# *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[1]. 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[2]). 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 *Fgf18*$^{CreERT2}$ 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 *Fgf18*$^{CreERT2}$ (from Dr. David Ornitz[3]) or to *Prg4CreERT2* transgenic[2], 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 RNAscope™, tissues were fixed in 4% paraformaldehyde, decalcified in Morse's Solution (10% Sodium Citrate, 22% Formic Acid), and paraffin processed. RNAscope™ for *Fgf18* (manual assay probe no. 495421) was performed per manufacturer's protocol (2.5 HD Assay-RED detection). For lineage tracing, tissues were fixed in 4% paraformaldehyde, decalcified in 20% EDTA, and cryopreserved. Tissues were stained with DAPI for confocal imaging.

**RESULTS:** Our RNAscope™ analyses revealed that *Fgf18* is solely restricted to the superficial cells (Fig 1B) through postnatal articular cartilage development compared to *Prg4* (Fig 1A[2]). 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[4]). At E16.5, the interzone expresses high levels of *Gdf5*[5] and *Col22a1*[6], and embryonic growth plate chondrocytes express high levels of Matn1/3[7]. *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*[4] (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 findings[2], *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 cells 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.

**REFERENCES:** [1]Kozhemyakina+ *Arthritis Rheum* 2015 [2]Decker+ *Dev Biol* 2017 [3]Hagan+ *Dev Dyn* 2019 [4]Haseeb+ *PNAS* 2021 [5]Rountree+ *PLoS Biol* 2004 [6]Feng+ *Stem Cell Rep* 2019 [7]Hyde+ *Dev Biol* 2007

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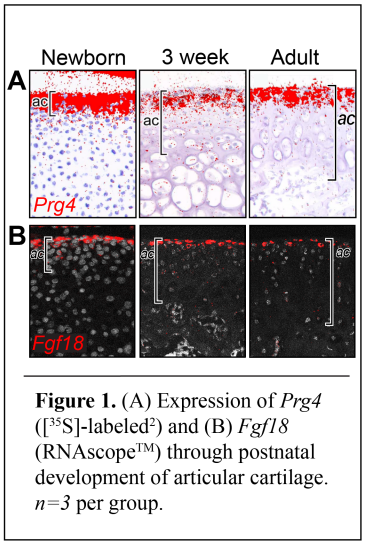


**Figure 1.** (A) Expression of *Prg4* ([$^{35}$S]-labeled[2]) and (B) *Fgf18* (RNAscope™) through postnatal development of articular cartilage. *n=3* per group.

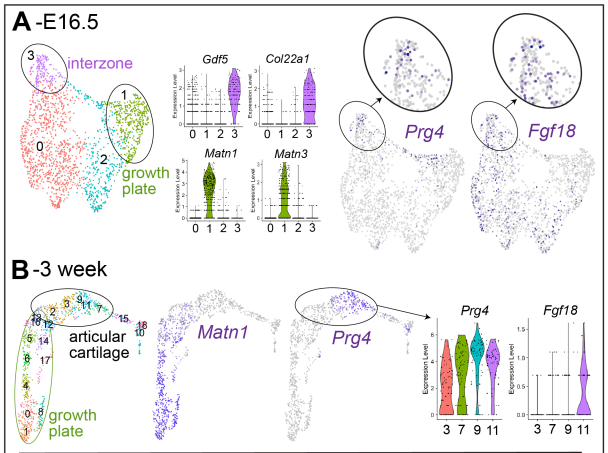


**Figure 2.** scRNA-seq of wildtype (A) knee interzone at E16.5 (*n=5*) and (B) knee articular and growth plate cartilage at 3 weeks (publicly available[4]).

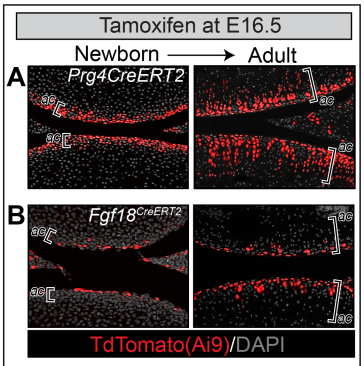


**Figure 3.** Lineage tracing in knee articular cartilage of (A) *Prg4CreERT2* and (B) *Fgf18*$^{CreERT2}$ with *Rosa26-Ai9* reporter. Tamoxifen induction at E16.5. Tissue collection at newborn and adult stages. *n=3* per group.