Spatial transcriptomics reveal unique molecular signatures of the developing Achilles Tendon

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Disclosures: None.

INTRODUCTION: Despite the vast knowledge of the structure and mechanical function of mature tendons, the understanding of tenogenic cell differentiation, including markers that define specific stages of the lineage, is limited. Cells within the tendon body (i.e., internal tendon fibroblasts) have traditionally been considered a homogeneous population. From the discovery of the tenogenic transcription factor Scleraxis (Scx) and other markers (e.g., Mkx, Tnmd) that are enriched in tendons, several advancements have been made in understanding this lineage. For instance, recent work utilizing single-cell RNA sequencing (scRNA-seq) has revealed greater cell heterogeneity than previously appreciated. Despite these advancements, we still lack an accepted list of markers that delineate cells within the different regions of tendon (e.g., myotendinous junction, peritenon, enthesis), and much less cells at multiple differentiation states (progenitor, tenoblast, tenocyte) [1]. Indeed, *in vivo* tools are needed to identify the progenitors and more mature cell types to better understand this lineage, as *in vitro* differentiation assays do not maintain the tenogenic fate of isolated cells. Spatial transcriptomics has been used to attempt to address this need. However, many methods lack true single-cell spatial resolution and are also cost-prohibitive for many labs. Our group has advanced the 10x Xenium platform to improve transcript quality while also markedly reducing costs. This study demonstrates our ability to define discrete cell populations based on anatomical location while also uncovering shifting tenogenic cell populations with age, all while using an "off-the-shelf" gene panel not tailored to musculoskeletal tissues.

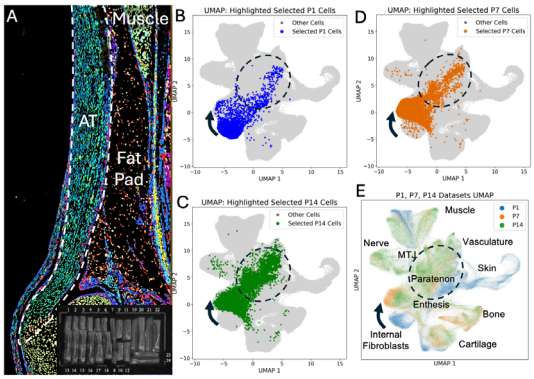

Figure 1: (A) Xenium Spatial Transcriptomics image of a P7 AT (dotted outline) with colored cells indicating different clusters. Inset shows 24 sections on 1 slide. (B) UMAP of selected AT at P1, (C) P7, and (D) P14. Arrow indicates shifting population and dotted line indicates growing population with age. (E) UMAP of all timepoints with cell types annotated.

METHODS: Animals: Hindlimbs were harvested from postnatal days 1 (P1), 7 (P7), and 14 (P14) wildtype mice (n=3/group with 2 slides per mouse, sex unknown for pilot study). Histology: Hindlimbs were fixed in 10% formalin, embedded in OCT, and Achilles tendon (AT) was sectioned (8µm thick) in sagittal plane with Cryofilm. Within the 12 x 24 mm space, 24 sections (inset in **Fig. 1A**) were glued to 10x Xenium slides using UV-curable adhesive (many more sections than used in the standard FFPE pipeline). Xenium spatial transcriptomics were performed using the mouse tissue atlassing panel containing probes for 379 genes and processed using standard 10x protocols through the University's single-cell sequencing core. Subsequent processing and analysis of 18 of the sections were performed using Xenium Explorer browser and Python scripts. For data presented in this abstract, we generated UMAPs from the Xenium data by normalizing and filtering low-abundance cells, performing principal component analysis to generate a probabilistic model of cell-to-cell relationships based on their similarity and differences in highly variable gene expression. Nearest neighbor analysis was also performed, where for a given "target" cell the cluster of the nearest "neighbor" cell was identified and cluster association percentages were computed.

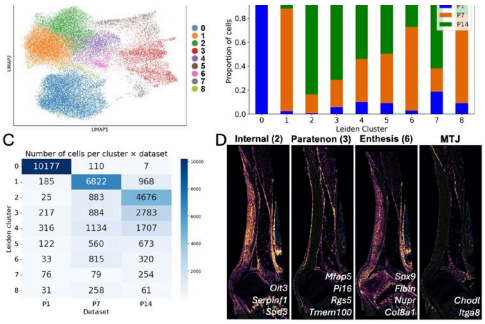

Figure 2: (A) UMAP of reanalyzed P1, P7, P14 AT selections. (B) Proportion of each timepoint per cluster. (C) Breakdown of cell numbers per cluster per age. (D) Representative image from a P7 AT highlighting genes from cluster 3 showing enrichment in the paratenon.

