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Diverse contribution of *Col2a1*-expressing cells to the craniofacial skeletal cell lineages

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Abstract

Objectives—Craniofacial skeletal development requires deliberate coordination of two distinct mechanisms of endochondral and intramembranous ossification. *Col2a1*-expressing cells encompass growth-associated skeletal progenitors in endochondral bones of the limb. The objective of this study is to determine the contribution of *Col2a1*-expressing cells to the craniofacial skeletal cell lineages. We hypothesize that *Col2a1*-expressing progenitors significantly contribute to various modes of ossification associated with the craniofacial development.

Methods—Cellular fates of *Col2a1*-expressing cells were studied based on a *cre-loxP* system using a *Col2a1-cre* transgene and an *R26R*-tdTomato reporter allele. We analyzed three distinct locations of the craniofacial skeletal complex representing unique ossification mechanisms; the cranial base, the calvaria and the mandibular condyle.

Results—*Col2a1-cre* consistently marked a majority of skeletal cells in the cranial base. Interestingly, *Col2a1-cre* also marked a large number of osteoblasts and suture mesenchymal cells in the calvaria, in addition to chondrocytes in the underlying transient cartilage. In the mandibular condyle, *Col2a1-cre* marked chondrocytes and osteoblasts only during the growth phase.

Conclusions—*Col2a1* is expressed by progenitors of the skeletal lineage in canonical endochondral bone formation occurring in the cranial base. In contrast, other ossification mechanisms of the craniofacial complex utilize *Col2a1*-expressing cells in a different manner, whereby *Col2a1* may be expressed in more differentiated or transient cell types of the skeletal lineage.

Keywords

Type II collagen; Cell lineage tracing; Endochondral ossification; Intramembranous ossification; Mandibular condylar cartilage

Introduction

Craniofacial skeletal development requires deliberate coordination of two distinct mechanisms of endochondral and intramembranous ossification. The cranial base is initially formed by fusion of three primordial (1). The synchondroses, unique bidirectional growth

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plates, are formed between these ossification centers as spheno-ethmoidal (SES), intersphenoidal (ISS) and spheno-occipital synchondrosis (SOS), which play an important role in the growth of the cranial base. Recent studies suggest that chondrocytes within the growth plate serve as a major source of other skeletal lineage cells in growing bones (2,3). Our recent study revealed that *Col2a1* promoter/enhancers are active in early growth-associated skeletal progenitors in the limb (4). The mandibular condylar cartilage of the skull is a secondary cartilage without growth plate structures. A recent study reports that direct transformation of chondrocytes into bone cells also occurs in these cartilages (5). Interestingly, subsets of osteoblast precursors in intramembranous ossification are known to express both 'osteogenic' and 'chondrogenic' markers; *Col2a1* mRNA is transiently expressed by preosteoblasts and 'chondrocyte-like osteoblasts' during early development and adulthood (6,7). However, it is unclear how significant *Col2a1*-expressing cells contribute to the craniofacial skeletal cell lineages. Here, we hypothesize that *Col2a1*-expressing cells significantly contribute to various modes of ossification occurring in the craniofacial skeletal complex. The state-of-the-art *in vivo* cell fate-mapping technique that we utilize in this study has the possibility to reveal novel mechanisms for craniofacial skeletal development.

Materials and Methods

Mice

Col2a1-cre and *Col1a1(2.3kb)*-GFP have been described elsewhere (8,9). *Rosa26-loxP-stop-loxP*-tdTomato (*R26R*-tdTomato, Ai14, JAX007914 (10)) mice were acquired from the Jackson laboratory. All procedures were conducted in compliance with the Guideline for the Care and Use of Laboratory Animals approved by the University of Michigan.

Histology and Flow cytometry

These procedures were performed as previously described (4).

