sup>+</sup> perichondrium. (e) E14.5; arrows: Osx<sup>+</sup> Col1<sup>+</sup> perichondrium, arrowheads: Osx<sup>+</sup> prehypertrophic chondrocytes. (f) E15.5; asterisks: Osx<sup>+</sup> cells in primary ossification centre. Green: EGFP, red: tdTomato, blue: Alexa647, grey: DAPI. Scale bars: 100 μm.

(g) Fate mapping of Col2-cre<sup>+</sup> cells in Runx2-deficient bone anlage at E14.5 was performed using Col1-GFP; Col2-cre; R26R<sup>Tomato</sup>; Runx2<sup>–/–</sup> mice. Middle and right panels: dotted area of left panel revealing perichondrium. Arrows: Tomato<sup>+</sup> perichondrial cells, arrowheads: Tomato<sup>+</sup> chondrocytes beneath perichondrium. Green: EGFP, red: tdTomato, grey: DAPI and DIC (differential interference contrast). Scale bars: 100 μm (left panel) and 10 μm (centre and right panels).

(h,j) Comparative fate mapping in P3 bones was performed using Col1-GFP; Col2-cre; R26R<sup>Tomato</sup> (h) and Col1-GFP; Osx-cre; R26R<sup>Tomato</sup> (j) mice. Green: EGFP, red: tdTomato, blue: DAPI. Scale bars: 500 μm.

(i,k) Comparative fate mapping in P3 bones was performed using Ocx12GF; Col2-cre; R26R<sup>Tomato</sup> (i) and Ocx12GF; Osx-cre; R26R<sup>Tomato</sup> (k) mice. Shown are diaphyseal endocortices and bone marrows, stained for CD31. Green: EGFP, red: tdTomato, blue: Alexa633, grey: DAPI. Scale bars: 50 μm.

(l,m) Flow cytometry analysis was performed using dissociated bone cells collected at P3. The CD45<sup>–</sup> fraction was gated for GFP. (l) Representative dot plots of cells from Col2-cre<sup>+</sup> mice that also carry Col1-GFP (left middle panel), Oc-GFP (right middle panel) or Ocx12-GFP (rightmost panel). Leftmost panel: no GFP control. Lower panels: histograms of the GFP<sup>+</sup> fraction developed for Tomato; blue lines: GFP<sup>+</sup>Tomato<sup>–</sup> control cells. (m) Representative dot plots of cells from Osx-cre::GFP; R26R<sup>Tomato</sup> mice that also carry Col1-GFP (left middle panel), Oc-GFP (right middle panel) or Ocx12-GFP (rightmost panel). Leftmost panel: Osx-GFP only control. Lower panels: histograms of the GFP<sup>+</sup> fraction developed for Tomato; blue lines: GFP<sup>+</sup>Tomato<sup>–</sup> control cells. Upper panels: x axis: tdTomato, y axis: GFP. Lower panels: x axis: tdTomato. n=3 mice per group. All data are presented as mean ± s.d.
Figure 2  Col2-creER marks cells earlier than Osx-creER+ cells in the osteoblast lineage in fetal mice. (a,b) Pregnant mice received 1 mg tamoxifen at E11.5, and Col1-GFP; Col2-creER; R26R<sup>Tomato</sup> mice were chased for 24 h (a; E12.5) or 4 days (b; E15.5). Shown are distal halves of growth cartilages. Perichondrium is on top. Femur sections were stained for nuclei and CD31. Arrows: Tomato+ perichondrial cells, asterisks: Tomato+ cells in primary ossification centre. Green: EGFP, red: tdTomato, blue: Alexa633, grey: DAPI. (c,d) Lineage-tracing of embryonic Col2-creER<sup>-</sup> and Osx-creER<sup>-</sup> cells was performed by injecting 1 mg tamoxifen into pregnant mice at E11.5, and Col2-creER; R26R<sup>Tomato</sup> (c) or Osx-creER; R26R<sup>Tomato</sup> (d) mice were chased until P0. Shown are femur sections with the distal side on the left. Col2<sup>-creER</sup>-E11.5 represents descendants of Col2<sup>-creER</sup> cells at E11.5. White indicates tdTomato. (e,f) Pregnant mice received 1 mg tamoxifen at E13.5, and Col2-creER; R26R<sup>Tomato</sup> mice were chased for 24 h (e; E14.5) or 3 days (f; E16.5). Femur sections were stained for nuclei and CD31. Asterisks: Tomato+ chondrocytes, sharps: Tomato+ cells in primary ossification centre. Red: tdTomato, blue: Alexa633, grey: DAPI. (g-j) Lineage-tracing of embryonic Col2-creER<sup>-</sup> and Osx-creER<sup>-</sup> cells was performed by injecting 1 mg tamoxifen into pregnant mice at E13.5, and Col2-creER; R26R<sup>Tomato</sup> (g,h) or Osx-creER; R26R<sup>Tomato</sup> (i,j) mice were chased until the indicated postnatal day (P0 or P21). Shown are femur sections with the distal side on the left. Col2<sup>-creER</sup>-E13.5 represents descendants of Col2<sup>-creER</sup> cells at E13.5. White indicates tdTomato. Scale bars: 100 μm (a,b,e,f) or 500 μm (c,d,g-j).