RESULTS: Shifting tenogenic cell populations with postnatal growth. We first defined our region of interest around the Achilles tendon, including the enthesis, peritenon, MTJ, and midsubstance to capture the tenogenic population (white dotted line in **Fig. 1A**). Next, we combined each timepoint into one UMAP highlighting the selected tendon regions to see how these three populations change with age (**Fig. 1B-E**). Largely, we identified a population that shifted (black arrows) and another population that grew in number (dotted lines) with age. Cell clusters mapping to specific tendon regions. To identify what cells were contributing to these growing populations and where in the tissue those cells are located, we reanalyzed the P1, P7, and P14 selected tendon cells as new clusters (**Fig. 2A-C**). We found the shifting population was clusters 0, 1, and 2 and were predominantly internal tendon fibroblasts, while the growing population was mostly in cluster 3 and localized to the peritenon. Additional clusters were enriched in the enthesis (cluster 6), while 2 genes *Chodl* and *Itga8* localized to the MTJ (**Fig. 2D**). Each of the three timepoints had a predominant internal fibroblast population: (Cluster 0 for P1, Cluster 1 for P7, and Cluster 2 for P14 (**Fig. 2B**). Internal fibroblast clusters have distinct spatial associations. Given the shift in the internal fibroblast population (clusters 0-2) with age (**Fig. 3A**), we next determined the spatial associations between these clusters using nearest neighbor analysis. We identified the cluster for the nearest "neighboring" cell of each "target" cell and then calculated percentages for each association. For example, 97% of cluster 1 cells were adjacent to cluster 0, whereas only 5% of cluster 0 cells were adjacent to cluster 1 at P1. We also found that clusters 1 and 2 were spatially intermixed in the tendon midsubstance with 17% of cluster 1 cells adjacent to cluster 2 cells and 70% of cluster 2 adjacent to cluster 1 cells at P7 and switches at P14 (**Fig 3B**).

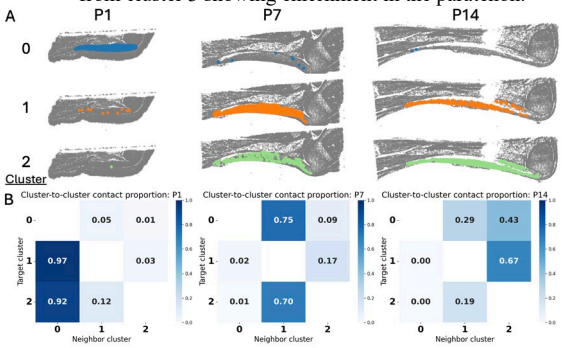

Figure 3: (A) Highlights of Clusters 0, 1, and 2 on representative AT section at P1, P7, and P14. (B) Cluster-to-cluster contact maps for Cluster 0, 1, and 2 for P1, P7, and P14 AT.

DISCUSSION: Taken together, these data highlight an innovative method to study tenogenic cell differentiation with precise spatial context at a truly single-cell level. Clustering data indicates that, as the tendon develops, there are major shifts in gene expression from P1 to P14 across the tendon. Currently, we are in the process of generating Xenium data with genes involved in tenogenesis and tendinopathy to further establish markers at different stages of the tenogenic lineage. In addition, we will use lineage tracing models to define clonal tendon cell arrays to establish how these arrays differentiate over time. In fact, given the close spatial association between clusters 1 and 2 in this study, there may indeed be a clonal relationship where cluster 1 cells differentiate into cluster 2.

SIGNIFICANCE/CLINICAL RELEVANCE: This study provides a novel understanding and establishment of a protocol for identifying distinct molecular signatures of tendon cells with single-cell spatial resolution with a marked increase in throughput with cryofilm sections. This technology can be applied to clinical samples with specific markers for studying tenogenesis and disease.

REFERENCES: 1. Huang, et al, J Orthop Res, 2023.

ACKNOWLEDGEMENTS: NIH P50-AR080581, P30-AR069619, and R01-AR082810.