Results

Col2a1-expressing cells contribute to the majority of skeletal cells in the cranial base

At E15.5, red cells occupied the synchondrosis (Fig.1a, asterisk: SOS), the ossification center (sharp) as well as the perichondrium (arrowheads). The vast majority of *Col1a1*-GFP$^+$ osteoblasts appearing in the inner perichondrium were yellow (Fig.1a, right panel: arrows), therefore derived from *Col2a1*-cre$^+$ cells. At P3, the synchondrosis generated a mirror-image growth plate composed of a common central resting zone (R) and proliferative (P) and hypertrophic (H) zones on both sides (Fig.1b). *Col1a1*-GFP$^+$ osteoblasts on the trabecular surface and in the cortical bones were yellow, indicating that they were derived from *Col2a1-cre*$^+$ cells (Fig.1b, right panels, arrowheads: osteoblasts). Although active cranial base growth had already ceased at P21, *Col2a1-cre*-derived red cells continued to contribute not only to chondrocytes and perichondrial cells in the synchondrosis, but also to osteoblasts, osteocytes and bone marrow stromal cells (Fig.1c, inset 3: arrows: osteocytes, right panel 4: arrowheads: osteoblasts, sharps: stromal cells). Flow cytometry analysis of dissociated cranial base cells revealed that *Col2a1-cre*-marked cells contributed to

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essentially all osteoblasts (Fig.1d, 85.6±4.7% and 97.7±3.5% of *Col1a1*-GFP^{high} cells at P3 and P21, respectively). The synchondrosis was patent at 8 weeks old, and surprisingly, even at 6 months of age in these mice, associated with consistent robust generation of *Col2al-cre*-derived red cells as osteoblasts and bone marrow stromal cells (Fig.1e,f). No Tomato^{+} cells were observed in the absence of *Col2a1-cre* (Fig.1g). Therefore, these findings indicate that a large majority of chondrocytes, osteoblasts/cytes and bone marrow stromal cells in the cranial base are derived from *Col2a1*-expressing cells, underscoring the identical developmental mechanisms that support both the growth plate in the limb and the synchondrosis in the cranial base.

Col2a1-expressing cells contribute to osteoblasts and suture mesenchymal cells in the calvaria

First, we verified the meshwork-like spotted cartilage in parietal bones near the midsagittal suture using whole-mount Alcian Blue staining and H&E staining at P0 in C57/BL6 mice (Fig.2a-a′, arrowheads: cartilage islands, arrows: underlying cartilage). Second, we took advantage of *Col1a1*-GFP; *Col2a1-cre*; *R26R*-tdTomato trigenic mice for more detailed fate mapping of *Col2a1*-expressing cells in the calvaria. At E15.5, a well-defined layer of cartilage underpinned the calvaria on the lateral portion of the parietal bones, in which chondrocytes and perichondrial cells were red, therefore marked by *Col2a1-cre* (Fig.2b, arrows: underlying cartilage). Many *Col1*-GFP^{+} calvarial osteoblasts were yellow, demonstrating that these cells were either derived from, or themselves were *Col2a1-cre*$^{+}$ cells (Fig.2b, right panel, arrowheads: GFP/tdTomato^{+} osteoblasts). Few red cells were observed in the midsagittal suture at this stage. At P3, a *Col2a1-cre*-marked small piece of the underlying cartilage appeared directly underneath the midsagittal suture (Fig.2c, asterisk). On the lateral portion, many *Col1a1*-GFP^{+} calvarial osteoblasts located on the boundary between the calvarial bone and the underlying cartilage were yellow (Fig.2c, right panel, arrowheads). However, virtually all *Col1a1*-GFP^{+} osteoblasts near the suture were green, suggesting that they were not derived from *Col2a1-cre*$^{+}$ cells (Fig.2c, arrows). Although the underlying cartilage had disappeared at P21, many *Col1a1*-GFP^{+} calvarial osteoblasts in the inner aspect of the calvaria and osteocytes were yellow and red, respectively (Fig.2d, right panel, arrowheads), thus marked by *Col2a1-cre*. In addition, *Col2a1-cre*-marked cells contributed to suture mesenchymal cells (Fig.2d, arrows), but not to *Col1a1*-GFP^{+} osteoblasts in proximity to the midsagittal suture. It could not be ascertained whether descendants of the underlying cartilage chondrocytes contributed to these suture mesenchymal cells, or *Col2a1* promoter/enhancer activities occurred *de novo* among these cells at this stage. Flow cytometry analysis of digested calvarial cells revealed that 21.4±7.6% and 60.9±8.4% of *Col1a1*-GFP^{high} osteoblasts were Tomato^{+} at P3 and P21, respectively (Fig.2f), indicating that the fraction of calvarial osteoblasts derived from *Col2a1*-expressing cells increased during active calvarial growth. This trend continued onto an adult and an aged stage at 8 weeks and 6 months of age (Fig.2g,h). Although osteoblasts on the bone surface were increasingly diminished in these later stages, osteoblasts on the endosteum and osteocytes were yellow and red, respectively (Fig.2g,h, right panels: arrowheads: osteoblasts, arrows: osteocytes). Abundant *Col2a1-cre*-marked suture mesenchymal cells continued to be observed, indicating persistent activities *Col2a1* promoters/enhancers in the late stages. No Tomato^{+} cells were observed in the absence of