Also Fig. 4b). After a month, Col2<sup>-creER</sup>-P3 cells became osteoblasts and stromal cells in the metaphysis and epiphysis, in addition to chondrocytes in the growth plate and articular cartilage (Fig. 3b, see also Fig. 4c). During an extended chase period over a year after the pulse, Col2<sup>-creER</sup>-P3 cells continued to yield chondrocytes, osteoblasts and stromal cells in the metaphysis, and also became adipocytes in the metaphyseal bone marrow (Fig. 3c,d and Supplementary Fig. 2f). Of note, Col2-creER does not mark marrow cells in the middle of the diaphysis at P3, even though Col2-cre marks all stromal and osteoblastic regions of bone. Presumably, this contrast results from the Col2 promoter driving the expression of creER no longer being active in marrow stromal cells in the diaphysis at P3.
Figure 3 Col2-creER<sup>C</sup> cells encompass early cells of the osteoblast lineage in postnatal mice. (a–h) Lineage-tracing of postnatal Col2-creER<sup>C</sup> and Osx-creER<sup>C</sup> cells was performed by injecting 0.1 mg tamoxifen into P3 mice. Col2-creER; R26R<sup>Tomato</sup> (a–d) or Osx-creER; R26R<sup>Tomato</sup> (e–h) mice were chased for a week (a,e), a month (b,f), a year (c,g) and 18 months (d,h). Shown are femur sections with the distal side on the left. Col2<sup>creER-P3</sup> represents descendants of Col2-creER<sup>C</sup> cells at P3. Arrowheads in a point to Col2<sup>creER-P3</sup> cells under the growth plate and beneath the perichondrium. White indicates tdTomato. (i–l) No-tamoxifen controls of Col2-creER; R26R<sup>Tomato</sup> (i,j) or Osx-creER; R26R<sup>Tomato</sup> (k,l) mice at 1 month of age (i,k) or 1 year of age (j,l). Shown are femur sections with the distal side on the left. White indicates tdTomato. (m) Osx-creER; R26R<sup>Tomato</sup> mice received 0.1 mg tamoxifen at P3 and were chased for a year. Shown are higher-magnification views of g. Osx<sup>creER-P3</sup> represents descendants of Osx-creER<sup>C</sup> cells at P3. Left and right two panels show the magnified views of the metaphysis and diaphysis, respectively. Subpanels on the right show a single-colour view of tdTomato. Blue indicates DAPI and red and white indicate tdTomato. Scale bars: 1 mm (a–l) or 500 μm (m).
Figure 4 Col2–creER<sup>R</sup><sup>O</sup><sup>b</sup> cells generate multiple mesenchymal lineages in postnatal growing bones. (a,b) Col1–GFP; Col2–creER; R26R<sup>T</sup> mice received 0.1 mg tamoxifen at P3 and were chased for 48 h (a) or a week (b). Shown are distal halves of growth cartilages at the junction of growth plate and primary spongiosa. Perichondrium is on top. Femur sections were stained for nuclei and CD31. Asterisks: Tomato<sup>+</sup> chondrocytes, sharps: Tomato<sup>+</sup> perichondrial cells, arrows: Tomato<sup>+</sup> cells in primary spongiosa, arrowheads: a group of Tomato<sup>+</sup> cells directly under the growth plate and beneath the perichondrium. (c,d) Cxcl12–GFP; Osx–creER; R26R<sup>Tomato</sup> mice received 0.1 mg tamoxifen at P3 and were chased for 48 h (c) or a week (d). Shown are distal halves of growth cartilages at the junction of growth plate and primary spongiosa. Perichondrium is on top. Femur sections were stained for nuclei and CD31. Arrows: Tomato<sup>+</sup> perichondrial cells, asterisks: Tomato<sup>+</sup> osteoblasts, arrowheads: GFP<sup>+</sup> Tomato<sup>+</sup> stromal cells. (e,f) Cxcl12–GFP; Col2–creER; R26R<sup>Tomato</sup> (e) or Cxcl12–GFP; Oox–creER; R26R<sup>Tomato</sup> (f) mice received 0.1 mg tamoxifen at P3 and were chased for a month. Shown are metaphyseal spongiosa (left panel) and diaphyseal endocortex (right panel). Femur sections were stained for nuclei and CD31. Asterisks: Tomato<sup>+</sup> osteoblasts, arrowheads: GFP<sup>+</sup> Tomato<sup>+</sup> stromal cells. For a-f, green: EGFP, red: tdTomato, blue: Alexa633, grey: DAPI. Scale bars: 100 μm. (g,h) Flow cytometry analysis was performed using dissociated bone cells collected from mice that received 0.1 mg tamoxifen at P3 (except no-tamoxifen controls) and were chased for the indicated periods. The CD45<sup>-</sup> fraction was gated for GFP. (g) Representative dot plots of cells from Col1–GFP; Col2–creER; R26R<sup>Tomato</sup> (upper panels) and Cxcl12–GFP; Col2–creER; R26R<sup>Tomato</sup> (lower panels) mice. (h) Representative dot plots of cells from Col1–GFP; Oox–creER; R26R<sup>Tomato</sup> (upper panels) and Cxcl12–GFP; Oox–creER; R26R<sup>Tomato</sup> (lower panels) mice. Left panels: 2 day chase, middle panels: 4 week chase, right panels: no-tamoxifen controls. x axis represents tdTomato, and y axis represents GFP. (i,j) Percentage of Col2<sup>in<sup>+</sup></sup>-P3 cells (i) or Oox<sup>in<sup>+</sup></sup>-P3 cells (j) among Col1–GFP<sup>+</sup> (blue lines) osteoblasts or Cxcl12–GFP<sup>+</sup> (red lines) stromal cells during the chase. x axis represents the duration of the chase, and y axis represents the percentage of Tomato<sup>+</sup> cells among total GFP<sup>+</sup> cells. Dashed lines represent no-tamoxifen controls. n = 3–6 mice per group (see Methods for precise n values). All data are presented as mean ± s.d.
ARTICLES

The contrast between the cells marked by Col2–creER and Osx–creER transgenes is instructive. Shortly after the pulse, most osteoblasts on the bone surface and cells in the perichondrium were marked by Osx–creER (Fig. 4c). After a week of chase, OsxcreER-P3 cells continued to be present on the bone surface and in the marrow space (Fig. 3e, see also Fig. 4d). After a month, OsxcreER-P3 cells became osteoblasts and stromal cell in the metaphysis and diaphysis, but not in the epiphysis (Fig. 3f, see also Fig. 4f). In contrast to the persistence of Col2creER-P3 cells in the one-year chase, OsxcreER-P3 cells gradually disappeared from the metaphysis and became increasingly present as stromal cells in the diaphyseal bone marrow and osteoblasts on the endocortical surface (Fig. 3g,h,m and Supplementary Fig. 2c). The Osx–creER; R26R–tdTomato system had little to no tamoxifen-independent activities (Fig. 3k,l). Thus, Osx–creER+ cells at P3 generate cells in the metaphysis only transiently, but persist in the marrow stroma for a prolonged period.

