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Exploring Cell Differentiation Vs. Localization in Engineered Ligament-to-Bone Entheses

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Exploring Cell Differentiation Vs. Localization in Engineered Ligament-to-Bone Entheses

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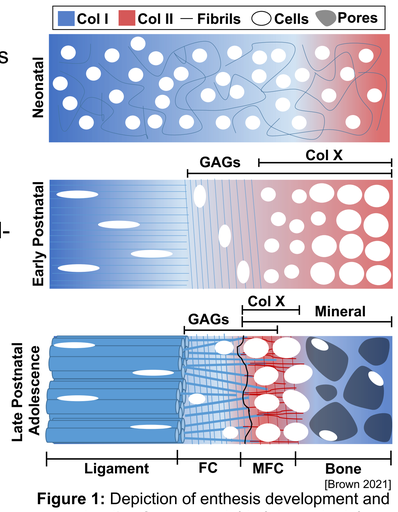
Introduction

- The ligament-to-bone attachment, or enthesis, is a complex tissue that translates load from elastic ligaments to stiff bone via gradients in organization, composition, and cell phenotype (Figure 1) [1]
  - Currently, no repair technique restores these gradients, yielding limited repair options & high failure rates [3]
- Recently, we developed a tensile-compressive culture system that guides cells isolated from the entire ACL to develop early postnatal-like zonal entheses with gradients in cell phenotype (Figure 2) [2]
  - These tissues hold great promise as functional replacements, but since they are made from a mixed population of cells, its unknown which cells are driving maturation
  - Understanding how the zonal cell phenotypes form is critical to developing functional ACL replacements and to driving regeneration of enthesis *in vivo* after graft repair
- Here we explored how ligament fibroblasts from ACL mid-substance and fibrochondrocytes from ACL entheses respond to mechanical cues in our culture system to assess if cells
  - Localize to specific tissue regions or
  - Differentiate in response to the local mechanical environment
- We hypothesize 50/50 co-culture of ligament and entheses cells will produce more native-like matrix gradients due to cells intrinsically localizing to their preferred mechanical microenvironment



[Brown 2021]

Figure 1: Depiction of enthesis development and maturation from neonatal to late postnatal.

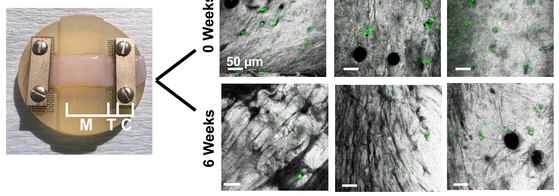


Figure 2: Novel static culture system drives early postnatal-like organization by 6 weeks. Grey = collagen, green = auto-fluorescent cells isolated from entire ACL [2].

Objective

Investigate how tissue-specific cells isolated from the ligament or enthesis produce zonal entheses with gradients in cell phenotype, matrix organization, and composition in our culture system

Methods

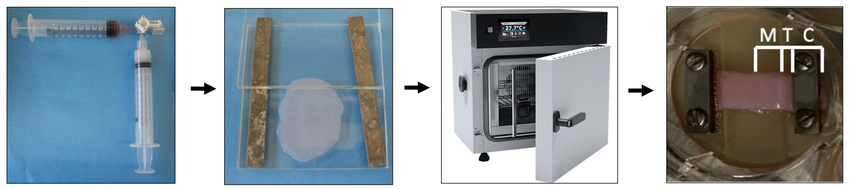


Figure 3. Depiction of construct creating and culture. Cells isolated from neonatal bovine ACLs are seeded into collagen and cast into a sheet gel. The gel is cut into rectangular constructs and cultured in the clamping device for up to 6 weeks.

