A distinct population of PTHrP+ cells is associated with the onset of supraspinatus tendon enthesis mineralization

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INTRODUCTION: The zonal nature of the developing tendon enthesis in many ways resembles the growth plate; unmineralized and mineralized fibrochondrocytes in the enthesis parallel the proliferative and hypertrophic zone chondrocytes of the growth plate, respectively. In the growth plate, the negative feedback loop between parathyroid hormone related peptide (PTHrP) and Indian hedgehog (Ihh) signaling has been well described. Pre-hypertrophic chondrocytes produce Ihh and stimulate PTHrP expression in the resting zone to balance chondrocyte proliferation, differentiation, and hypertrophy during expansion. Hedgehog signaling is also required for enthesis formation and hedgehog-responsive (*Gli1*+) cells make up the entire lineage of the fibrocartilage region cells [1]. In contrast to the growth plate, however, the enthesis lacks regions of resting and proliferative chondrocytes, and cells within the mineralized fibrocartilage maintain collagen X expression and a non-hypertrophic state that is not replaced by bone. Furthermore, single-cell RNA sequencing and ligand-receptor analysis of Gli1-lineage cells identified interactions between enthesis cells expressing PTHrP and its associated receptor, PTH1R [2]. This suggests that PTHrP may play a role in regulating enthesis mineralization. Using mouse models to label *PTHrP*+, *Gli1*-lineage, and *Col10a1*+ cells, our objective was to define the landscape of PTHrP expression in the developing supraspinatus tendon enthesis and contrast it to the humeral head growth plate.

METHODS: To localize *PTHrP*+ cells within the enthesis, *PTHrP*-mCherry [3] reporter mice were sacrificed at postnatal (P) days 0, 5, 8, 11, 14, 18, 28, and 56 (n=2-5/age). Approximately equal numbers of male and female mice were used. Mice were injected with EdU 3 hours prior to sacrifice. To identify parallels of *Gli1*-lineage and *Col10a1*+ cells within the growth plate, *Gli1*-CreER^{T2};Ai14 [4] and *Col10a1*-mCherry [5] models were also evaluated. *Gli1*-CreER^{T2};Ai14 mice were injected with tamoxifen at P5, and sacrificed at P11, P18, and P56 (n=3/age). *Col10a1*-mCherry mice were sacrificed at P11, P18, and P56 (n=3/age). To identify PTHrP-lineage cells, *PTHrP*-CreER^{T2};Ai14 mice were injected with tamoxifen at P5 and P7, and sacrificed at P18. Shoulders were prepared for cryohistology as previously described [6]. Briefly, shoulders were fixed and sectioned. PTHrP-mCherry signal was detected using immunohistochemistry for mCherry. Sections were stained with calcein blue to mark mineralized regions and alkaline phosphatase (ALP) to label mineralizing cells.

RESULTS: In the developing glenohumeral joint, *PTHrP*+ cells were initially restricted to the epiphyseal cartilage of the humeral head at P0 and P5 (not shown) and emerged at the supraspinatus tendon enthesis at P8, near the secondary ossification center (**Fig 1A**). At P11 and P14, mineralizing *PTHrP*+ cells localized to the mineralization front of the tendon enthesis, where populations were observed both outside and within the mineralized zone (**Fig 1B-C**). At P18, *PTHrP* + cells became more localized to the non-mineralizing zone proximal to the mineralization front (**Fig 1D**). No *PTHrP*+ cells were observed in the supraspinatus tendon enthesis at P28 (**Fig 1E**) or P56. We next compared the profiles of *Gli1*-lineage, *Col10a1*+, and *PTHrP*+ cells within the enthesis and humeral growth plate. At P18, *Gli1*-lineage cells spanned the length of both the enthesis and growth plate (**Fig 2A-B**), and collagen X was restricted to mineralized fibrochondrocytes and hypertrophic chondrocytes (**Fig 2C-D**). The identification of mineralizing *PTHrP*+ cells within the enthesis contrasted to the growth plate, where *PTHrP*+ cells were exclusive to resting zone chondrocytes (**Fig 2D-E**). EdU+ proliferating cells were also absent from the supraspinatus tendon enthesis at all ages, while proliferative zone chondrocytes were labeled in the growth plate (not shown). Additionally, while PTHrP-mCherry expression was localized to cartilaginous regions (**Fig 3A**), PTHrP-lineage cells were surprisingly identified in the tendon midsubstance when cells were labeled at P5 and chased to P18 (**Fig 3B, B-1**). PTHrP-lineage cells were also observed in the growth plate (**Fig 3B-2**) and the perichondrium.