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Col2a1-cre (Fig.2e). Therefore, these findings indicate that a large number of osteoblasts, osteocytes and suture mesenchymal cells in the calvaria are either derived from *Col2a1*-expressing cells or themselves express *Col2a1*, revealing a significant contribution of *Col2a1*-expressing cells in the intramembranous skeletal lineage in the calvaria.

Transient contribution of Col2a1-expressing cells to the mandibular condyle

At E15.5, a number of *Col2a1-cre*-marked red cells were observed in the condyle anlage, distinct from sheet-like *Col1a1*-GFP$^+$ osteoblasts of the mandible body (Fig.3a, asterisk: condylar cartilage). At P3, the condylar cartilage significantly increased in length, particularly in the proliferating and hypertrophic cell layers. These chondrocytes were red thus marked by *Col2a1-cre* (Fig.3b, asterisk). Abundant trabecular bone formation took place underneath the cartilage at this stage, where a majority of *Col1a1*-GFP$^+$ trabecular osteoblasts were yellow thus derived from *Col2a1*-cre$^+$ cells (Fig.3b, arrowheads: GFP/tdTomato$^+$ osteoblasts). Flow cytometry analysis of digested whole mandible cells revealed that 19.4±5.8% of *Col1a1*-GFP^{high} osteoblasts were Tomato$^+$ at P3 (Fig.3g). The condylar cartilage reduced in its thickness but developed distinct structures at P21, whereas the domain for the subchondral trabecular bones expanded further. Most of condylar chondrocytes were red; in addition, and many *Col1a1*-GFP$^+$ osteoblasts and osteocytes were yellow and red, respectively, thus derived from *Col2al-cre*$^+$ cells (Fig.3c, arrowheads: osteoblasts, arrows: osteocytes). At 8 weeks of age, less red chondrocytes were noted in the condylar cartilage (Fig.3d, asterisk), and only a small fraction of *Col1a1*-GFP$^+$ trabecular osteoblasts were marked by tdTomato (Fig.3d, arrowheads), indicating that contribution of *Col2a1-cre*$^+$ cells to these osteoblasts diminished at this stage. At 6 months of age, *Col2a1*-marked cells largely disappeared from the condylar cartilage, with only a few yellow osteoblasts present in the trabecular bone (Fig.3e). No Tomato$^+$ cells were observed in the absence of *Col2a1-cre* (Fig.3f). Therefore, these findings indicate that, while *Col2a1*-expressing cells make significant contribution to chondrocytes and subchondral osteoblasts during the early stages, these cells are replaced by other types of cells during the late stages. This underscores a transient contribution of *Col2a1*-expressing cells to the mandibular condylar cartilage and its subchondral bones.

