Supraspinatus and Long Head of the Biceps Tendon Profiling through Multiomics Data Integration

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INTRODUCTION: Tendon degeneration and rupture is common and causes high levels of functional impairment[1]. Tendon heals by fibrosis and fails to restore normal structure and function even after surgical repair[2,3]. Additionally, individual systemic factors may further impair healing[4]. Over 500,000 rotator cuff tears are repaired each year in the U.S.[5] but retear rates remain above 20%, increasing with the size of the tear up to 90%[6]. Despite substantial progress, there are many unanswered questions about tendon biology that could inform strategies to improve outcomes. In this regard, tendon structure varies significantly with anatomic location, reflecting the specific function of the tendon-muscle unit[7]. However, the molecular and epigenetic differences between tendons with divergent structure are not well understood, but could inform tendon cell identity. The supraspinatus tendon is subjected to heterogeneous multiaxial loading patterns and has heterogeneous structure[8,9]. In contrast, most of the long head of the biceps brachii tendon is subjected to homogeneous uniaxial tensile loading and has a homogeneous archetypal tendon matrix[10] but is not often engaged in activities of daily living[11]. The aim of this study was to evaluate the DNA methylome and mRNA and miRNA transcriptome from bilateral paired long head of the biceps brachii (BT) and supraspinatus tendons (SST) from human cadaveric donors in order to better understand tendon cell identity in tendons with very different organizational hierarchy, and to ascribe the molecular characteristics of divergent tendon relative to form and function.

METHODS: SST and BT were dissected from paired cadaveric shoulders with the shortest feasible postmortem interval, and with no prior history of shoulder injury or disease, among other exclusion criteria. All procedures were approved as Exempt from Reivew by the Institutional Review Board. DNA and complete (smallRNA and totalRNA) RNA extraction was performed simultaneously using Quick-DNA/RNA MagBead columns (Zymo) after Proteinase K treatment. Grossly healthy samples with RNA and DNA that passed quality control (n=24-SST, n=14-BT), were subjected to small (50bp single reads) and total (150bp paired end) RNA sequencing, and whole-genome bisulfite sequencing (150bp paired end) (Zymo Research). After data cleaning and mapping to the human genome (GRCh38), paired healthy samples (10-SST, 10-BT from left and right shoulders of 5 donors) were compared. Differentially Expressed Genes (DEG) were identified with DESeq2 for miRNA and RNA with qvalue<0.05. Differentially methylated regions (DMR) were identified with MethylKit[12]. Differential methylation required qvalue <=0.01 and methylation difference >10%. Additional analyses identified potential biomarkers using Partial Least Squares-Discriminant Analysis (PLS-DA), and related gene clusters using Weighted Gene Co-expression Network Analysis (WGCNA). Biological meaning of the data was attributed with WebGestalt, Ingenuity Pathway Analysis (IPA), and Annotatr[13].

RESULTS: There were 57 DEG mRNA biotypes, with 13 additional differentially expressed miRNA (**Fig. 1**), and 203,066 DMR between SST and BT. PLS-DA analysis showed that while the transcriptomic datasets showed some separation between tissue, sex and side (left vs. right), this separation was more pronounced at the level of the DNA methylome (**Fig. 2**). Total RNA analysis showed that the SST transcriptome was enriched in muscle-related transcripts and membrane regulators (*IGFN1, TRIM63, ENO3, SPTB*), and developmental genes (*EMX2, LMX1B*), while BT was enriched in developmental genes and ECM regulators (*MAB21L2, SHOX, SIX3, EN1, OGN).* For SST, coding RNA biotypes with the greatest numbers of DMR were those impacting neural development (*RBFOX1, LRRC4C, CNTNAP2, DAB1, CLSTN2*). WGCNA results (**Fig. 3**) followed by TopHubGenes on significant modules for transcriptome found 4 significant gene modules involved in histone acetylation, muscle development, citrate transport and neurotransmitter regulation for SST vs BT(*MBIP, NRAP, SFXN5, NAALAD2*). WGCNA for the DNA methylome for SST vs BT instead found 14 significant modules, with TopHubGenes particularly associated with in neural system development, embryonic development, and cellular processes as proliferation, differentiation and transcription regulation (*CELF, FMN2, MAPK10, ROBO2, INTU, SFRP4*).