To assess quantitatively how cells marked by Col2–creER or Osx–creER contribute differentially to defined cell types over time, we performed flow cytometry analysis of cells isolated from enzymatically digested bones from triple transgenic mice (Col1–GFP or Cxcl12–GFP, Col2–creER or Osx–creER and R26R–tdTomato) with a single tamoxifen injection at P3. Shortly after the pulse, only a small fraction of cells marked by Col2–creER expressed these GFP reporters (48 h after tamoxifen injection, Col1–GFP+; 3.2 ± 1.2% (n = 4), Cxcl12–GFPhigh, 0.3 ± 0.1% (n = 3), data are presented as mean ± s.d.), suggesting that Col2–creER+ cells were largely distinct from osteoblasts or Cxcl12-expressing stromal cells (Fig. 4g, left panels). The fraction of Col2creER-P3 cells among osteoblasts and Cxcl12-expressing stromal cells increased progressively as the chase period extended (Col1–GFP+: from 8.7 ± 2.1% (n = 3), 23.7 ± 1.7% (n = 5), 27.6 ± 1.7% (n = 5) to 29.3 ± 4.6% (n = 6), Cxcl12–GFPhigh: from 1.9 ± 0.3% (n = 4), 7.3 ± 3.1% (n = 4), 9.9 ± 1.2% (n = 4) to 13.8 ± 0.5% (n = 4), for the first, second, third and fourth week, respectively, Fig. 4g, middle panels, and Fig. 4i, blue and red lines, data are presented as mean ± s.d.). No increase of tdTomato+ cells was observed without tamoxifen injection (Fig. 4g, right panels). In contrast, 87.9 ± 1.4% (n = 4) and 11.6 ± 3.5% (n = 3) of cells marked by Osx–creER shortly after the pulse were Col1–GFP+ osteoblasts and Cxcl12-expressing stromal cells, respectively (Fig. 4h, left panels, data are presented as mean ± s.d.). The fraction of OsxcreER-P3 cells decreased among osteoblasts (58.1 ± 10.9% (n = 7) and 14.9 ± 0.1% (n = 3) for the first and fourth week, respectively, Fig. 4h, upper middle panel, and Fig. 4j, blue line), and increased among Cxcl12-expressing stromal cells (49.2 ± 6.3% (n = 3) and 32.9 ± 1.1% (n = 4) for the second and fourth week, respectively, Fig. 4h, lower middle panel, and Fig. 4j, red line, data are presented as mean ± s.d.). These studies reinforce the results from the histological findings that Col2–creER-marked cells robustly contribute to osteoblasts and stromal cells, whereas Osx–creER-marked cells transiently become osteoblasts and persist longer in bone marrow as stromal cells.

Relationship of growth skeletal progenitor cells and adult bone marrow stromal/mesenchymal progenitor cells

To determine the relationship of the growth skeletal stem/progenitor cells that we have defined here with the more traditionally defined BMSCs, we conducted CFU-F assays using unfractionated bone marrow cells isolated from P5 mice. Quantification of tdTomato+ colonies revealed that Col2–cre targeted a higher proportion of CFU-Fs than Osx–cre did (Tomato+ CFU-F: Col2–cre: 42.9 ± 6.3%, Osx–cre: 12.0 ± 4.0%, Fig. 5a,b, see also Supplementary Fig. 3a, n = 4 per group, data are presented as mean ± s.d.), indicating that a larger fraction of BMSCs were derived from Col2–cre+ cells than from Osx–cre+ cells during development. To understand whether BMSCs themselves express Col2 or Osx, CFU-F assays were performed using bone marrow cells collected from Col2–creER or Osx–creER; R26R–tdTomato mice 2 days after the pulse. Only a small proportion of CFU-Fs were tdTomato+ using this protocol (Tomato+ CFU-F: Col2–creER; 1.9 ± 1.7%, Osx–creER; 3.0 ± 3.5%, Fig. 5c, see also Supplementary Fig. 3b, n = 4 per group, data are presented as mean ± s.d.), suggesting that the majority of BMSCs were rather descendants of cells expressing Col2–cre. Second, we conducted flow cytometry analysis of a P0S fraction using dissociated bone cells collected from these mice at P5 (Supplementary Fig. 3c). A P0S fraction represents <1% of the CD45–Ter119– fraction and is enriched for CFU-Fs (ref. 6). Col2–cre targeted a higher proportion of P0S cells than Osx–cre did (Tomato+ P0S cells: Col2–cre: 44.0 ± 6.9%, Osx–cre: 15.6 ± 6.9%, Fig. 5d,e, n = 4 per group, data are presented as mean ± s.d.), as anticipated from the fraction of CFU-Fs marked by these cre lines. A fraction of P0S cells were Cxcl12–GFPhigh, and a small percentage of Cxcl12–GFPhigh cells were positive for Scal; these fractions seemed to decrease with age (Supplementary Fig. 3d,e). A great portion of Cxcl12–GFPhigh cells were PDGFRα–Scal− cells (Supplementary Fig. 3f). Nes–GFP+ cells include all CFU-Fs (ref. 5) and are descended from Col2–creER+ cells during bone development18. Analysis of Nes–GFP; Col2–cre; R26R–tdTomato bone cells revealed that 46.7 ± 5.1% of Nes–GFP+ cells were targeted by Col2–cre (Supplementary Fig. 4a, n = 5, data are presented as mean ± s.d.). We further interrogated the P0S fraction using Nes–GFP; Nes–creER; R26R–tdTomato mice and a single tamoxifen injection at P3. A high fraction of P0S cells were marked by Nes–creER and their descendants throughout the entire first month of chase (48 h after the pulse, 41.6 ± 17.8% (n = 5), then 50.4 ± 12.4% (n = 3) and 44.9 ± 2.8% (n = 4) for the second and fourth week of chase, respectively, Fig. 5f,g). We further interrogated the Nes–creER-marked cells contributed to Cxcl12–GFPhigh cells transiently (50.1 ± 4.2% (n = 3), 11.6 ± 2.3% (n = 4) then 6.0 ± 0.8% (n = 3), for the second day, the first and fourth week of chase, respectively, data are presented as mean ± s.d., Supplementary Fig. 4c), whereas their contribution to endothelial cells is predominant and persistent18. Virtually no P0S cells were marked by Col2–creER or Osx–creER shortly after the pulse (0.3 ± 0.4% (n = 4) and 0.7 ± 0.3% (n = 6), respectively, Fig. 5f,g, data are presented as mean ± s.d.). During the chase, the fraction of P0S cells derived from OsxcreER-P3 cells gradually increased (5.2 ± 2.6% (n = 5) and 10.5 ± 6.0% (n = 8) for the second and fourth week, respectively), whereas those derived from Col2creER-P3 cells only temporarily increased (0.4 ± 0.3% (n = 5), 5.3 ± 2.8% (n = 7), 1.3 ± 1.6% (n = 5) and 1.4 ± 1.3% (n = 9) for the first to fourth week, respectively, data are presented as mean ± s.d.; Fig. 5f,g).