Discussion

Two distinct types of skeletal progenitors, growth-associated skeletal progenitors and adult mesenchymal stromal progenitors, are present to promote growth, maintenance and repair of endochondral bones. The former cells reside within or in the proximity to the growth plate cartilage and can be marked by promoter/enhancers of chondrocyte-related genes such as *Sox9* and *Col2a1*. We have previously reported that *Col2a1-cre* transgene can mark such early skeletal progenitors in the limb (4). The relationship of skeletal progenitors between endochondral and intramembranous bone formation is unknown. Here in this study, we first asked whether *Col2a1-cre* could mark similar early cells of the skeletal lineage in the cranial base where endochondral bone formation takes place. As expected, *Col2a1-cre* marked a great majority of skeletal cells, including chondrocytes, osteoblasts/cytes and bone marrow stromal cells. As *Col2a1* is also reported to be present in intramembranous osteoblast precursors and/or preosteoblasts (6,7), we next asked whether *Col2a1-cre* could mark similar

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early cells in the calvaria where intramembranous bone formation takes place. *Col2a1* mRNA is transiently expressed by preosteoblasts and 'chondrocyte-like osteoblasts' during early development of intramembranous bones (6), as well as by calvarial bone osteoblasts (7) . In addition, previous reports have demonstrated the existence of transient cartilages in the calvaria (11,12). We found that, during early stages, *Col2a1-cre* marked a number of osteoblasts/cytes especially in the lateral calvaria, where the underlying cartilage underpinned the bone structure during early stages (12). Subsequently, *Col2a1-cre*-marked osteoblasts/cytes increased during the postnatal growth phase. We could not determine whether their progenitor cells or osteoblasts themselves express *Col2a1*. More notably, *Col2a1-cre* did not mark osteoblasts/cytes near the midsagittal suture. Instead, a distinct group of suture mesenchymal cells were consistently marked by *Col2a1-cre*, also suggesting the possibility that suture mesenchymal cells might also express *Col2a1*. Third, we asked whether *Col2a1-cre* marks early skeletal progenitors in a secondary cartilage present in the mandibular condylar cartilage. The mandibular condyle cartilage is a secondary cartilage lacking growth plates (13), where direct transformation of chondrocytes into osteoblasts occurs (5). Development of the mandibular condyle starts at around E14.5 (14), when Sox9-positive mesenchymal cells condense on the posterior side of the mandible body (5). The proximal portion of the mandible has been suggested to contain osteo-chondroprogenitors (15). Secondary cartilages are derived from mesenchymal progenitor cells that express markers of early osteoblast differentiation, such as alkaline phosphatase (ALP) and runt-related transcription factor 2 (Runx2). This is different from the growth plate, where *Runx2*-expressing precursors are derived from *Sox9*-expressing progenitor cells (16). This reversal expression pattern of *Runx2* and *Sox9* is a unique feature of mandibular condylar cartilage formation; skeletal progenitor cells in the mandibular condylar cartilage are necessary to differentiate down an osteogenic lineage prior to chondrogenic differentiation (17). In contrast, some reports advocate that chondrocytes in the mandibular condylar cartilage can transform into osteoblasts (5); therefore the mechanism of development of mandibular condyle is complex. We found that, as expected, *Col2a1-cre* marked a majority of chondrocytes and osteoblasts/cytes during the early phase. However, *Col2a1-cre* only marked a small fraction of these cells in the later post-growth phase, suggesting that *Col2a1*-expressing cells only transiently contributed to the mandibular condylar formation. This discrepancy might be attributed to the unique complexity of the mandibular condylar cartilage. Our findings collectively suggest that, while *Col2a1* is expressed by early progenitors of the skeletal lineage in 'canonical' endochondral bone formation occurring in the cranial base, other mechanisms of craniofacial bone formation utilizes *Col2a1*-expressing cells in a different manner. Deliberate considerations would be necessary in extrapolating cell lineage and molecular findings obtained from the limb to the craniofacial skeletal structures.

