Fibro/Adipogenic Progenitor (FAP) Trajectory Analysis Reveals Adipogenic Commitment that is not Reflected in Elevated Fat Infiltration in the Quadriceps after ACL reconstruction.

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INTRODUCTION: Anterior cruciate ligament (ACL) tears are common knee injuries that result in unresolved quadriceps weakness and atrophy despite rehabilitation[1]. The mechanisms responsible for this poor muscle recovery are not thoroughly characterized; however, strength and physical function deficits in skeletal muscle have been previously associated with an accumulation of intramuscular adipose tissue (IMAT)[2]. This deposition of adipose tissue between muscle fibers is mediated via the adipogenic differentiation of a small population of progenitor cells, fibro-adipogenic progenitors (FAPs). FAPs are known to produce soluble factors that stimulate satellite cells to sustain myogenesis, and they also have the potential to adopt adipogenic or fibrogenic differentiation lineages. Our purpose in the current project was to perform unbiased single nuclear transcriptomic and fate assessment of quadriceps FAPs to identify molecular targets that uncover FAP behavior as it affects muscle integrity and IMAT deposition during recovery following ACL injury.

METHODS: Muscle biopsy specimens from the healthy (non-injured) and ACL-injured vastus lateralis were collected from 16 young adults (8M,8F) following ACL injury. Follow-up biopsies were obtained from the injured limb one week and 4 months after ACL reconstruction surgery (ACLR). Samples were immediately flash frozen in liquid nitrogen for RNA sequencing or embedded in OCT and frozen for immunohistochemistry. For bulk RNA-seq analysis, total RNA was isolated and sequenced on an Illumina NovaSeq 6000 at Novogene, and data were analyzed using Partek Flow. For single nucleus RNA-seq, muscle was mechanically minced and homogenized; subsequently, nuclear suspensions were filtered and loaded onto a 10x chromium controller. RNA was sequenced and output data were aligned in CellRanger and analyzed in R with the Seurat package. The metadata from Partek Flow was imported into R for analysis with the Monocle 3 package. Briefly, data preprocessing, dimensionality reduction, clustering, and trajectory construction were performed and FAPs were ordered in pseudotime with respect to THY1+ progenitor status. Genes that changed as a function of pseudotime were identified using the graph_test() function and selected if significant ($q<0.05$). To evaluate IMAT development and deposition, immunohistochemistry analyses were conducted on 7µm sections. The sections were labeled with Perilipin-1 primary antibody, followed by secondary antibody, DAPI and a lipophilic fluorescent dye (Bodipy). IMAT% was determined by dividing the total area positive for IMAT (perilipin+) by the overall section area. Statistical analyses for all IMAT assessments were performed using a one-way ANOVA, with no sphericity assumption to account for repeated observations within the same subject.

RESULTS: snRNA-seq analysis revealed that a majority of FAPs (67.1%) adopt an adipogenic commitment one week after ACLR, denoted by enriched expression of adipogenic differentiation specific genes (e.g. *ITGA8, MME, DLK1*). Subsequently, ACLR induced a decline in the relative number of THY1+ mesenchymal progenitor FAPs (-6.1%) within the quadriceps muscle. Trajectory analysis exhibited a bifurcation in the differentiation lineage of FAPs, strongly matching their known potential to adopt adipogenic or fibrogenic lineages. Further, trajectory analysis showed ACLR promoted the adoption of a FAP adipogenic lineage within the quadriceps muscle. Interestingly, immunohistochemistry analysis showed a significant decrease ($p<0.01$) in IMAT% in the injured limb 4 months after ACLR (Healthy IMAT: 2.3±1.6%, 4 Mo post ACLR IMAT: 0.7±0.8%), indicating that the adipogenic transcriptome profile of FAPs does not directly translate to a phenotypic increase in fat deposition within the muscle following ACLR.

DISCUSSION: Our data show that ACLR induces substantial changes in FAP transcriptional profiles, with FAPs adopting an adipogenic lineage. Conversely, the elevated adpiogenic commitment of FAPs was not associated with accumulation of IMAT within the muscle. Previous literature has reported no significant differences in intramuscular fat between healthy and ACLR limbs when using Magnetic Resonance Imaging (MRI) to denote fat within the muscle[4]. Importantly, IMAT was not predictive of quadriceps strength several years after ACLR[4]. Our data identify a unique transcriptomic signature for FAPs following ACLR but suggest that the predicted adipogenic commitment does not meaningfully alter IMAT abundance within the quadriceps. Based on these findings, there is a discontunuity between the single-cell transcriptomic data and the decline in IMAT% in the muscle samples, underscoring the need for further investigation into the distinctive FAP differentiation stages and overall behavior following ACL injury.

SIGNIFICANCE/CLINICAL RELEVANCE: The biological underpinnings for protracted quadriceps weakness following ACLR remain poorly understood. This work provides evidence that FAP adipogenesis and IMAT accumulation are not critical effectors of quadriceps dysfunction following ACLR. Future work is needed to continue refining our understanding of the molecular and cellular source of poor quadriceps quality following ACLR.

REFERENCES: 1. Brightwell, CR et al. Sci Adv. (2023), 2. Biltz, NK et al. J Physiol. (2020), 3. Sciorati, C et al. Cell Mol Life Sci. (2015), 4. White, MS et al. J Orthop Res. (2024).

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IMAGES AND TABLES:

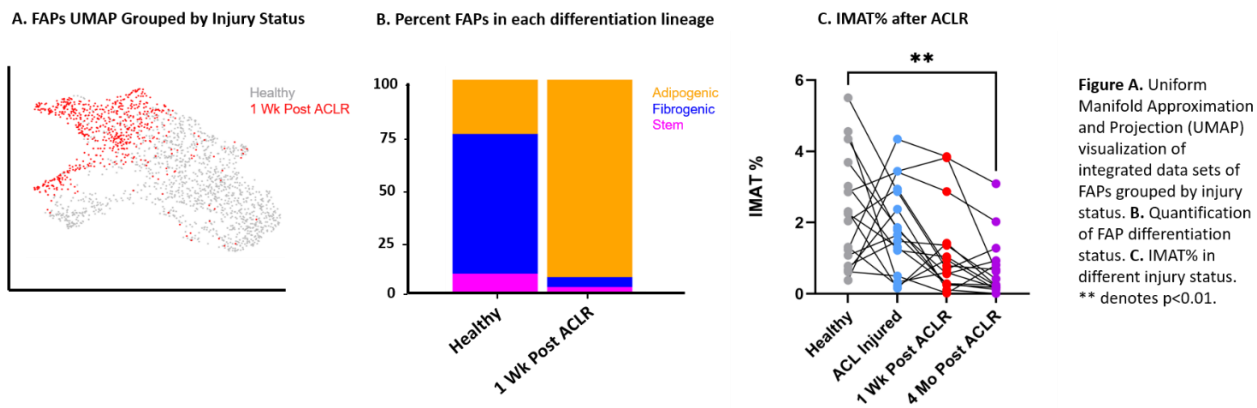

Figure A. Uniform Manifold Approximation and Projection (UMAP) visualization of integrated data sets of FAPs grouped by injury status. **B.** Quantification of FAP differentiation status. **C.** IMAT% in different injury status. ** denotes $p<0.01$.