DISCUSSION: Unbiased evaluation of the DNA methylome and transcriptome showed that the key differences between SST and BT may be mediated by what are considered 'developmental pathways' with an emphasis on neural development and embryonic development maintained in the adult DNA methylome. Notably orientation patterning of these tendons during embryonic development is conserved for many decades of adult life. Further, while tendons generally have minimal motor innervation, afferent innervation of tendons provides important sensory information[14]. The largest pool of neurons in tendons are the Golgi tendon organs (GTO) that are important for proprioception and are located near the myotendinous junction[14], although other mechanoreceptors are also identified.[15] At the bulk sequencing level, the GTO may drive the dominance of neural related pathways in the SST vs BT comparison since the former is shorter, wider and more proximate to the musculotendinous junction, while BT is almost 10 times its length[16,17] Therefore, it is possible that SST may have higher levels of GTO due to the heterogeneous structure and the proximity of the myotendinous junction. However, the role of GTO and the afferent function of mechanoreceptors to the SST has not been well examined relative to shoulder function, tendon homeostasis, or to the propensity of the SST to develop tendinopathy. Nonetheless, the extensive differential methylation of coding genes associated with neural development suggest that tendon homeostasis and response to environment may be partially regulated by neurons and since SST is subjected to greater magnitudes and frequencies of loading may contain more mechanoreceptors, or these genes may be more sensitive to differential expression in SST [18]. This hypothesis could be supported with the differential expression of genes involved in the differentiation and migration of the neural crest (*MAB21L2*[19], *EN1*[20], *LMX1B*[21], *EMX2*[22]). However, SYN2 is the only gene differentially expressed that is associated with mature neurons. Together, these results show substantial differences in the epigenetic programming of SST and BT, especially in innervation. However, the transcriptome of SST and BT is surprisingly close, suggesting that fine tuning of novel therapeutic strategies to match site-specific tendon cell identity may not be necessary. Further studies analyzing the proteome and metabolome of these tissues could further validate these conclusions.

SIGNIFICANCE/CLINICAL RELEVANCE: This research highlights the divergent DNA methylome but remarkably similar transcriptome of SST and BT and highlights the potential implications of these differences for tendon homeostasis and novel therapeutic approaches.

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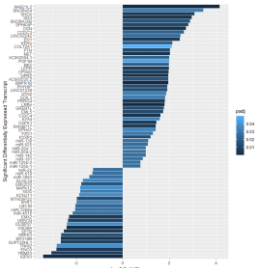

Figure 2. Significant differentially expressed transcripts for mRNA and microRNA, colored by p-adjusted values.

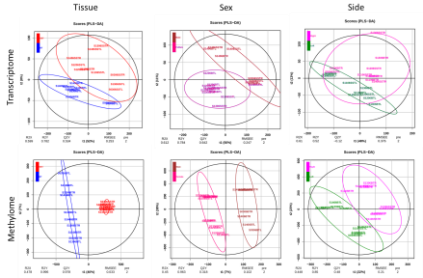

Figure 2. PLS-DA score plot for transcriptome(top) and methylome(bottom) for tissue (left), sex (center) and side (right)

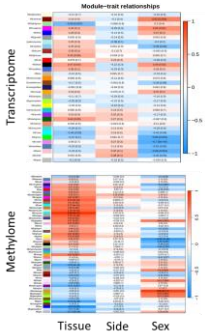

Figure 1. WGCNA module trait tables for transcriptome(top) and methylome(bottom) for tissue (left), sex (center) and side (right)