Conclusions

Col2a1 is expressed by early skeletal progenitors of the skeletal lineage in canonical endochondral bone formation occurring in the cranial base. In contrast, other ossification mechanisms of the craniofacial complex utilize *Col2a1*-expressing cells in a different

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manner, whereby *Col2a1* may be expressed in more differentiated or transient cell types of the skeletal lineage.

Acknowledgments

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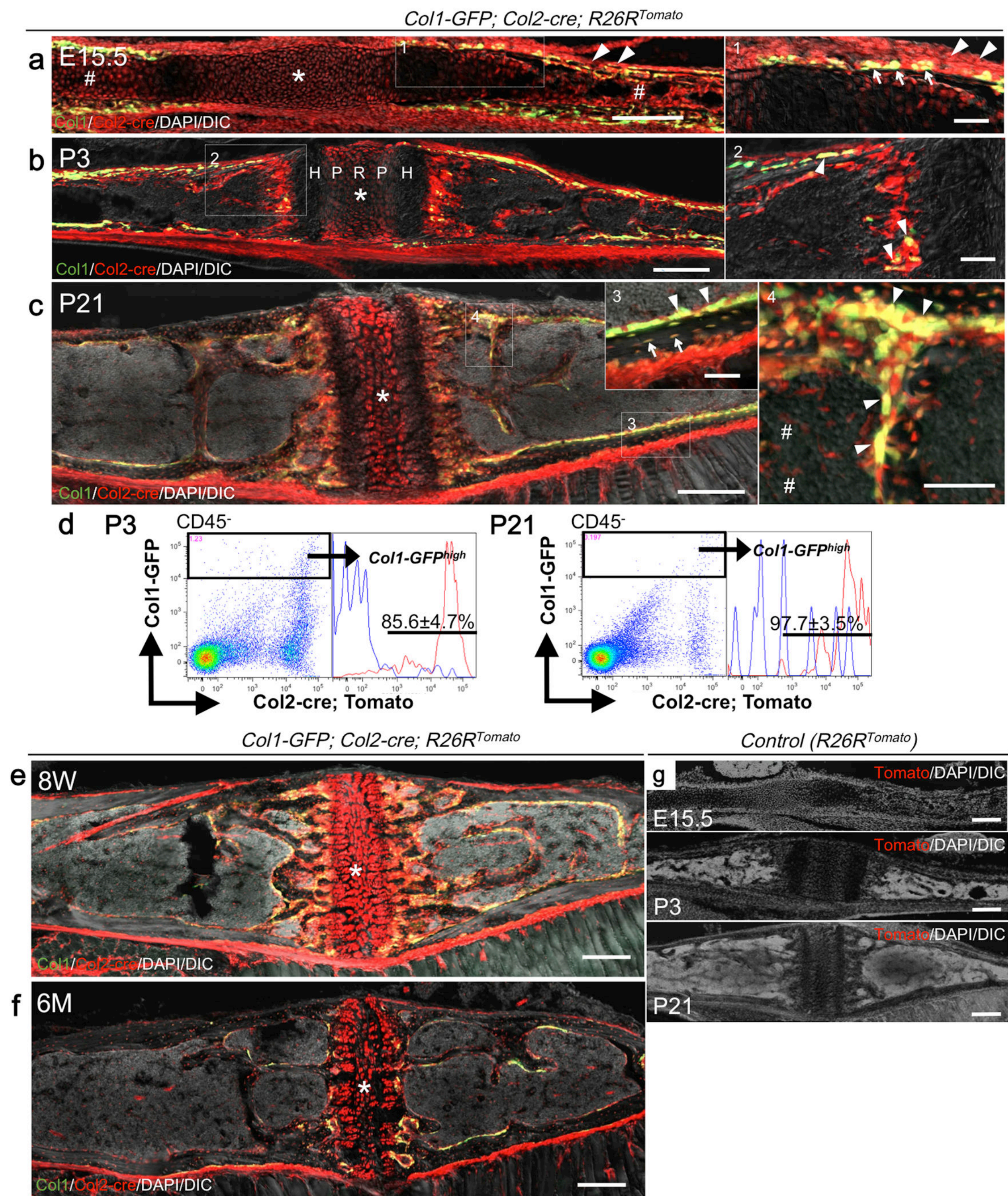

Fig.1.