Similarly, an E13.5 pulse to Col2–creER mice marked virtually no P0S cells after two weeks of chase at P7 (Supplementary Fig. 4b). Therefore, although postnatal Col2–creER+ cells robustly contributed to Col1–GFP+ osteoblasts and Cxcl12high stromal cells during bone
Figure 5 Relationship of growth skeletal progenitor cells and adult bone marrow stromal/mesenchymal progenitor cells. (a) CFU-F assay was performed using unfractionated P5 bone marrow cells plated at a clonal density (10^4 cells per 9.6 cm^2) and cultured for 10 days. Cells were collected from Col2-cre, R26^R;Osx-cre (left panel), Osx-cre;R26^R (right panel) mice, Col2-creER; R26^R or Oss-creER; R26^R mice that received 0.1 mg tamoxifen at P3. White indicates tdTomato. Insets highlight examples of Tomato^+ colonies. Scale bars: 2 mm. (b,c) Percentage of Tomato^+ colonies among total colonies (>50 cells) was enumerated. P5 bone marrow cells from Col2-cre, R26^R;Osx-cre or Oss-cre; R26^R mice (b), and Col2-creER; R26^R or Oss-creER; R26^R mice that received 0.1 mg tamoxifen at P3 (c) were subjected to CFU-F assay. n = 4 mice per group. *P < 0.05. All data are presented as mean ± s.d. (d,e) Flow cytometry analysis was performed using dissociated bone cells stained for CD45, Ter119, PDGFRα (CD140a) and Sca1. Representative contour plots of CD45^Ter119^-CD140a^+; Sca1^+ (PuS) (d) or CD45^-Ter119^-CD140a^+; Sca1^- (PuS^-) (e) fraction of the indicated genotype. x axis represents tdTomato, and y axis represents Cxcl12^-GFP. (f,g,i) Percentage of Tomato^+ PuS cells (f) and Tomato^+ PuS^- cells among total PuS cells. n = 4 mice per group. *P < 0.05. All data are presented as mean ± s.d. (h) Flow cytometry analysis was performed using dissociated bone cells stained for CD45, Ter119, PDGFRα (CD140a) and Sca1. Representative contour plots of CD45^-Ter119^-CD140a^-Sca1^- (PuS^-) mice (h) or CD45^-Ter119^-CD140a^-Sca1^- (PuS^-) cells during the chase was plotted. (j,k) Flow cytometry analysis was performed using dissociated bone cells stained for CD45, Ter119, PDGFRα (CD140a) and Sca1. Representative contour plots of CD45^-Ter119^-CD140a^-Sca1^- (PuS^-) or CD45^-Ter119^-CD140a^-Sca1^- (PuS^-) cells among total PuS^- cells. n = 3–9 mice per group (see Methods for precise n values). All data are presented as mean ± s.d.
growth, their contribution to a P0S fraction seems to be minimal. As Col2–creER cells do contribute substantially to the P0S population, it is possible that they do so at a time that we have not tested with Col2–creER mice. A PDGFRα+ Sca1- fraction encompasses most BMSCs (ref. 7). Col2–cre and Oss–cre also targeted a similar proportion of PDGFRα+ Sca1- cells (Tomato+ PDGFRα+ Sca1- cells: Col2–cre; 57.7 ± 14.6%, Oss–cre; 57.2 ± 5.7%, Fig. S5i,j, n = 4 per group, data are presented as mean ± s.d.). A sizable fraction of PDGFRα+ Sca1- cells was marked by Col2–creER or Oss–creER shortly after the pulse (16.1 ± 5.8% (n = 4) and 34.1 ± 10.0% (n = 6), respectively), whereas only a marginal fraction of the cells was marked by Nes–creER (1.8 ± 1.3% (n = 5), Fig. S5j,k, data are presented as mean ± s.d.).

Table 1

| Group                          | No tamoxifen | +2 days | +4 weeks | +2 days +4 weeks |
|-------------------------------|--------------|---------|----------|------------------|
| Sox9–creER; R26R;Tomato       | A             | B        | C        | D                |
| Acan–creER; R26R;Tomato       | A             | B        | C        | D                |

Figure 6  Sox9–creER+ and Acan–creER+ cells encompass early growth skeletal progenitor cells in postnatal growing bones. (a–f) Lineage-tracing of postnatal Sox9–creER+ and Acan–creER+ cells was performed by injecting 0.1 mg tamoxifen into P3 mice. Sox9–creER; R26R;Tomato (a–c) or Acan–creER; R26R;Tomato (d–f) mice were chased for a week (a,d), a month (b,e) and 6 months (c,f). Shown are femur sections with the distal side on the left. Sox9–creER-P3 represents descendants of Sox9–creER+ cells at P3, and Acan–creER-P3 represents descendants of Acan–creER+ cells at P3. White indicates tdTomato. Scale bars: 1 mm. (g,h) No-tamoxifen controls of Sox9–creER-P3 cells (red bars) or Acan–creER-P3 cells (green bars) among Col1–GFP+ osteoblasts after 2 days of chase (left group) or 4 weeks of chase (right group) was enumerated. n = 3 mice for 2 day chase of Sox9–creER-P3 and Acan–creER-P3 cells, n = 4 mice for 2 day chase of Col2–creER-P3 cells, n = 8 mice for 4 week chase of Sox9–creER-P3 cells, n = 5 mice for 4 week chase of Col2–creER-P3 and Acan–creER-P3. All data are presented as mean ± s.d.
We investigated further whether cells defined by promoter/enhancer activities of other genes involved in early chondrocyte development could, like Col2–creER, be used to mark early cells of the osteoblast lineage in postnatal bone development. For this purpose, we took advantage of Sox9–creER (ref. 26) and aggreCan (Acan)–creER (ref. 27) knock-in alleles and performed similar pulse-chase experiments. Shortly after the chase, Sox9–creER marked populations comparable to those marked by Col2–creER (Supplementary Fig. 5a), whereas Acan–creER marked a larger number of perichondrial cells (Supplementary Fig. 5c). After a week of chase, both Sox9CreER–P3 and AcanCreER–P3 cells increased in the metaphyseal spongiosa and endocortical surface (Fig. 6a,b, see also Supplementary Fig. 5b,d). We further conducted flow cytometry analysis using mice carrying Col1–GFP, Sox9–creER or Acan–creER and R26R–tomato reporter. A small fraction of cells expressed Col1–GFP shortly after the pulse (Sox9–CreER; 3.6 ± 0.8%, Acan–creER; 5.0 ± 2.1%, n = 3 per group, data are presented as mean ± s.d.), and the fraction among osteoblasts increased to 55.1 ± 8.2% (Sox9–creER, n = 8) and 65.9 ± 8.2% (Acan–creER, n = 5, data are presented as mean ± s.d.) after 4 weeks of chase (Fig. 6i,j). Both Sox9CreER–P3 and AcanCreER–P3 cells continued to generate osteoblasts and stromal cells at least for 6 months in a pattern that resembled those of Col2CreER–P3 cells (Fig. 6c,f). Little to no tamoxifen-independent activities was noted with the use of these knock-in lines (Fig. 6g,h).

