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The Critical Role of Mesenchymal Progenitors in Initiating the Secondary Ossification Center at the Epiphyseal Cartilage

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Introduction

Endochondral ossification in long bones proceeds via the development of a diaphyseal primary ossification center (POC) in embryo and an epiphyseal secondary ossification center (SOC) after birth. While POC formation has been extensively studied, the initiation and expansion of SOC are largely uncharacterized. In particular, whether mesenchymal progenitors, which have been shown to move into developing POC along with invading blood vessels,[1] play a similar role in the SOC formation is not known. Tomato (Td)+ cells of *Col2-Cre Rosa-Td* mice were recently identified as mesenchymal progenitors that constantly replenish osteoblasts and osteocytes in POC.[2] In this study, we used this lineage tracing method to investigate the potential actions of mesenchymal progenitors in SOC formation and development.

Methods

All animal work was approved by the Institutional Animal Care and Use Committee at the University of Pennsylvania. *Whole mount staining of bone tissues- Col2-Cre Rosa-Td* mice were generated by breeding *Col2-Cre* mice with Rosa-Td homozygous mice. Femurs and tibiae were harvested at various time points after birth, fixed in 2% PFA, decalcified in 15% EDTA, immersed into 20% sucrose and 2% polyvinylpyrrolidone, and processed for thick frozen sections, which underwent immunofluorescent staining with antibodies against Endomucin, VEGF, PDGFRβ, osterix (Osx), and DIPEN. Fluorescent signals were acquired from Zeiss LSM-710 laser scanning confocal microscope with a depth of 100 μm and 3D images were processed by Volocity. *Cell migration-* Bodyen chamber was used to study the migration of mouse primary endothelial cells towards the conditioned media from mouse bone marrow mesenchymal progenitors. *Fibroblast colony-forming cells (CFU-F) and differentiation assays-* Td^{high} cells from the epiphysis of *Col2-Cre Rosa-Td* pups were sorted and seeded at a density of 6,000 cells/T25 for CFU-F assay. Those cells after expansion were cultured in osteogenic and adipogenic media for differentiation assays. *Statistics-* Data are expressed as means ± SEM and analyzed by unpaired, two-tailed Student's t-test.

Results

We observed that in P4 *Col2-Cre Rosa-Td* pups, while most epiphyseal chondrocytes were Td+, those located at cartilage surface (Td^{high} cells) had much higher Td signal than those inside cartilage and therefore were the only visible cells after elevating the threshold of Td signal (Figure 1A). Since later all osteoblasts, osteocytes, and Osx+ osteoprogenitors within SOC were Td^{high} (Figure 1B), those Td^{high} cells at cartilage surface likely contain mesenchymal progenitors responsible for subchondral bone formation. Indeed, sorted Td^{high} cells had very high CFU-F frequency (6×10^{-3}) and were able to differentiate into

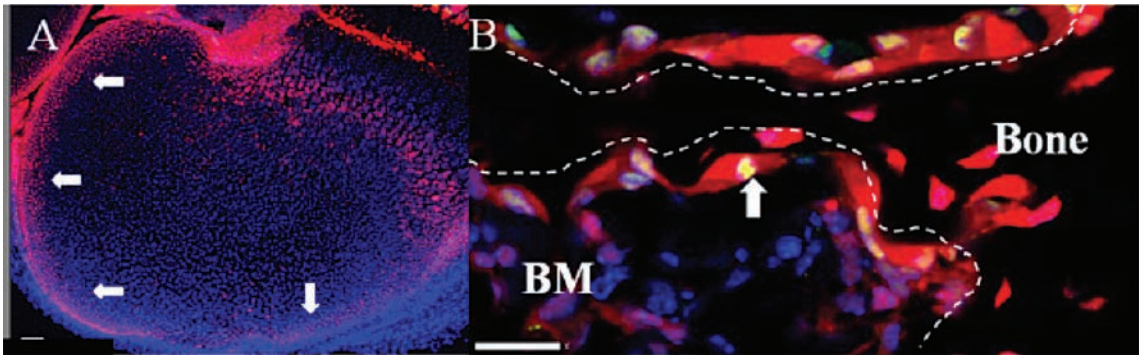

Figure 1. Td^{high} cells in the epiphyseal perichondrum contain mesenchymal progenitors that reconstitute subchondral bone after SOC formation. A. At P4, chondrocytes at perichondrium (arrow) express a high level of Td. B. At P18, osteoblasts on the bone surface (dash line), osteocytes within bone, and Osx+ cells (arrow) were all Td^{high} cells (Td: red; Osx: green; DAPI: blue).

