Non-biased analysis of ACL tissue shows distinct differences in cell type and gene expression between male and female populations

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DISCLOSURES: Authors have no relevant disclosures.

INTRODUCTION: The anterior cruciate ligament (ACL) provides rotational stability to the knee. The rate of ACL tears has increased over the past 20 years, occurring in approximately 16 per 1000 high school athletes. One discrepancy not well understood is the differences in tear rate between sex. Females have been found to experience tear rates that are 2-8 times higher than their male counterparts. This has been attributed to physical conditioning, muscular strength, neuromuscular control, differences in pelvis and lower extremity alignment, increased laxity in ligaments, and the effects of estrogen on ligament properties. However, the statistically significant difference in terms of tear rate is lost between sex following ACL reconstruction (ACLR) suggesting an intrinsic difference between male and female ACL tissue. We hypothesized there is a biological sex difference in the composition of the ACL at the cellular and transcriptomic level that can help explain the increased ACL tear rate in females.

METHODS: ACL samples were collected from four male and six female patients (n = 10) following ACL reconstruction surgery (under IRB and ethics board approval). Live cells were collected through flow cytometry and sent for single cell RNA-sequencing utilizing 10x Genomics 3' kits. The datasets were analyzed using Cellranger, Freemuxlet, and Seurat, to perform quality control, cluster cells, and determine differential gene expression between male and female groups. Significant expression was set at greater than a 25% increase in expression level ($log_2FC > 0.32$ and $p < 0.05$).

RESULTS: We identified seven distinct cell types by gene expression including fibroblasts (ACTA2), pericytes (RGS5), endothelial cells (PECAM1), M1/M2 macrophages (CD86/MRC1), T-cells (CD3E) and a unique PDGFRA+ TPPP3+ ligament progenitor population (LPCs) that share several genotypic similarities with muscle fibroadipogenic progenitor stem cells (Figure 1A/B). The greatest difference in cell composition between male and females were endothelial cells with a 13.9% increase in males and LPCs with a 9.5% increase in females (Figure 2B). There was little difference in terms of M1/2 macrophages and T-cells (0.3%, 2.8%, 2.7%, respectively) (Figure 2B). Differential gene expression of collagen genes found COL5A2/3, COL14A1, and COL12A1 to be upregulated in females ($log_2FC = 0.78, 0.33, 0.36, 0.63$, respectively), with COL12A1 being significantly upregulated in LPCs, COL5A2 in endothelial cells, and both moderately upregulated in fibroblasts and pericytes. COL15A1 was the only collagen gene downregulated in females ($log_2FC = -0.44$) (Figure 2C). Expression of the collagen assembly gene, SOX5, was significantly upregulated in the LPCs, fibroblasts, and pericytes of females ($log_2FC = 1.93$). ECM degradation genes, FAP and SULF1, were significantly upregulated in female endothelial cells, LPCs, fibroblasts, and pericytes. Metalloproteases, ADAMTS1/4, were significantly downregulated in female endothelial cells, fibroblasts, and pericytes ($log_2FC = -0.85, -0.96$). The cell adhesion gene, THBS4, was upregulated in female endothelial cells and LPCs ($log_2FC = 0.51$), while THBS1 was downregulated in endothelial cells, fibroblasts, and pericytes($log_2FC = -0.69$) (Figure 2C). LPC and FAP progenitor marker,TPPP3 and CD74, was significantly upregulated in male LPC ($log_2FC = 0.47, 0.60$)(Figure 3A). PDGFRA+ cells were cultured from digested ACL harvested during ACL reconstruction (Figure 3B).

DISCUSSION: These results demonstrate that sex differences exist between male and female ACL at a cellular and transcriptomic level. In the LPC and endothelial cell populations, we see significant upregulation of COL5A2, COL12A1, SOX5, SULF1, and FAP, genes that encode various types of collagens as well as proteins that assemble and degrade them in females. COL5A2 is a major structural component of ligament while COL12A1 encodes type 12 collagen, another structural component of ligament fibril. Increased expression of both genes has been seen to be associated with ACL rupture in females.[1,2] The upregulation of these genes provides evidence for increased collagen turnover in the ACL of female patients, pointing to a possible difference in the composition of the collagen/ ECM network that make up male and female ACLs. Additionally, we found a unique PDGFRa+TPPP3+ LPC population that expresses many highly differentiated genes in male and female populations. Given that these LPCs resemble fibroadipogenic progenitor stem cells, a highly modifiable cell type in muscle, we could leverage this population in the future and study the role of different LPC pathways on ACL biomechanical properties in order to decrease ACL rupture rates.

SIGNIFICANCE: A novel ligament progenitor population provides a possible target population for studying ligament injury and regeneration. Differential expression of collagen and ECM related genes provide evidence for specific genes that could be therapeutically targeted to strengthen the ACL and reduce the risk of rupture, particularly in female athletes.

ACKNOWLEDGMENTS: The authors would like to thank the following funding sources: UCSF Department of Orthopaedic Surgery Seed Grant 5014

FIGURES:

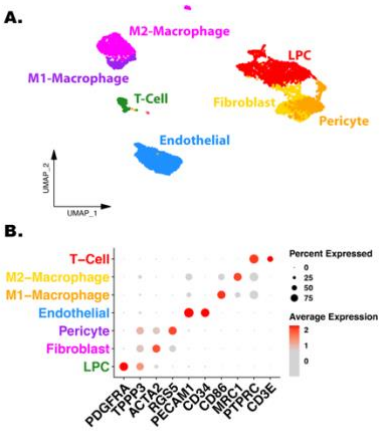

Figure 1: A. UMAP plot of ACL cells clustered by cell type B. Dot plot of gene markers used for identification of clusters

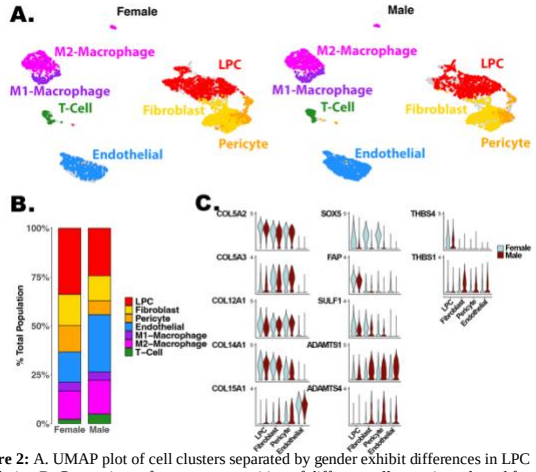

Figure 2: A. UMAP plot of cell clusters separated by gender exhibit differences in LPC population B. Comparison of percent composition of different cell types in male and female C. Violin plots of differential gene expression between male and female of collagen genes, collagen/ ECM assembly + degradation genes, and cell adhesion genes

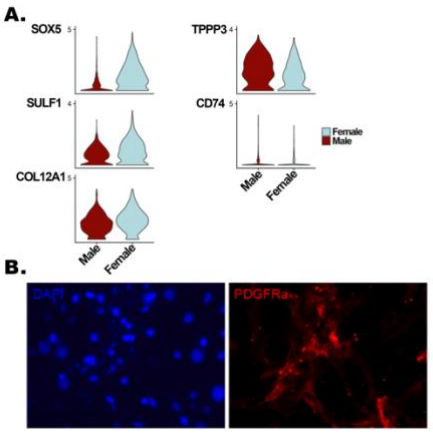

Figure 3: A. Violin Plot of gene expression in male and female LPCs B. IHC staining of PDGFRA+ cells cultured from ACL