DISCUSSION

Our data collectively indicated that cells defined by promoter/enhancer activities of genes associated with chondrocytes and their precursors, such as Sox9, Col2 and Acan, encompass early mesenchymal progenitors that continue to become chondrocytes, osteoblasts, stromal cells and adipocytes during endochondral bone development. Cre/creER-recombinases driven by the Sox promoter mark a downstream population with limited lineage potential to generate osteoblasts and stromal cells (Supplementary Fig. 6, see the diagram). Cells marked by Oss–creER at P3 are not continually generated in the metaphysis, in contrast to cells marked by Sox9–creER, Col2–creER and Acan–creER, but do persist as stromal cells in metaphyseal bone marrow. Any inconsistency between our data and that of ref. 28 is probably due to the length of the chase, because we chased these cells for a longer period of time, for up to 18 months. In addition, when postnatal Col2–creER+ cells generated osteoblasts and stromal cells progressively in the metaphysis, there was no comparable increase of Col2–creER+ descendants among a PoS fraction. We speculate that this last observation may be relevant to findings recently published in ref. 7. In that study, it was found that, in adult mice, PoS cells overlapped considerably with those marked by leptin receptor (LepR)–cre and that cells marked with the LepR–cre; R26R–tdTomato progressively marked osteoblasts, but only starting at two months of age. We suggest that the growth-associated progenitors that we have identified here may be the source of osteoblasts during the rapid phase of bone growth before LepR–cre-marked cells provide precursors for the osteoblast lineage. The demonstration of Col2–cre; R26R–tdTomato mice marking a large fraction of postnatal osteoblasts and stromal cells, including PoS cells, suggests that the adult mesenchymal precursor cells might derive from the progenitors that we have identified. Transition of these growth-associated progenitors into adult BMSCs seems to be infrequent and requires continuous inputs to make an easily discernible contribution. Further experiments will be required to establish the relationship between the growth-related mesenchymal progenitors identified here and adult mesenchymal precursors. The existence of these two populations provides opportunities for distinct regulation of the explosive growth of bone in early life and the much slower remodelling that occurs in adulthood.

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AUTHOR CONTRIBUTIONS

N.O. and H.M.K. conceived the project and designed the experiments. T.N. provided mice. N.O. and W.O. performed the experiments. N.O. and W.O. analysed the data. N.O. and H.M.K. wrote the manuscript; T.N. critiqued the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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METHODS

Mice. Col1(2.3kb)–GFP (ref. 15), Osx–GFP (ref. 15), Nestin–GFP (ref. 29), Cxcl12–GFP/null (ref. 20), Col2a1–creERT2 (ref. 13), Osx–creERT2 (ref. 16), Col2a1–creERT2 (ref. 25), Osx–creERT2 (ref. 2), Nestin–creERT2 (ref. 30), Sox9–creERT2 (ref. 26), Acan–creERT2 (ref. 11) or Rosa26-loxP-stop-loxP–tdTomato (R26R–tdTomato, IAX7914) mice were acquired from Jackson laboratory. All procedures were conducted in compliance with the Guideline for the Care and Use of Laboratory Animals approved by Massachusetts General Hospital’s Institutional Animal Care and Use Committee (IACUC). All mice were housed in a specific pathogen-free condition, and analysed in a mixed background. Mice were identified by micro-tattooing or ear tags. Tail biopsies of mice were used by a HotShot protocol (incubating the tail sample at 95 °C for 30 min in an alkaline lysis reagent followed by neutralization) and used for PCR-based genotyping (Qiaq Green Master Mix, Promega, and C1000 Touch Cycler, Bio-rad). Perinatal mice were also genotyped fluorescently (BLS miner’s lamp) whenever possible. Mice were euthanised by over-dosage of carbon dioxide or decapitation under inhalation anaesthesia in a drop jar (Aerarre solvent, Henry Schein).

For the experiments in Fig. 1a–f, male mice (Col1–GFP; Col2–cre; R26R–tdTomato or Osx–cre; GFP; R26R–tdTomato) were mated to female CD1 mice (>8 weeks old, Charles River Laboratories) and the vaginal plug was checked in the morning. For the cell proliferation assay, 1 mg of 5-ethynyl-12-deoxyuridine (EdU; Invitrogen A10044) dissolved in PBS was administered to pregnant mice three hours before euthanasia at the indicated embryonic days. Embryos were used for analysis regardless of the sex. At least three embryos of the indicated genotype were examined at each time point shown in the figure.

For the experiments in Fig. 1g, male mice (Col1–GFP; Col2–cre; R26R–tdTomato or Osx–cre; GFP; Col2–cre; R26R–tdTomato or Cxcl12–GFP; Col2–cre; R26R–tdTomato or Col1–GFP; Osx–cre; GFP; R26R–tdTomato or Osx–cre; GFP; R26R–tdTomato or Cxcl12GFP; Osx–cre; GFP; R26R–tdTomato) were mated to female CD1 mice. Pups at postnatal day 3 were used for analysis regardless of the sex. Three pups of the indicated genotype were examined at the time point shown in the figure. n = 3 mice per group.

For the experiments in Fig. 1h–m, male mice (Col1–GFP; Col2–cre; R26R–tdTomato or Col2–cre; R26R–tdTomato or Osx–cre; GFP; Col2–cre; R26R–tdTomato or Cxcl12–GFP; Col2–cre; R26R–tdTomato or Col1–GFP; Osx–cre; GFP; R26R–tdTomato or Osx–cre; GFP; R26R–tdTomato or Cxcl12GFP; Osx–cre; GFP; R26R–tdTomato) were mated to female CD1 mice and the vaginal plug was checked in the morning. Pregnant mice received 1 mg tamoxifen (Sigma T5648) and progesterone (Sigma P4972) intraperitoneally at an indicated embryonic day. Embryos or pups were used for analysis regardless of the sex. At least three embryos or pups of the indicated genotype were examined at the time point shown in the figure.