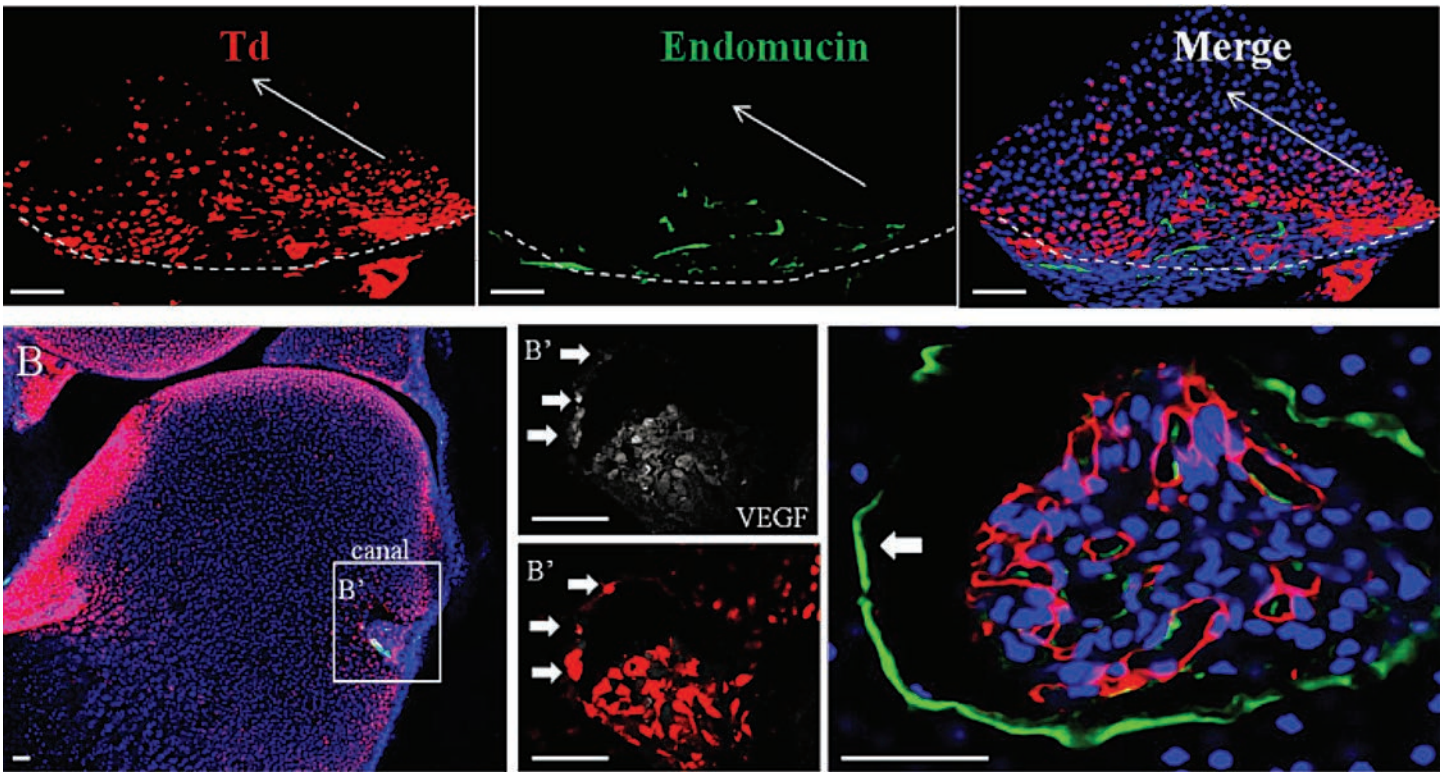

Figure 2. Td^{high} cells promote vasculogenesis during the initial stage of cartilage canal formation. A. Td^{high} cells penetrate into cartilage, followed by individual Endomucin+ endothelial cells. Arrow points to the direction of canal advancement, and perichondrium is labeled with dash lines. B. Td^{high} cells at the leading edge were VEGF+. B': zoom in images. Arrows point to Td+VEGF+ cells at the front edge of a cartilage canal. C. DIPEN staining at the front edge (arrow) and wall of a cartilage canal (DIPEN: green; Endomucin: red; DAPI: blue; scale bar = 50 μm).

osteoblasts and adipocytes in culture. At P5, using a whole mount staining with 3D image reconstruction, we observed that Td^{high} cells started to penetrate into cartilage at discrete surface sites, followed by individual Endomucin+ endothelial cells (Figure 2A). TUNEL staining identified that chondrocytes within these sites undergoes apoptosis. At P6, cartilage canals were initiated at these sites. The canal wall, which consists of Td^{high} cells, endothelial cells, and chondrocytes, was again preceded by VEGF+ Td^{high} cells leading the front edge (Figure 2B). At this stage, surrounding chondrocytes were VEGF−. Inside the canal, there was a cone-shaped and dense cell cluster, including Td^{high} cells, single endothelial cells, vessels, and