- Type I collagen was extracted from rat tails and reconstituted at 30 mg/ml [2,4]
- 1-6 day old bovine anterior cruciate ligament fibroblasts and enthesis fibrochondrocytes were isolated from the ACL mid-substance and ACL entheses, respectively, and labeled with CellTrace Fluorescence
- Fluorescently labeled cells were mixed into collagen, injected between glass sheets, and gelled at 37°C for 1 hour to obtain 20 mg/ml constructs at $5x10^6$ cells/ml (Figure 3) [2,4]
  - Seeded with 100% ligament fibroblasts, 100% enthesis fibrochondrocytes, or a 50/50 mixture
  - 30 x 6 mm rectangular constructs were cut from sheet gels and statically clamped for 0 to 6 weeks to induce early postnatal-like enthesis development [2,4]
- Samples were analyzed in the middle (M), transition (T), and clamped (C) regions (Figure 3) [2,4]
- Confocal reflectance was used to assess collagen hierarchical organization [2,4]
- DNA, collagen, glycosaminoglycan (GAG), alkaline phosphatase (ALP) activity were measured using PicoGreen, hydroxyproline (hypro), DMMB, and paranitrophenol assays, respectively [2,4]
- 2-way ANOVAs with Tukey post-hoc ($p<0.05$ significant) were used for data analysis [2,4]

References

[1] Lu, H et al., Annu Rev Biomed Eng, 15:201-226, 2013. [2] Brown, E et al., Acta Biomaterialia, 2021. [3] Patel, S et al., J Orthop Res, 36:1069-1077, 2018. [4] Puetzer, J et al., Biomaterials, 2021. [5] Wang+ *J Orth Res*, 2006.

Results

Gross Morphological Changes & Zonal Collagen Concentrations

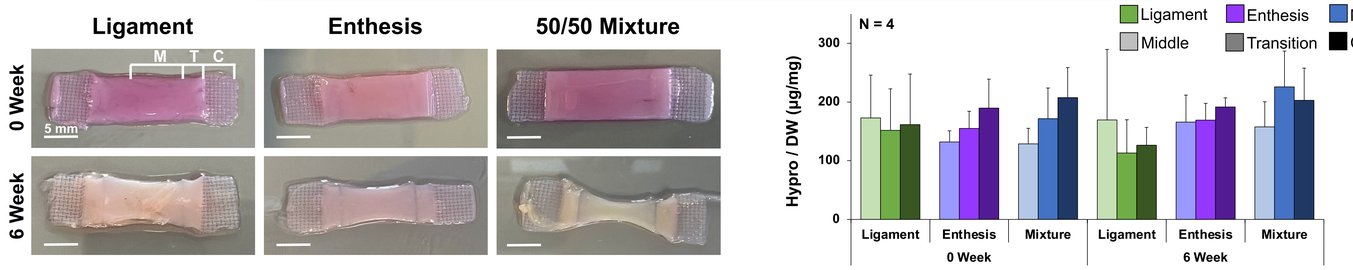


Figure 4. High-density collagen constructs at 0 & 6-weeks and zonal collagen represented by hydroxyproline (hypro) normalized to dry weight (DW). 50/50 constructs appears to contract more than 100% ligament or enthesis constructs; however there are no significant differences in zonal collagen concentration by 6 weeks.

