The distinct development of fibrous and fibrocartilaginous entheses

James Y. Chen[1], Ashley K. Fung[1], Noriaki Ono[2], Nathaniel A. Dyment[3], Stavros Thomopoulos[1]
[1]Columbia University, New York, NY, [2]University of Texas Health Science Center at Houston School of Dentistry, Houston, TX, [3]University of Pennsylvania, Philadelphia, PA
sat2@columbia.edu

Disclosures: The authors have no disclosures.

Introduction: Injuries to the enthesis, the specialized tissue that connects tendon and ligament to bone, prevalently cause pain and limit joint function. Most entheses are fibrocartilaginous and consist of a mineralized gradient across tendon, unmineralized fibrocartilage, mineralized fibrocartilage, and bone. However, this native architecture is not regenerated following injury or with surgical reattachment and is instead replaced by fibrous scar tissue, resulting in high rates of reinjury. A second type of enthesis—termed fibrous—consists of perforating collagen fibers that attach directly to the bone cortex or the periosteum; these attachments resemble the fibrous attachments that form post-injury at the healing tendon-to-bone interface[1]. The medial collateral ligament (MCL) of the knee uniquely has a fibrocartilaginous enthesis at its femoral insertion and a fibrous enthesis at its tibial insertion,[2] offering a model for examining the development of these distinct structures. Two key factors were previously reported as important for enthesis mineralization: Hedgehog (Hh) and parathyroid hormone-related protein (*PTHrP*); these two factors form a feedback loop in endochondral growth plates that drives bone growth[3]. Previous work at the enthesis identified a Hh-responsive cell population marked by *Gli1*[4,5] and a role for PTHrP in enthesis mineralization[6]. As the distinct developments of fibrocartilaginous and fibrous entheses are insufficiently understood, we hypothesized that PTHrP is differentially expressed in the two insertions.

Methods: Knee samples were collected from two mouse lines. In one model, a *Gli1*-CreER^{T2} mouse line was crossed with an Ai14 tdTomato reporter line to yield *Gli1*-CreER^{T2};Ai14 mice (N=11 female mice and 12 male mice). These mice were injected with tamoxifen on postnatal day 5 (P5) to label *Gli1*-positive cells for lineage tracing. The second model was a *PTHrP*^{mCherry} mouse line[7] allowing for localization of *PTHrP*-positive cells. Mice were sacrificed on P11, P14, P18, and P28. The samples were fixed, embedded, and cryosectioned. Sequential sections were evaluated for reporter expression, deposited mineral (calcein blue), bone remodeling (tartrate-resistant alkaline phosphatase [TRAP] activity), and fibrocartilaginous morphology (toluidine blue). PTHrP-mCherry signal was detected using immunohistochemistry for mCherry. Three biological replicates were used per assay, except for the P14 and P18 timepoints of the *Gli1*-CreER^{T2};Ai14 mouse model for which 2 biological replicates were used.

Results: Whereas the fibrocartilaginous femoral enthesis of the MCL underwent extensive postnatal maturation that included the deposition of proteoglycans and mineral between P14 and P18, the fibrous tibial enthesis showed limited compositional changes (**Figures 1A-B**). At this time of mineralization, the fibrocartilaginous enthesis showed more bone remodeling activity than the fibrous enthesis. Instead, periosteal osteoclast activity was greater in the fibrous enthesis later in the postnatal timeline (**Figure 1C**). Persistently across all timepoints observed, *Gli1*-lineage cells were found to extensively populate both entheses (**Figure 2A**). These *Gli1*-lineage cells were also present throughout the adjacent periosteum. Meanwhile, *PTHrP*-positive cells were expressed in both entheses only at earlier timepoints (P11); subsequently, *PTHrP* expression declined in the fibrocartilaginous enthesis while it remained durable in the fibrous enthesis (**Figure 2B**). In contrast to the substantial presence of *Gli1*-lineage cells, a smaller proportion of the fibrous enthesis cells were positive for *PTHrP* at each timepoint.