CD45+ cells, with the base at the cartilage surface. Those Td^{high} cells became proliferative (BrdU+) and migrated either as perivascular cells or as individual cells. Interestingly, they only expressed mesenchymal progenitor marker PDGFRβ, but not other markers such as Osx, CD44, and CD105, implying that they are probably at the early stage of stem cells. There was a space about 30 um long between the canal wall and the cell cluster where only erythrocytes were detected. The canal wall was distinctly labeled by antibodies against the aggrecan-degraded product DIPEN (Figure 2D) and apoptotic chondrocytes. The unique initiation and structure of cartilage canal strongly suggest that mesenchymal progenitors play a leading role in promoting vasculogenesis, a de novo vessel formation process, and together with chondrocytes create a path for canal protrusion. In vitro, mesenchymal progenitors greatly stimulate the migration of endothelial cells (Figure 3). After P8, all hypertrophic chondrocytes were VEGF+ so the expansion of SOC was led by blood vessels growth into chondrocytes followed by Td^{high} progenitors (Figure 4), the same mechanism by which POC is developed.

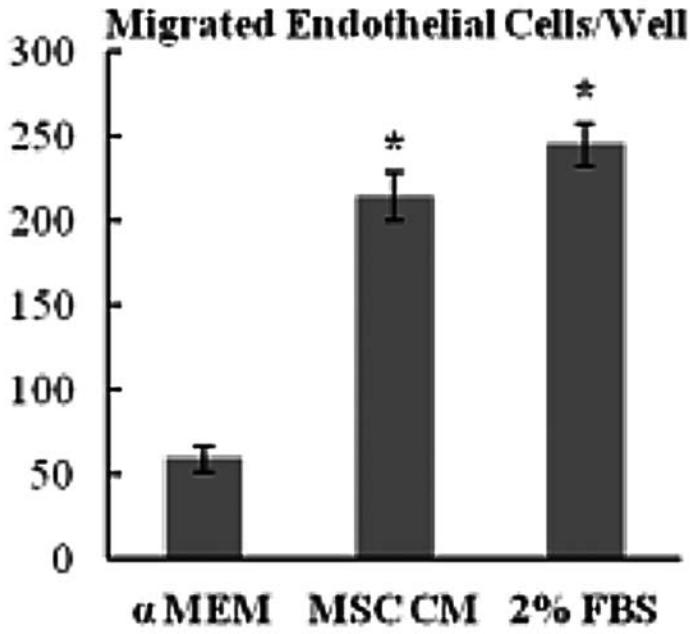

Figure 3. Endothelial cells migrate towards the conditioned media (CM) from mesenchymal progenitors (Mean ± SEM, n = 4, *: $p < 0.01$ vs αMEM).