Zonal Collagen Organization

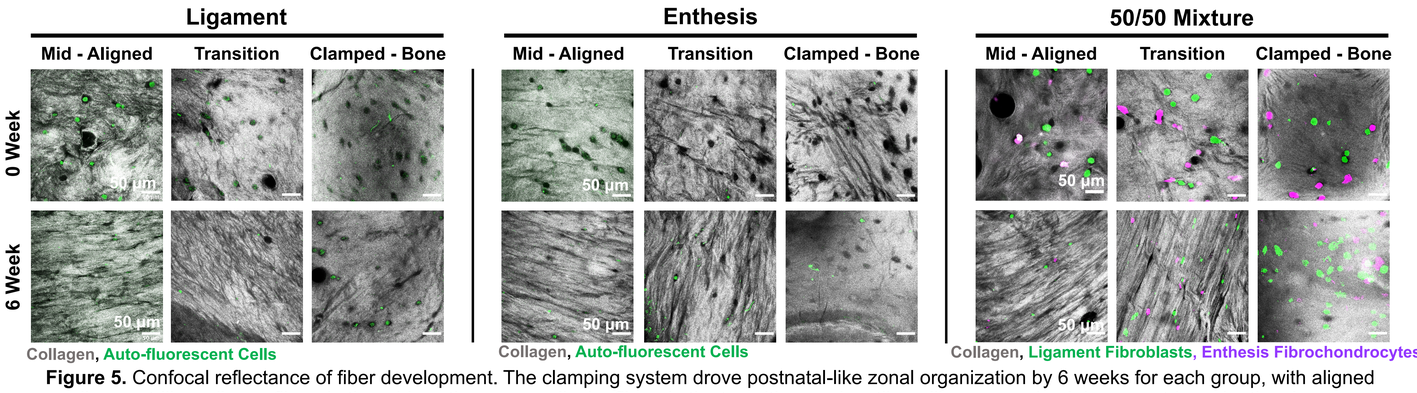


Figure 5. Confocal reflectance of fiber development. The clamping system drove postnatal-like zonal organization by 6 weeks for each group, with aligned ligament-like collagen fibers in the mid-section, perpendicular fibers in the transition, and cartilage-like unorganized dense matrix under the clamp.

Zonal Biochemical Properties

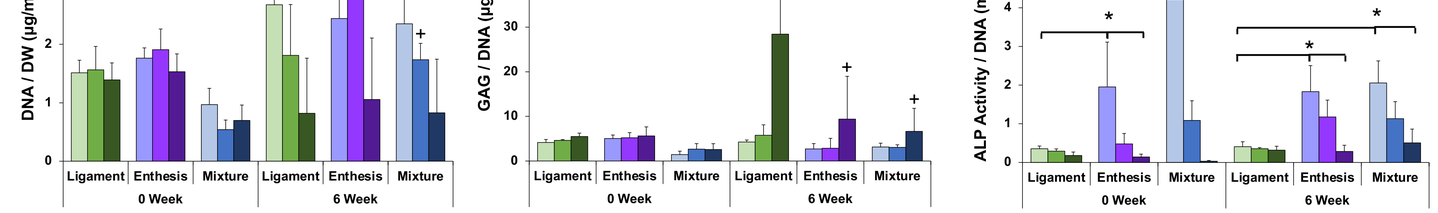


Figure 6. A) DNA normalized to dry weight (DW); B) GAG / DNA, and C) ALP Activity / DNA. By 6 weeks, DNA significantly increased in the middle and transition regions and GAG significantly increased under the compressive clamp for all cell types, suggesting chondrogenic-like tissue formation under the clamp. ALP activity was elevated in enthesis fibrochondrocytes and 50/50 mixture constructs. Significance to *bracket or +0 week region ($p < 0.05$). N = 4

Conclusions

- The tensile-compressive culture device drove postnatal-like organization with 3 unique zones of collagen organization in all groups, with aligned collagen fibers in the mid-section, perpendicular fibers in the transition, and cartilage-like unorganized matrix under the clamp
  - 50/50 mixture appeared to drive more accelerated and well-defined organization suggesting cells synergistically drive maturation
  - Both ligament fibroblasts and enthesis fibrochondrocytes are present in each zone by 6 weeks suggesting cells differentiate due to mechanical cues and do not localize to specific regions
  - On-going work is evaluating cell shape and cell number in each region to better assess cell localization and phenotypic changes
- Biochemical analysis revealed little differences between cell populations
  - All groups developed zonal DNA and GAG distribution, with maintained DNA and significantly increased GAG accumulation under the compressive clamp by 6 weeks, suggesting a shift toward chondrogenic-like tissue formation, similar to postnatal entheses [5]
  - ALP activity, a marker for mineralized fibrocartilage formation [1,2], remained low in all zones of ligament constructs, however was significantly increased in enthesis cultures and 50/50 mixtures, suggesting enthesis fibrochondrocytes play a significant role in mineralized fibrocartilage formation in the developing enthesis
- This study provides new insight into how different cell populations of the ACL individually and synergistically contribute to enthesis formation, allowing for more targeted therapies for regenerating the enthesis in engineered tissues and after surgical repair
- Ongoing work is evaluating constructs at 2 & 4 wks and performing mechanical analysis to evaluate functional properties of entheses

Acknowledgments

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