Fate mapping of *Col2a1-cre*$^{+}$ cells in the cranial base synchondrosis.

(a) Embryonic day 15.5 (E15.5). Asterisk: SOS. Sharp: the ossification center. Arrowheads: perichondrium.

(b) At postnatal day 3 (P3). Arrowheads: GFP/tdTomato^{+} osteoblasts.

(c) P21. Arrows: Tomato^{+} osteocytes. Arrowheads: GFP/tdTomato^{+} osteoblasts. Sharps: stromal cells.

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(d) Flow cytometry analysis at P3 (left panels) and P21 (right panels). Right subpanels: histograms of the GFP^{high} fraction developed for Tomato; blue lines: GFP^{high} Tomato^{neg} control cells. X-axis: tdTomato, Y-axis: GFP. *n*=3 (P3), *n*=4 (P21). All data represented as mean ± S.D.

(e, f) 8 weeks (e) and 6 months (f) of age. Asterisk: SOS.

(g) Negative controls (*R26R*^{Tomato}) at E15.5, P3, P21.

Scale bars: 200μm in (main panels in a-c, e, and f), 100 μm in (right panels 1, 2 and 4 in a-c) and 50μm in (inset 3 in c)

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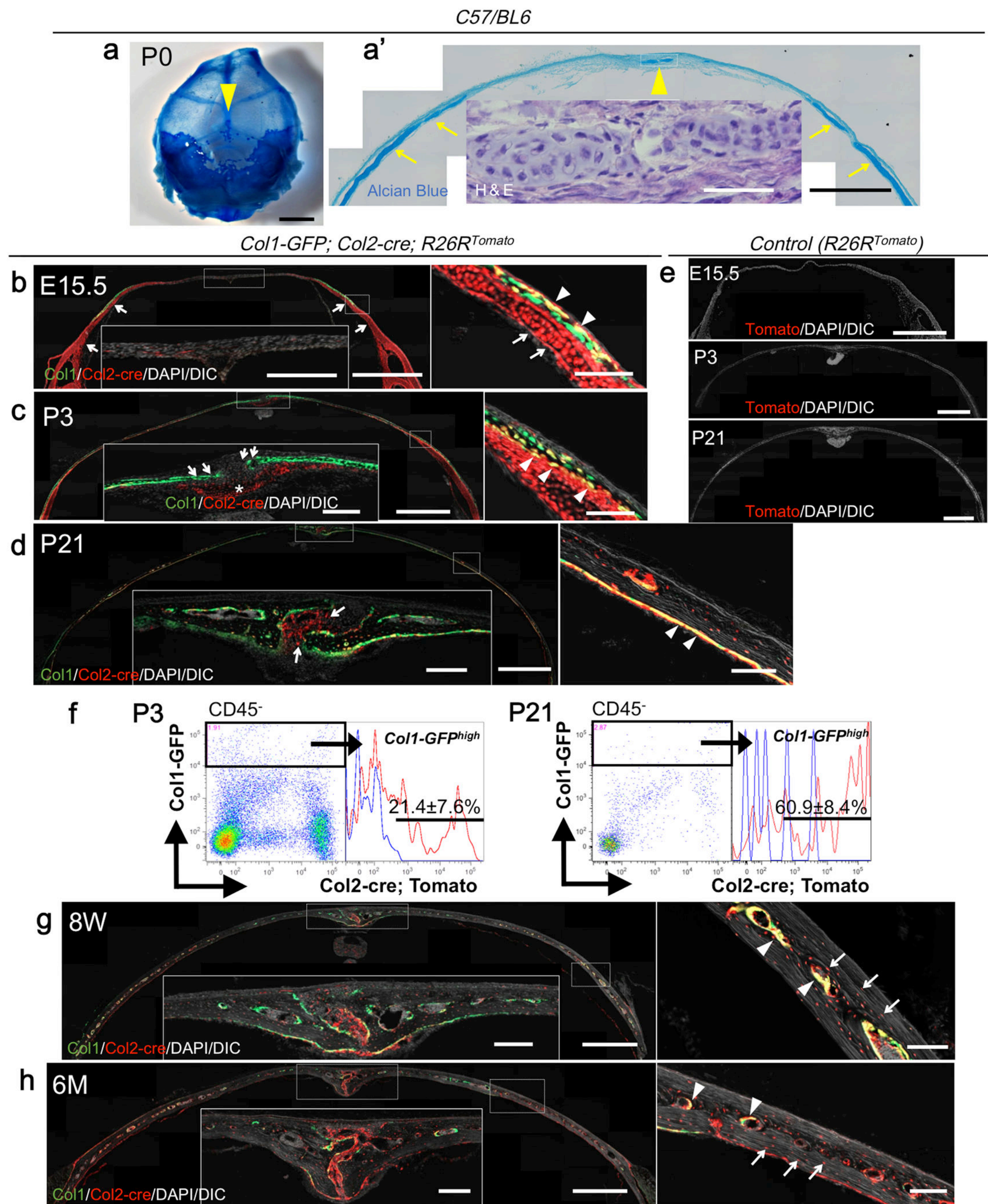