Discussion: Across the distinct developments of fibrocartilaginous and fibrous entheses, Hh-associated *Gli1*-lineage cells populate and persist in both fibrocartilaginous and fibrous entheses while *PTHrP* is differentially expressed more strongly and durably in the fibrous enthesis. *PTHrP* may shape these processes through positive regulation of *Sox9*[8] and antagonization of *Runx2*[9], both of which inhibit chondrocyte hypertrophy and downstream mineralization. Furthermore, previous studies have also implicated *PTHrP* in promoting periosteal osteoclast activity enabling migration of fibrous insertions along bone surfaces to accommodate linear growth[6,10], consistent with the observation of increased osteoclast activity in the fibrous enthesis at P28 during peak linear growth. Further examination with gene expression assays and knockout models is warranted to better elucidate and discern these mechanisms. Lastly, the prevalence of *Gli1*-lineage cells throughout both entheses and the periosteum, in contrast to the expression of *PTHrP* in a smaller subset of enthesis cells, motivates future inclusion of more postnatal timepoints to better explore these temporal patterns.

Significance: Reestablishing the zonal architecture of fibrocartilaginous entheses after injury remains a principal challenge. This work forms a basis for further study of the role of Hh and *PTHrP* in these insertion structures that may inform the design of therapeutic approaches for enhancing tendon-to-bone repair.

References: [1]Oguma+, *J Ortho Res*, 2001. [2]Thambyah+, *Anat Rec*, 2014. [3]Fang+, *Matrix Biol*, 2022. [4]Schwartz+, *Development*, 2015. [5]Felsenthal, *Development*, 2018. [6]Wang+, *JBMR*, 2014. [7]Mizuhashi+, *Nature*, 2018. [8]Huang+, *PNAS*, 2001. [9]Li+, *Exp Cell Res*, 2004. [10]Wang+, *Anat Rec*, 2013.

Acknowledgements: This work was supported by the NIH (R01 AR080717 to ST) and the Columbia VP&S Office of Medical Education (JYC).

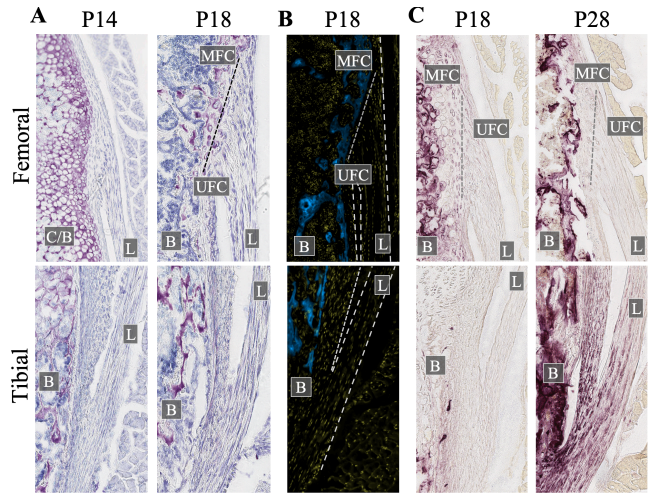

Figure 1. MCL insertions stained for **(A)** fibrocartilage morphology (toluidine blue), **(B)** deposited mineral (blue: calcein blue; pale green: DRAQ5), and **(C)** bone remodeling (purple: TRAP).

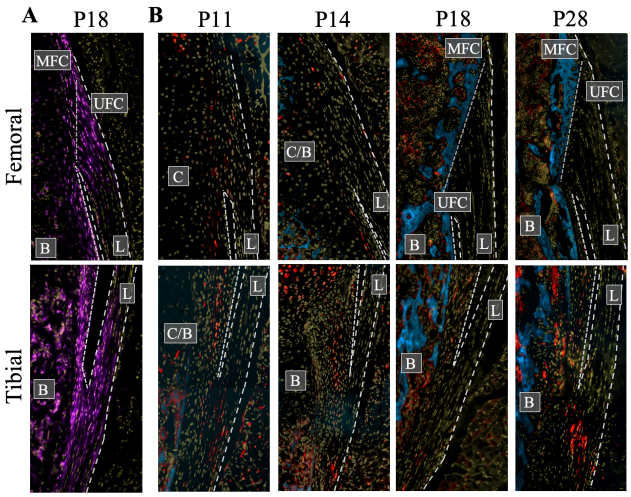

Figure 2. MCL insertions imaged for **(A)** Gli1-lineage cells (purple: Gli1;Ai14, pale green: DRAQ5) and **(B)** PTHrP+ cells and deposited mineral (red: PTHrP-mCherry, blue: calcein blue, pale green: DRAQ5).

B = Bone, C = Cartilage, L = Ligament, MFC = Mineralized Fibrocartilage, UFC = Unmineralized Fibrocartilage.