For the experiments in Figs 2 and 3, male mice (Col2–cre; R26R–tdTomato or Osx–cre; GFP; R26R–tdTomato or Osx–cre; GFP; R26R–tdTomato or Cxcl12–GFP; Osx–cre; GFP; R26R–tdTomato or Cxcl12–GFP; Osx–cre; GFP; R26R–tdTomato or Col1–GFP; Acan–cre; GFP; R26R–tdTomato or Col1–GFP; Acan–cre; GFP; R26R–tdTomato) were mated to female FVB/N mice to generate pups. Three-day-old mice received 0.1 mg of tamoxifen intraperitoneally. Mice were used for analysis regardless of the sex. For the cell proliferation assay, three mice of the indicated genotype were examined at 4 (1 week chase), n = 3 (2 week chase) and n = 4 (4 week chase) mice; Col2–cre; R26Rtomato; n = 4 (2 day chase), n = 5 (1 week chase), n = 7 (2 week chase), n = 5 (3 week chase) and n = 9 (4 week chase) mice; Osx–cre; GFP; R26Rtomato; n = 6 (2 day chase) and n = 5 (4 week chase) mice.

For the experiments in Figs 4a, 6, male mice (Sox9–cre; R26R–tdTomato or Col1–GFP; R26R–tdTomato or Col–GFP; R26R–tdTomato or Acan–cre; GFP; R26R–tdTomato or Col1–GFP; Acan–cre; GFP; R26R–tdTomato or Col1–GFP; Acan–cre; GFP; R26R–tdTomato) were mated to female FVB/N mice to generate pups. Three-day-old mice received 0.1 mg of tamoxifen intraperitoneally. Mice were used for analysis regardless of the sex. At least three mice of the indicated genotype were examined at the time point shown in the figure.

For the experiments in Supplementary Fig. 4a, male mice (Col1–GFP; Col2–cre; R26R–tdTomato or Osx–cre; GFP; R26R–tdTomato) were mated to female FVB/N mice to generate pups. Mice were used for analysis regardless of the sex. One pup of the indicated genotype was examined at P7.

For the experiments in Supplementary Fig. 4b, male mice (Col1–GFP; Col2–cre; R26R–tdTomato or Osx–cre; GFP; R26R–tdTomato) were mated to female FVB/N mice to generate pups. Three-day-old mice received 0.1 mg of tamoxifen intraperitoneally. Mice were used for analysis regardless of the sex. At least three mice of the indicated genotype were examined at the time point shown in the figure.

Tamoxifen. Tamoxifen (Sigma T5648) was mixed with 100% ethanol until dissolved. Subsequently, a proper volume of sunflower seed oil (Sigma S907) was added to the tamoxifen–ethanol mixture and rigorously mixed. The tamoxifen–ethanol–oil mixture was incubated at 60 °C in a chemical hood until the ethanol evaporated completely. The tamoxifen–oil mixture was stored at room temperature until use.

Histology. Samples were dissected under a stereomicroscope (Nikon SMZ-10A) to remove soft tissues, and fixed in 4% paraformaldehyde, overnight at 4 °C, then decalcified in 15% EDTA for a proper period, typically ranging from 1 to 14 days. Decalcified samples were cryoprotected in 30% sucrose/PBS solutions and then in 30% sucrose/PBS/OCT (1:1) solutions, each overnight at 4 °C. Samples were embedded in an OCT compound (TissuTek, Sakura) under a stereomicroscope and transferred on a sheet of ice to solidify the compound. Embedded samples were cryosectioned at 15 μm using a cryostat (Leica CM1850). Images were captured with a wide-field fluorescence microscope (Nikon Eclipse E800) with prefigured triple-band filter settings for DAPI/FITC/TRITC, and merged with Spot Advanced Software (Spot Imaging) or an automated fluorescence microscope with a whole-slide scanning platform (TissueFAXS, TissueGnostics). Confocal images were acquired using LSM510 and Zeiss 2009 software (Zeiss) with lasers and corresponding band-pass filters for DAPI (Ex. 405 nm, P420-480), GFP (Ex. 488 nm, BP505-530), tdTomato (Ex. 543 nm, BP565-595) and Alexa633 (Ex. 633 nm, LP650). LSM Image Viewer and Adobe Photoshop software were used to capture and align images. Representative images of at least three independent biological samples are shown in the figures.

Flow cytometry. Femurs, tibias, iliac crests and humeri were carefully dissected and gently crushed in 5 ml Ca2+–Mg2+–free HBBS (Sigma) with a pestle and a mortar (Coors Tek), and then supernatants were filtered through a 70 μm cell strainer (Fisher) into a 50 ml tube on ice. Bone fragments were crushed for two additional times in 5 ml HBSS and supernatants were filtered into the same tube. Tissue remnants were incubated with 2 Wünsch units of Liberase TM (Roche) at 37 °C for 45 min on a shaking incubator (Thermomixer, Eppendorf). For experiments in Fig. 5a, cell collect osteoblasts, 0.25% trypsin–EDTA (Gibco) was added. Cells were mechanically triturated using an 18-gauge needle and a 1 ml syringe (BD) and filtered into the same tube. Cells were pelleted, resuspended and layered over Ficoll-Paque PLUS (GE Healthcare) to collect low-density cell fractions. Cells were stained
**METHODS**

with anti-mouse CD45–APC, CD45–eFluor 450, Ter119–eFluor 450, Sca1–Alexa Fluor 700 (1:500, eBioscience), PDGFRI (CD140a)–APC (1:250, eBioscience) or their isotype controls (1:500, eBioscience) in DPBS/2%FBS on ice for 30 min. Flow cytometry was performed using a four-laser BD LSRII (Ex.355/407/488/633nm) and FACSDiva, and analysed on FlowJo (TreeStar). Representative plots of at least three independent biological samples are shown in the figures.

**Immunohistochemistry.** Cryosections were stored at –20°C in freezers until use. Sections were postfixed in 4% paraformaldehyde for 15 min, blocked with 2% BSA/TBST for 30 min and incubated with rat anti-CD31 monoclonal antibody (1:100, AbD Serotec MCA2388), overnight at 4°C, and subsequently with Alexa Fluor 633-conjugated goat anti-rat IgG (1:400, Invitrogen A21087) for 3 h at 4°C. For lipid staining, cryosections were gently rinsed with TBS and incubated with LipidTOX Deep Red (1:200, Invitrogen H34477) for 30 min at room temperature. Sections were further incubated with DAPI (4',6-diamidino-2-phenylindole, Invitrogen D1306) to stain nuclei. Stained samples were coverslipped and mounted in a mounting medium for fluorescence imaging (Vectashield H-1000, Vector Labs). The edge of the coverslip was coated with commercially available transparent nail polish.

**Cell proliferation assay.** To evaluate cell proliferation, 1 mg of 5-ethynyl-2'-deoxyuridine (EdU; Invitrogen A10044) dissolved in PBS was administered to pregnant mice three hours before euthanization at the indicated embryonic days. Click-iT Imaging Kit (Invitrogen, C10337) with Alexa Fluor 647-azide (Invitrogen A10277) was used to detect EdU in cryosections.

