Role of mechanical force from muscle activity in establishing spatial and functional tenocyte diversity

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INTRODUCTION: Tendons are essential for proper transfer of contractile force from muscle activity to tissues of varying firmness that include, bones, cartilage, muscles, skin and eyes. Our current understanding of tendon development and function is largely based on tendons that attach to bones to form an osteo-tendinous enthesis and we do not know much about the processes that lead to formation of tendon attachments with other tissues. We also know little about diversity in tendons in terms of their ECM composition and structure. Recent studies have shown important roles for mechanical force in tenocyte morphogenesis and transcriptional dynamics at the enthesis. Does varying force from muscle activity affect all tendons and their respective attachments similarly? To address these questions, we have conducted a scRNA-seq analysis of transcriptional changes in tenocytes between wild type and paralyzed cranial tendons in zebrafish. We have validated many of the transcriptional differences identified by these single cell studies using semi-quantitative in situ expression experiments using hybridization chain reaction (*is*HCR).

METHODS: Cranial tendons from dissected heads of developing transgenic zebrafish larvae (*Tg(BAC scx:mCherry)*) expressing mCherry under the *scleraxis* promoter (*scx)* are dissociated using a cold protease protocol that utilizes subtilisin enzyme from *B.licheniformis* at 8ºC. Tenocytes expressing mCherry are sorted from the cell suspension using FACS. Sorted tenocytes are analyzed for their individual transcriptional signature using single cell RNA sequencing (scRNA-seq) and 10X Genomics. The sequence data is aligned with reference genome (version 10) from Ensembl and Seurat is used to perform unsupervised cell clustering. Using marker gene expression, identities are assigned to the clusters. Further validation of marker genes are performed using *is*HCR. To study the effect of force on tenocyte differentiation and patterning, tenocytes expressing mCherry driven by an *scx* promoter are sorted from cranial tendons of developing paralyzed mutants lacking the functions of a *β-subunit of a voltage-dependent L-type calcium channel* (*cacnb1*$^{-/-}$) or injected with alpha-bungarotoxin (*αBtx)*- injected in the background of Tg*(BAC scx:mCherry)* zebrafish larvae using FACS. Sorted tenocytes are analyzed similar to the WT siblings described above. The zebrafish adults and larvae are treated in accordance with approved protocols by IACUC at UCI.

RESULTS SECTION: We sorted tenocytes from three biological replicates of wild type, two biological replicates of *cacnb1*$^{-/-}$ and one sample of *aBtx*-injected embryos at 3 days post fertilization (dpf). We performed unsupervised clustering of the cells from their expression data to obtain the UMAP dimensional plot. We validated expression of tendon genes (*scxa*, *mkxa*, *thbs4b*) and mCherry across all the clusters. We also validated the expression of tendon genes and markers for each cluster using *is*HCR. Based on the data from *is*HCR and expression of known cluster markers, we manually assigned identities to cell clusters. Our analyses showed heterogeneity in tenocyte populations based on their functions both between and within individual tendons such as enthesis, mid-substance, ligaments, myotendinous junctions (MTJ) and joints, and also spatial heterogeneity such as extraocular, fin, and jaw tendons. KEGG pathway analyses of the clusters showed enrichment of downstream effectors of growth signaling pathways such as FGF, Wnt, TGFbeta, and retinoic acid in specific subsets of tenocytes suggesting differences in patterning and differentiation of tenocyte subpopulations. Using the analyses from our WT dataset we are currently investigating how force regulates the differentiation and patterning of different tenocyte clusters by comparing with paralyzed dataset (*cacnb1*$^{-/-}$ and *αBtx*-inj). We will validate the changes in expression of key genes and differentiation of specific tenocyte subclusters using *is*HCR and live-imaging of Tg(*BAC scx:nucEOS*) zebrafish embryos to visualize the patterning of different cranial tenocyte populations.

DISCUSSION: We have performed a global analyses of tenocyte heterogeneity both within a tendon (intra-tendon) and between different tendons (inter-tendon) during early embryonic development. Our analyses have revealed transcriptional variations correlating with spatial and functional differences among tenocyte clusters. Our in silico analysis is further strengthened by *is*HCR expression validation in whole embryos. Our ongoing analyses of WT and paralyzed datasets will reveal how force serves as an essential cue in differentiation and patterning of key tenocyte clusters. In combination with KEGG analysis this will help us elucidate the key signaling pathways that play a vital role in tendon mechanotransduction during development.

SIGNIFICANCE/CLINICAL RELEVANCE: (1-2 sentences):
This is the first scRNA-seq study comparing tenocytes broadly across the entire set of cranial tendons to determine spatial and functional heterogeneity. The findings from this study will help us establish a clear signature for tendons based on their type of attachments and further our understanding of how these tendons are patterned. This will help us develop and refine regeneration and bioengineering protocols for tendon repair and treatments.

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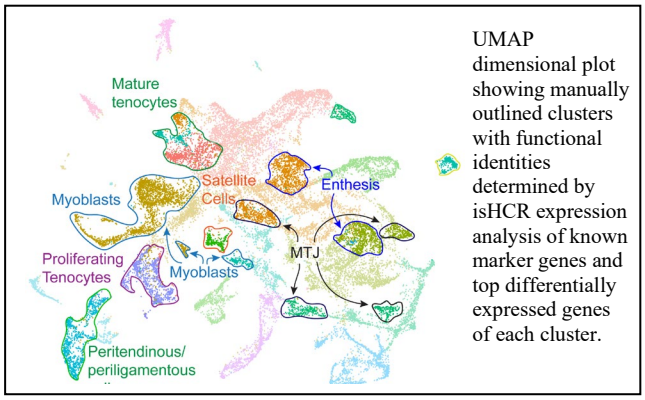
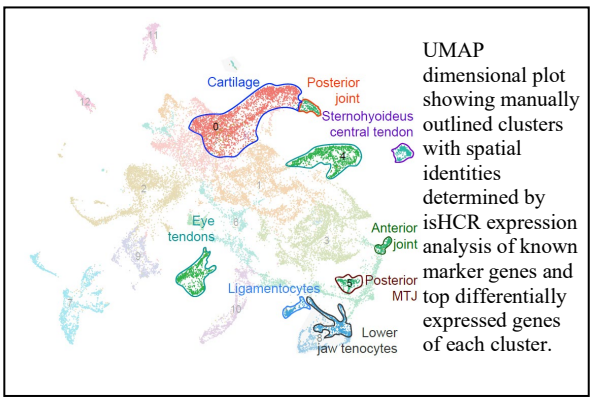
IMAGES AND TABLES



UMAP dimensional plot showing manually outlined clusters with functional identities determined by isHCR expression analysis of known marker genes and top differentially expressed genes of each cluster.



UMAP dimensional plot showing manually outlined clusters with spatial identities determined by isHCR expression analysis of known marker genes and top differentially expressed genes of each cluster.