**Fgf18-expressing cells in developing articular cartilage are progenitors for the superficial zone.**

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**INTRODUCTION:** Articular cartilage morphogenesis is a dynamic process that occurs over several weeks during postnatal growth. The mature tissue is characterized by a multi-zonal organization consisting of: a top zone of small, flat superficial cells; an intermediate/deep zone of large, column-organized articular chondrocytes flanked by resilience-providing matrix; and a bottom zone of mineralized chondrocytes attached to subchondral bone. The superficial zone cells are often referred to synonymously with the expression of Prg4 and production of Lubricin. It has also been postulated that these Prg4-expressing superficial cells are developmental progenitors for all mature articular chondrocytes. However, work by our group shows that Prg4 is expressed broadly in articular cartilage progenitors at birth, not just the superficial layer, before becoming increasingly restricted to superficial cells at weaning and to maturity (Fig 1A). Recent work by David Ornitz led to the generation of a novel inducible Cre allele targeting Fibroblast growth factor 18 (Fgf18)3. The study revealed that Fgf18 cell lineage expression was observed at the articular cartilage surface in neonatal animals and that cells were maintained in adult animals. These data suggested that Fgf18 is an additional marker for superficial zone cells and that Fgf18creERT2 is a novel inducible Cre allele for lineage tracing of these cells. In this study, we aimed to validate Fgf18 as a marker for superficial zone cells throughout articular cartilage morphogenesis and test whether these cells are progenitors for all zones in mature articular cartilage.

**METHODS:** Animal work was approved by the CHOP IACUC. Mouse Models: To assess Cre recombination, Rosa26-TdTomato (Ai9, Jax #007909) was crossed to Fgf18creERT2 (from Dr. David Ornitz3) or to Prg4CreERT2 transgenic2, Tamoxifen (4mg) was given at E16.5 (with 2mg Progesterone to minimize dystocia) and collected at birth or at 9 weeks (n=3 per time point). Single-cell RNA sequencing (scRNA-seq): Dissected E16.5 wildtype knee interzones (n=5) were digested and quantified before encapsulation by Chromium Controller (10X Genomics). After library construction and sequencing with Cell Ranger, analyses were performed using Seurat. E16.5 and publicly available 3-week datasets were analyzed independently. Histologic Analyses: For RNAscopeTM, tissues were fixed in 4% paraformaldehyde, decalcified in 20% EDTA, and cryopreserved. Tissues were sliced in 4% paraformaldehyde, decalcified in 20% EDTA, and cryopreserved. Tissues were stained with DAPI for confocal imaging.

**RESULTS:** Our RNAscopeTM analyses revealed that Fgf18 is solely restricted to the superficial cells (Fig 1B) through postnatal articular cartilage development compared to Prg4 (Fig 1A). To gain further insight, we performed scRNA-seq analyses on wildtype E16.5 knee interzone (Fig 2A) and on a published dataset of 3-week-old knee joints and growth plate cartilage (Fig 2B). At E16.5, the interzone expresses high levels of Gafs and Coll2a1, and embryonic growth plate chondrocytes express high levels of Matn1/3. Prg4 and Fgf18 were both expressed in interzone cells (Fig 2A). At 3 weeks, growth plate chondrocytes were also distinguished by Matn1/3 and in articular chondrocytes 4 clusters of ‘superficial cells’ were identified based on expression of Prg4 (Fig 2B). Intriguingly, we found that only one cluster expresses high levels of Fgf18 consistent with spatial histologic analyses (Fig 1). These data suggest Fgf18-expressing cells represent a subpopulation of superficial cells. To test this, we performed lineage tracing of Fgf18- and Prg4-expressing cells from E16.5. As expected from previous findings2, Prg4-lineage cells constituted all layers of articular cartilage from embryonic to adult stages (Fig 3A). By contrast, Fgf18-lineage cells were restricted to the superficial layer at birth and produced mostly superficial zone cells in mature articular cartilage (Fig 3B).

**DISCUSSION:** These studies reveal that Fgf18 is a marker for superficial zone cartilage throughout articular cartilage development and that Fgf18-lineage cells marked from embryonic development are not progenitors for all zones in mature articular cartilage but are largely restricted to superficial zone cells. Indeed Fgf18 appears to be a more specific marker to the superficial cells at all stages compared to Prg4 and our data suggest that Fgf18/Prg4 co-expressing cells are “true” superficial cells defining only the first layer cells in articular cartilage. In addition, our lineage tracing data reveal that these Fgf18-expressing embryonic cells only rarely give rise to deep zone chondrocytes compared to Prg4-expressing cells induced at the same stage that constitute all layers of articular cartilage from embryonic stages on. Our data provide new insight into the morphogenesis of articular cartilage zones during postnatal development and suggest that distinct subpopulations of progenitors exist in the embryonic joint that contribute to cartilage zonal organization.

**SIGNIFICANCE/CLINICAL RELEVANCE:** Fgf18 is a novel and specific marker of superficial zone cells and lineage labeling reveals that the differentiation potential of Fgf18+ superficial zone progenitors may be more limited in vivo than previously believed. This knowledge is important to guide strategies for articular cartilage regeneration and also for understanding mechanisms of degeneration.


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