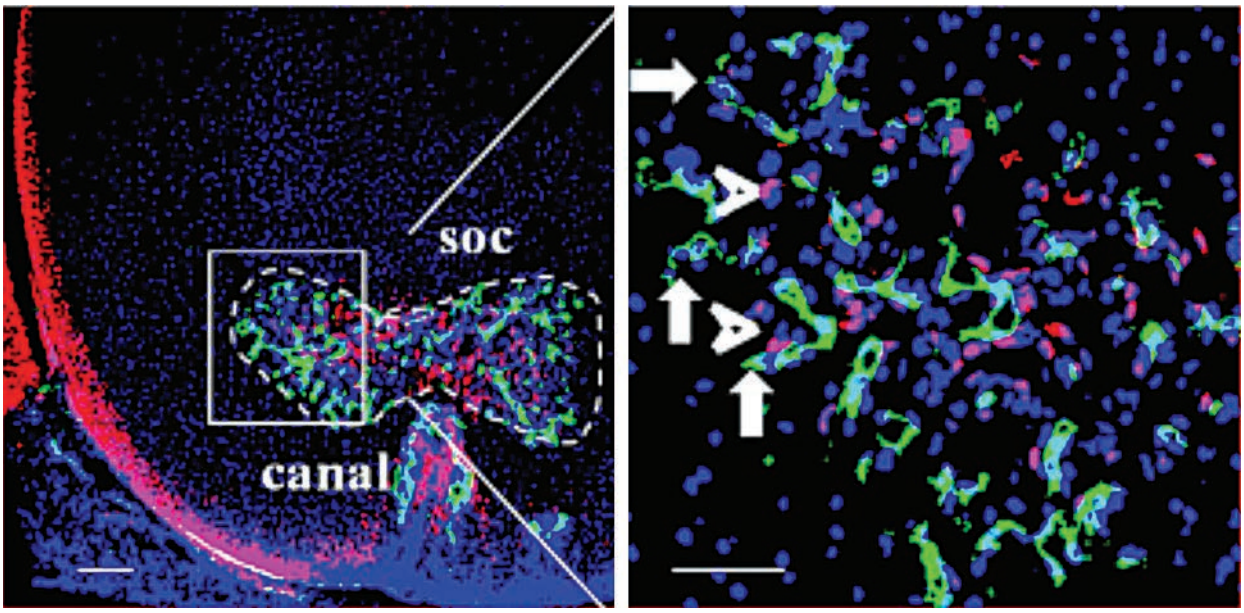

Figure 4. Vessel protrusion (arrows) precedes Td^{high} cells (arrow head) during SOC expansion at P9 (Endomucin: green; Td: red; DAPI: blue).

Discussion:

Our studies demonstrate a critical function of mesenchymal progenitors within the perichondrial Td^{high} cells in initiating SOC formation. Those cells are the first to move towards the center of epiphyseal cartilage and are part of the front edge of cartilage canal. They also chemoattract endothelial cells to promote vasculogenesis, possibly through a VEGF− dependent pathway, followed by cartilage canal formation. This mechanism is distinct from how POC is formed, by which angiogenesis precedes the moving of mesenchymal progenitors into cartilage.[1] This difference might be due to the different expression pattern of VEGF. In cartilage, only hypertrophic chondrocytes are VEGF+ cells. In contrast to POC where mesenchymal progenitors and vessels move into VEGF+ hypertrophic chondrocytes, the superfacial layer of articular cartilage before SOC formation does not contain VEGF+ chondrocytes. Instead, VEGF+ Td^{high} cells play a pivotal role in promoting vessel invasion. In addition, our studies revealed those mesenchymal progenitors undergo phenotypical changes during their migration from perichondrum to cartilage canal, and eventually become osteoblasts and osteocytes within SOC.

Significance

Using lineage tracing and 3D imaging approaches, we discovered a novel mechanism for mesenchymal progenitors to initiate SOC formation.

References

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2. Ono, N., *et al.,* *Nat Cell Biol*, 2014. 16 (12): p. 1157-1167.