**Colony-forming unit fibroblast (CFU-F) assay.** Bone marrow cells were collected as described in the Flow cytometry section. Bone marrow nucleated cells (10⁶) were plated into a 9.6 cm² glass-bottom chamber slide (Lab-Tek II, Nunc) and cultured in MesenCult Proliferation medium for mouse cells (StemCell Technologies) for 10 days. Cells were fixed in 4% paraformaldehyde, for 30 min at 4°C and counterstained with wheat germ agglutinin–Alexa Fluor 488 conjugate (Invitrogen W11261) for 15 min. Representative images from at least three independent biological samples are shown in the figures.

**Statistical Analysis.** Results are presented as mean values ± s.d. Statistical evaluation was conducted using the Mann–Whitney’s U-test. A P value of <0.05 was considered significant. 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.

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Supplementary Figure 1  Fate mapping of Col2-cre<sup>+</sup> and Osx-cre<sup>+</sup> cells during endochondral ossification.  

**a.** E11.5 femur sections of Col2-cre; R26<sup>R</sup>Tomato mice were stained for nuclei and CD31. Shown is mesenchymal condensation. Perichondrium is on top. Red represents tdTomato, blue represents Alexa633 and gray represents DAPI. Scale bars: 100 µm.

**b, d.** P3 femur sections of Col1-GFP; Col2-cre; R26<sup>R</sup>Tomato (b) or Col1-GFP; Osx-cre; R26<sup>R</sup>Tomato (d) mice were stained for nuclei. Shown is trabecular bone. Arrowheads: Col1-GFP<sup>+</sup>Tomato<sup>+</sup> osteoblasts. Green: EGFP, red: tdTomato, gray: DAPI and DIC (differential interference contrast). Scale bars: 50 µm.

**c, e.** P3 femur sections of Cxcl12-GFP; Col2-cre; R26<sup>R</sup>Tomato (c) or Cxcl12-GFP; Osx-cre; R26<sup>R</sup>Tomato (e) mice were stained for nuclei. Shown are diaphyseal endocortices and bone marrows. Arrows: Cxcl12-GFP<sup>+</sup>Tomato<sup>+</sup> stromal cells. Green: EGFP, red: tdTomato, gray: DAPI and DIC (differential interference contrast). Scale bars: 50µm.

**f.** E14.5 femur sections of Osx-cre::GFP; R26<sup>R</sup>Tomato mice were stained for nuclei and EdU, which was administered 3 hours prior to analysis. Shown are higher magnification views of Figure 1e. Arrowheads: proliferating Osx<sup>+</sup> cells in the perichondrium. Green: EGFP, red: tdTomato, blue: Alexa647, gray: DAPI. Scale bars: 50 µm.

**g.** Shown is the percentage of EdU<sup>+</sup> cells among tdTomato<sup>+</sup> cells in the E14.5 perichondrium of Col2-cre; R26<sup>R</sup>Tomato (blue dots) and Osx-cre::GFP; R26<sup>R</sup>Tomato (red dots). n=3 mice for Col2-cre; R26<sup>R</sup>Tomato, n=4 mice for Osx-cre::GFP; R26<sup>R</sup>Tomato.
**Supplementary Figure 2** Col2-creER<sup>T</sup> cells encompass early cells of the osteoblast lineage and generate multiple mesenchymal lineages. 

a. Pregnant mice received 1mg tamoxifen at E11.5, and Col2-creER; R26<sup>ERTomato</sup> mice were chased until postnatal day 21 (P21). Shown are femur sections with the distal side on the left. Col2creER-E11.5 represents descendants of Col2-creER<sup>T</sup> cells at E11.5. White indicates tdTomato. Scale bars: 500 μm.

b. Pregnant mice received 1mg tamoxifen at E13.5, and Osx-creER; R26<sup>ERTomato</sup> mice were chased until P0 (left panel), P7 (middle panel) or P14 (right panel). Shown are femur sections with the distal side on the left. OsxcreER-E13.5 represents descendants of Osx-creER<sup>T</sup> cells at E13.5. Red indicates tdTomato. Scale bars: 500 μm.

c. Lineage-tracing of postnatal Osx-creER<sup>T</sup> cells was performed by injecting 0.1mg tamoxifen into P3 mice. Pulsed Osx-creER; R26<sup>ERTomato</sup> mice were chased for a month (left panel), two months (middle panel) and six months (right panel). Shown are femur sections with the distal side on the left. OsxcreER-P3 represents descendants of Osx-creER<sup>T</sup> cells at P3. Red indicates tdTomato. Scale bars: 500 μm.

d,e. No tamoxifen controls of Col2-creER; R26<sup>ERTomato</sup> mice were shown. Shown are femur sections with the distal side on the left. White indicates tdTomato. Scale bars: 500 μm. d,e. No tamoxifen controls of Col2-creER; R26<sup>ERTomato</sup> mice at P5 (d) and P10 (e). month of age (g,i) or 1 year of age (h,j) are shown. Shown are femur sections with the distal side on the left. White indicates tdTomato. Scale bars: 100 μm.

f. Oc-GFP; Col2-creER; R26<sup>ERTomato</sup> mice received 0.1mg tamoxifen at P3 and were chased for 2 months. Shown are metaphyseal spongiosa. Femur sections were stained for nuclei and LipidTOX deep red. Asterisks: Tomato<sup>+</sup> osteoblasts, arrowheads: Tomato<sup>+</sup> adipocytes. Green: EGFP, red: tdTomato, blue: LipidTOX Deep Red, gray: DAPI. Scale bars: 100μm (left panel) or 20μm (right panel).
Supplementary Figure 3. Colony-forming unit fibroblasts (CFU-F) assay and flow cytometry analysis of CD45-Ter119-CD140a+Sca1+ (PαS) fraction.

a, b. Colony-forming unit fibroblast (CFU-F) assay was performed using unfractionated P5 bone marrow cells plated at a clonal density (10^6 cells per 9.6cm^2) and cultured for 10 days. Cells were harvested from Col2-cre; R26R<sup>tm</sup> (a; left panel), Osx-cre; R26R<sup>tm</sup> (a; left panel) mice, Col2-cre<sup>ER</sup>; R26R<sup>tm</sup> (b; left panel) or Osx-cre<sup>ER</sup>; R26R<sup>tm</sup> mice that received 0.1mg tamoxifen at P3 (b; right panel). Cells were stained for Wheat Germ Agglutinin (WGA)-Alexa488 conjugate. Red: tdTomato, green: Alexa488. The number of colonies (>50 cells) of each color was also shown. Scale bars: 2mm.