Fig.2.

Contribution of *Col2a1-cre*+ cells to osteoblasts and suture mesenchymal cells in the calvaria.

(a, a′) Whole-mount Alcian Blue (a) and H&E staining (a′) of P0 C57/BL6 mice. Arrowheads: cartilage islands. Arrows: underlying cartilage.

(b) E15.5. Arrows: underlying cartilage. Arrowheads: GFP/tdTomato+ osteoblasts.

(c) P3. Asterisk: underlying cartilage underneath the midsagittal suture. Arrows: GFP+/tdTomato- osteoblasts near the midsagittal suture. Arrowheads: GFP/tdTomato+ osteoblasts.

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(d) P21. Arrows: suture mesenchymal cells derived from *Col2a1-cre*$^{+}$ cells. Arrowheads: GFP/tdTomato^{+} osteoblasts in the inner aspect of the calvaria.
(e) Negative controls (*R26R*^{Tomato}) at E15.5, P3, P21.
(f) Flow cytometry analysis at P3 (left panels) and P21 (right panels). Right subpanels: histograms of the GFP^{high} fraction developed for Tomato; blue lines: GFP^{high} Tomato^{neg} control cells. X-axis: tdTomato, Y-axis: GFP. n=3 (P3), n=4 (P21). All data represented as mean ± S.D.
(g, h) 8 weeks (g) and 6 months (h) of age. Arrowheads: GFP/tdTomato^{+} osteoblasts on the endosteum. Arrows: tdTomato^{+} osteocytes.
Scale bars: 5mm in (a), 1mm in (a′,b-e, g and h) ,50μm in (inset in a′) ,200μm (insets in b-d, g and h) and 100μm in (right panels in b-d, g and h).

