Heterogeneity of fibroadipogenic progenitors (FAPs) in the mouse rotator cuff and their response to massive rotator cuff tear

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Introduction: The rotator cuff is composed of 4 muscles that stabilize the shoulder and control upper arm range of motion. Tears in the tendons of these muscles, known as rotator cuff tears (RCTs), are among the most common and debilitating shoulder injuries. Despite surgical repairs being common, they post significant rates of retear—seen as high as 94% for certain tear types and sizes. The causes of retear of multi-variable, but there are strong correlations between retear and intramuscular fat deposits (known as fatty infiltration) and fibrosis. Despite this long-standing clinical observation, little is known about 1) the precise cellular origins of the fatty infiltration and fibrosis and 2) the mechanisms/signaling events leading to these pathologies. A heterogenous population of muscle resident non-myogenic mesenchymal cells, known as fibroadipogenic progenitors (FAPs), have been implicated in many skeletal muscle pathologies—particularly those where intramuscular fat and fibrosis are abundant. Since FAPs are defined by their expression of *Pdgfra*, we performed lineage tracing and reporter analysis in a mouse model of massive RCT and demonstrated that FAPs give rise to mature adipocytes and fibroblasts associated with the RCT pathology. After this determination we further sought to understand FAP heterogeneity and early cellular and molecular changes following massive RCT. To examine the cellular and molecular responses we performed bulk RNA and single cell RNA-sequencing (scRNA-seq) on rotator cuff FAPs.

Methods: Massive RCT injury was surgically produced in mice through transections of the supra- and infraspinatus tendons from the head of the humorous coupled with transection of the suprascapular nerve. Lineage Tracing: Supra- and infraspinatus muscles were assessed in *PdgfraCre^{ERT2}; R26-tdTomato$^{f/+}$* mice at 8-weeks post-injury (wpi) following massive RCT surgery. Immunofluorescent staining assessed colocalization of TOMATO and PERELIPIN (fat) or FIBRONECTIN (fibrosis). Bulk RNA-seq: Using *PdgfraCre; R26-tdTomato$^{f/+}$* mice, TOMATO+ cells from 2wpi supra- and infraspinatus muscles and contralateral controls were sorted via FACS, RNA isolated and sequenced. Bioinformatic comparisons between tear and contralateral control assessed up and down-regulated genes. Single Cell RNA-seq: Uninjured and 2-wpi supra- and infraspinatus muscles were harvested from adult *Pdgfra-H2B-GFP* mice. GFP+ cells were isolated following tissue digestion and sorted via FACS for single cell RNA library preparation and sequencing. Reads were aligned to the mouse reference genome and unsupervised clustering in the Seurat-R platform was performed on the integrated control/tear datasets. Statistical analyses include log-fold change analyses, t-tests, and matched pair t-tests (when contralateral, uninjured muscles were utilized). All experiments we performed under an approved IACUC protocol.

Results: Reporter/lineage tracing analysis showed RCT induced adipocytes and large fibrotic plaques are derived from *Pdgfra*+ FAPs in the rotator cuff at 8-wpi. After assessing FAP numbers at multiple time points following RCT, we identified 2-wpi to be the optimal time point for the assessment of cellular and molecular changes in FAPs that may drive the massive RCT pathology. We analyzed 2,556 uninjured control FAPs and 1994 FAPs at 2-wpi and identified 9 clusters of *Pdgfra*+ cells (Figure 1) that could be validated using specific subpopulation markers. FAP subpopulation numbers shifted following massive RCT that coincided with changes in differentiation trajectories and altered gene expression. Of note, a pre-adipogenic (*Pprag+,* Cluster 7) and injury responsive subpopulation of FAPs undergoes further adipogenic differentiation. Additionally, a cluster of tenocyte-like myofibroblasts (Cluster 9) also respond to massive RCT with population size increases and enhanced differentiation and extracellular matrix (ECM) gene expression changes, while nerve-associated FAP subpopulations (Clusters 4,5,6) exhibit decreased numbers and altered neuronal signaling gene expression. Further Gene Set Enrichment Analysis (GSEA) of bulk and scRNA-seq comparisons at 2-wpi from injured and contralateral rotator cuff muscle FAPs showed significant changes in a number of specific signaling pathways that regulate adipogenesis, ECM synthesis, inflammation, apoptosis, axon guidance, neuromuscular junction (NMJ) function, and skeletal muscle atrophy.

Discussion: Rotator cuff tear induced pathologies including fatty infiltration, fibrosis, and muscle atrophy are correlated to the high rates of retear and poor clinical outcomes. Muscle resident FAPs have been implicated in these pathologies, but the heterogeneity of FAP subpopulations and their specific cellular/molecular roles in the rotator cuff has never been explored. Our lineage tracing and reporter analysis shows *Pdgfra*+ FAPs are the cellular origins of the adipocytes and fibrotic cells responsible for the massive RCT pathology present at 8-wpi. Additionally, this work is the first to generate robust datasets of the genetic subpopulations of FAPs in the adult mouse rotator cuff of uninjured animals as well as single cell and bulk RNA-seq analyses at 2 weeks following massive RCTs. In a normal, regenerative injury (ex. $BaCl_2$) FAPs peak 3-5 days post injury and return to homeostatic levels by 14-days post-injury (dpi); however, following RCTs the FAP levels remain high at 2-wpi yet have not fully differentiated into intramuscular adipocytes nor induced an excessive fibrotic ECM that is observed at 8-wpi. Through analyses of these integrated datasets at this early, yet critical time point, we identified specific FAP subpopulation population changes, novel and altered differentiation trajectories, as well as both enhanced and suppressed signaling pathways known to regulate adipogenesis, ECM synthesis, inflammation, apoptosis, axon guidance, neuromuscular junction (NMJ) function, and skeletal muscle atrophy that likely contribute to the massive RCT pathology. The results of these studies are crucial to our development of targeted therapies to reduce the intramuscular fat, fibrosis, and muscle atrophy that occurs following RCT injury, thus reducing the rates of retear and patient burden.

Significance/Clinical Relevance: Currently, there are no treatments for muscular fatty infiltration, fibrosis, or the rotator cuff muscle atrophy observed following RCTs. With the prevalence and debilitating effects of RCTs, as well as the high rates of retears and complications, it is critical to better understand the origins of this fat and fibrosis. Our study is the first to define FAP subpopulations in the rotator cuff and assess the cell type and molecular pathway responses to massive RCTs. This work provides vital information for generation of cell and molecular based therapies for the amelioration of RCT pathologies.

Figure 1: Single cell RNA sequencing UMAP generated from FAPs of uninjured and 2wpi massive RCT