c. Scheme to identify a CD45-Ter119-CD140a+Sca1+ (PαS) fraction. Dissociated bone cells were stained for CD45, Ter119, PDGFRα (CD140a) and Sca1, or CD45, Ter119 and isotype antibodies, and subjected to flow cytometry analysis. First, cells were gated on forward scatter and scatter profiles to identify single cells (left three subpanels). Second, cells were gated for a CD45<sup>-</sup>Ter119<sup>-</sup> fraction to analyze a non-hematopoietic fraction (left fourth subpanel, red box). This fraction was expanded for CD140a and Sca1. Red box designates PαS fraction (upper right panel; upper left and lower right subpanels indicate single-color staining, and lower and left subpanel indicates isotype control). d-f. Flow cytometry analysis was performed using dissociated bone cells harvested from Cxcl12-GFP mice at P14 (left panels), P32 (middle panel) and 8 weeks old (right panels). Cells were stained for CD45, Ter119, PDGFRα (CD140a) and Sca1 or their corresponding isotype controls. Representative dot plots of CD45<sup>-</sup>Ter119<sup>-</sup> fraction are shown on the leftmost panels. (d) Histograms of CD45<sup>-</sup>Ter119<sup>-</sup>Cxcl12<sup>GFP<sup>high</sup></sup> fraction developed for Sca-1; blue lines: isotype control. (e) Histograms of CD45<sup>-</sup>Ter119<sup>-</sup>CD140a+Sca1+ (PαS) fraction developed for Cxcl12<sup>GFP</sup>; blue lines: GFP- controls. (f) Dot plots of CD45<sup>-</sup>Ter119<sup>-</sup>Cxcl12<sup>GFP<sup>high</sup></sup> fraction developed for CD140a and Sca1. CD45<sup>-</sup>Ter119<sup>-</sup>CD140a+Sca1+ (PαS) and CD45<sup>-</sup>Ter119<sup>-</sup>CD140a-Sca1+ (PαS) fractions are boxed. n=4 mice for P14 and P32, n=3 mice for 8 weeks old mice. All data are represented as mean ± SD.
Supplementary Figure 4 Relationship of growth skeletal progenitor cells and adult bone marrow stromal/mesenchymal progenitor cells. a. Representative dot plots of cells from Nes-GFP; Col2-cre; R26R<sup>Tomato</sup> (left panel) and histograms of Nes-GFP<sup>+</sup> fraction (right panel). X-axis represents tdTomato, and Y-axis represents GFP (left panel) or percentage of maximum (right panel). n=5 mice per group. All data are represented as mean ± SD. b. Flow cytometry analysis was performed using dissociated bone cells harvested from P7 Cxcl12-GFP; Col2-creER; R26R<sup>Tomato</sup> mice after administration of tamoxifen at E13.5 (1mg into pregnant mice). Cells were stained for CD45, Ter119, PDGFR<sub>a</sub> (CD140a) and Sca1, and CD45-Ter119<sup>-</sup> fraction (left panel) was gated for PDGFR<sub>a</sub> and Sca1 (PoS, right panel). X-axis represents tdTomato, and Y-axis represents GFP. c. Flow cytometry analysis was performed using dissociated bone cells harvested from Cxcl12-GFP; Nes-creER; R26R<sup>Tomato</sup> mice that received 0.1mg tamoxifen at P3 and were chased for indicated periods. CD45<sup>-</sup> fraction was gated for GFP. Left subpanel: 2 days chase, middle subpanel: 4 weeks chase. X-axis represents tdTomato, and Y-axis represents GFP. Percentage of Nes<sup>creER</sup>P3 cells (purple line) among CD45<sup>-</sup>Cxcl12-GFP<sup>high</sup> stromal cells during the chase was plotted. X-axis represents the duration of the chase, and Y-axis represents the percentage of Tomato<sup>+</sup>GFP<sup>+</sup> cells among total GFP<sup>+</sup> cells. n=3 mice for 2 days chase and 4 weeks chase, n=4 mice for 1 week chase. All data are represented as mean ± SD.
Supplementary Figure 5 Sox9-creER\(^{+}\) and Acan-creER\(^{+}\) cells encompass early growth skeletal progenitor cells in postnatal growing bones. a,b, Sox9-creER; R26\(^{+}\)Tomato mice received 0.1mg tamoxifen at P3 and were chased for 48 hours (a) or a week (b). Shown are distal halves of growth cartilages at the junction of growth plate and primary spongiosa. Perichondrium is on top. Femur sections were stained for nuclei and CD31. Asterisks: Tomato\(^{+}\) chondrocytes, sharps: Tomato\(^{+}\) perichondrial cells, arrows: Tomato\(^{+}\) cells in primary spongiosa, arrowheads: a group of Tomato\(^{+}\) cells directly under the growth plate and beneath the perichondrium. Red: tdTomato, blue: Alexa633, gray: DAPI. Scale bars: 100\(\mu\)m. c,d, Col1-GFP; Acan-creER; R26\(^{+}\)Tomato (c) or Acan-creER; R26\(^{+}\)Tomato (d) received 0.1mg tamoxifen at P3 and were chased for 48 hours (c) or a week (d). Femur sections were stained for nuclei and CD31. Asterisks: Tomato\(^{+}\) chondrocytes, sharps: Tomato\(^{+}\) perichondrial cells, arrows: Tomato\(^{+}\) cells in primary spongiosa, arrowheads: a group of Tomato\(^{+}\) cells directly under the growth plate and beneath the perichondrium. Red: tdTomato, blue: Alexa633, gray: DAPI. Scale bars represent 100\(\mu\)m.
Supplementary Figure 6 A subset of chondrogenic cells provides early mesenchymal progenitors in growing bones. Diagram of a growing endochondral bone is shown. Shown on the left is growth plate, and shown on the right is diaphysis. As bones rapidly grow (to the left in this diagram), a subset of Sox9+Col2+Acan+ cells self-renews and provides a stable source of Osx+ precursors in the metaphysis. While Osx+ precursors become Col1+ differentiated osteoblasts on the bone surface and Cxcl12+ stromal cells in bone marrow, these precursors are transient and not indefinitely self-renewing in this actively growing portion of bones. A subset of these chondrogenic cells also provides a source of bone marrow mesenchymal stromal progenitor cells (BMSCs), which are highly enriched among bone marrow pericytes such as PDGFRα+Sca1+ “PαS” cells and Nes-GFP+ cells. LepR+ stromal cells are likely to overlap with Cxcl12+ stromal cells and PαS cells.