DISCUSSION: This study identified a previously uncharacterized population of *PTHrP*+ cells within the developing supraspinatus tendon enthesis. *PTHrP*+ cells appeared at the enthesis shortly after the emergence of the secondary ossification center, localized to the mineralization front during active mineral deposition, and diminished by P28. This transient expression pattern contrasted with the presence of *PTHrP*+ cells in the resting zone of the growth plate, where they function as a stem/progenitor cell reservoir and regulate chondrocyte maturation [3]. Our findings suggest that while the enthesis shares key molecular programs with the growth plate—such as Hedgehog signaling through *Gli1*+ cells and *Col10a1*+ hypertrophic or mineralized fibrochondrocyte cells— PTHrP expression at the enthesis is uniquely associated with early mineralization events. PTHrP may therefore play a role in modulating the timing or extent of mineral deposition at the fibrocartilaginous enthesis rather than suppressing hypertrophy. Though the presence of PTHrP-lineage cells within the tendon midsubstance along with an absence in the enthesis region was unexpected, this expression is similar to PTHrP+ cells in the growth plate resting zone. The differential expression patterns of PTHrP-mCherry and PTHrP-CreER^{T2};Ai14 may be attributed to PTHrP-mCherry marking only actively expressing cells, as opposed to PTHrP-lineage descendants, suggesting that dynamic and transient PTHrP expression plays a critical role in lineage specification. Future studies are required to identify the origin of PTHrP-lineage cells, define the mechanistic and functional role of PTHrP in modulating enthesis mineralization, and explore transcription factors downstream of PTHrP, such as the *Mef2* family, in regulating fibrochondrocyte maturation.

SIGNIFICANCE: Restoring the native zonal fibrocartilaginous enthesis following rotator cuff tendon injury remains a significant clinical challenge. We identified a population of PTHrP+ cells that is associated with enthesis mineralization, suggesting that modulation of PTHrP may enhance tendon-to-bone healing during rotator cuff repair.

REFERENCES: [1] Schwartz+ Development 2015, [2] Fang+ NPJ Regen Med 2025, [3] Mizuhashi+ Nature 2018, [4] Ahn+ Cell 2004, [5] Maye+ Genesis 2011, [6] Dyment+ JoVE 2016

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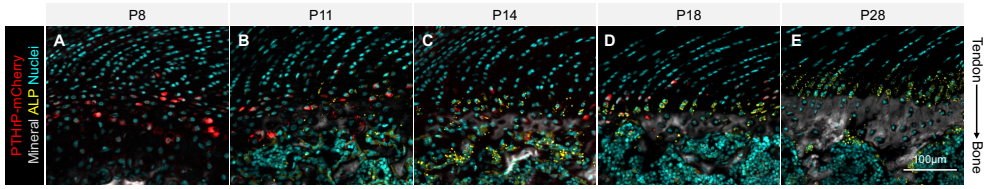

Figure 1: PTHrP-mCherry expression at A) P8, B) P11, C) P14, D) P18, and E) P28. Sections were stained with calcein blue and alkaline phosphatase (ALP) to label mineralized regions and mineralizing cells, respectively.

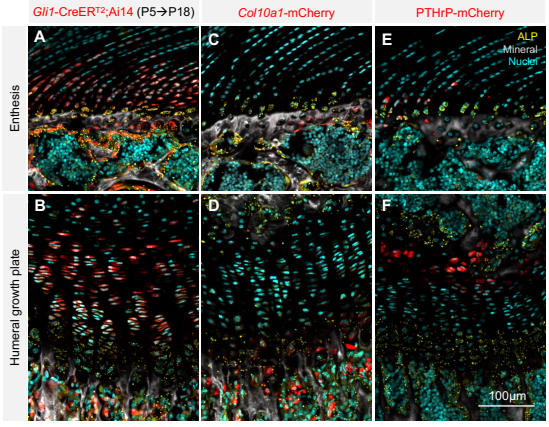

Figure 2: A) *Gli1*-lineage, B) *Col10a1*+, and C) *PTHrP*+ cell expression profiles at P18 within the supraspinatus tendon enthesis and humeral growth plate. Sections were stained with calcein blue and alkaline phosphatase (ALP) to label mineralized regions and mineralizing cells, respectively.

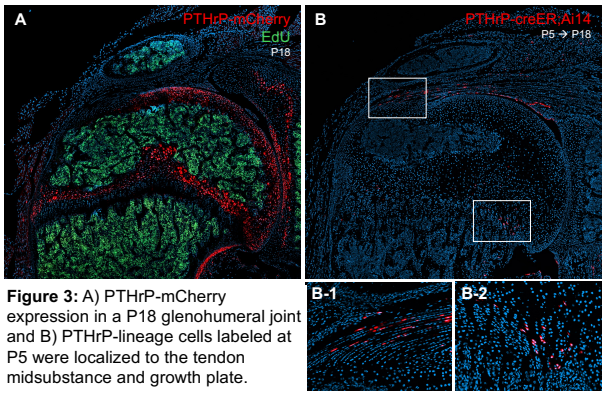

Figure 3: A) PTHrP-mCherry expression in a P18 glenohumeral joint and B) PTHrP-lineage cells labeled at P5 were localized to the tendon midsubstance and growth plate.