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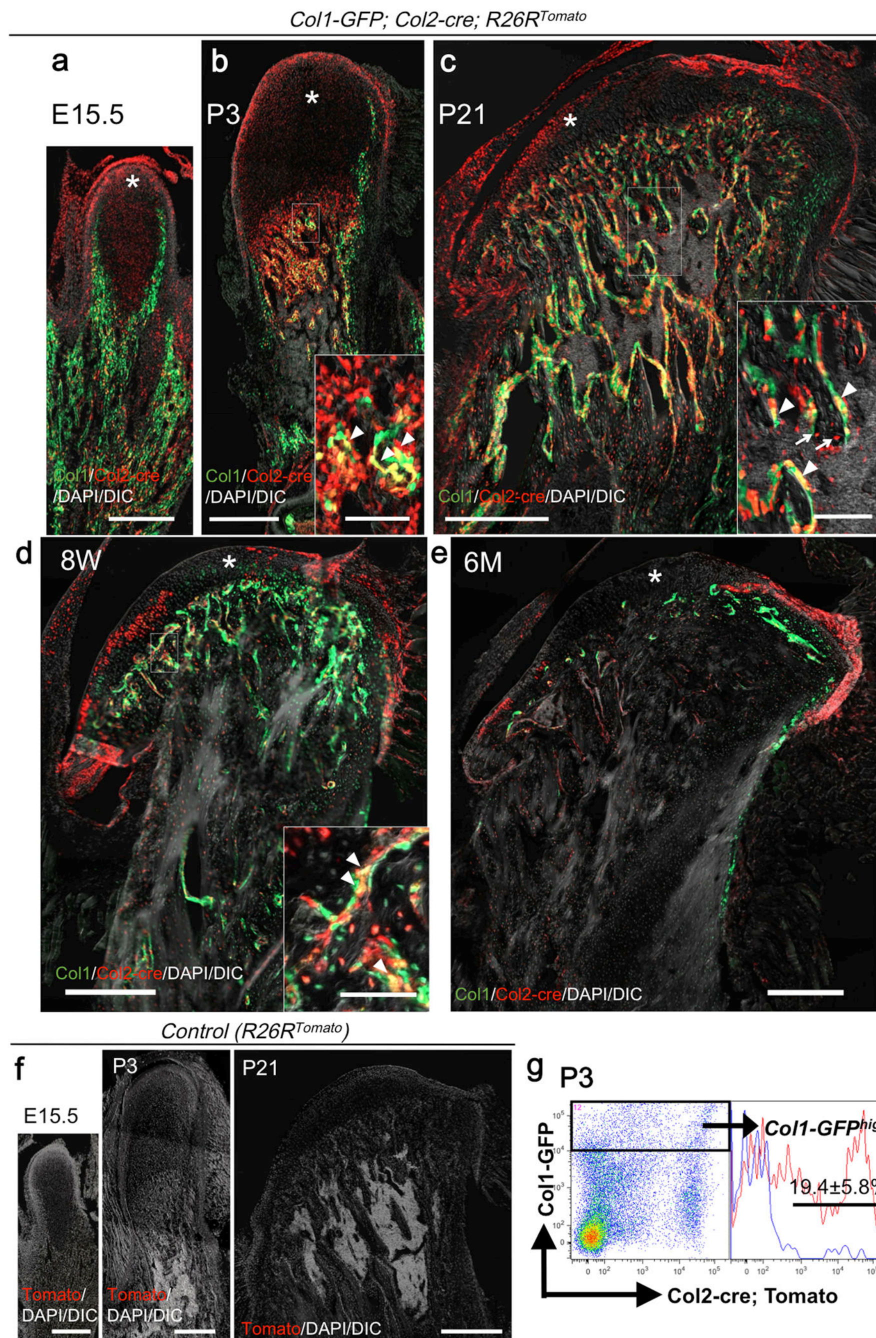

Fig.3.

Transient contribution of *Col2a1-cre*$^{+}$ cells to chondrocytes and subchondral osteoblasts in the mandibular condyle.

(a-e) E15.5 (a), P3 (b), P21 (c), 8 weeks (d) and 6 months (e) of age. Asterisk: condylar cartilage, arrowheads: GFP/tdTomato^{+} osteoblasts. Arrows: tdTomato^{+} osteocytes.

(f) Negative controls (*R26R*$^{\text{Tomato}}$) at E15.5, P3, P21.

Scale bars: 400μm in (a-f), 100 μm in (insets in b,c).

(g) Flow cytometry analysis at P3. Right subpanels: histograms of the GFP^{high} fraction developed for Tomato; blue lines: GFP^{high} Tomato^{neg} control cells. X-axis: tdTomato, Y-axis: GFP. n=3. All data represented as mean ± S.D.

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