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Fulcrum Therapeutics

Use of snRNA-seq to characterize the pathogenic skeletal muscle microenvironment

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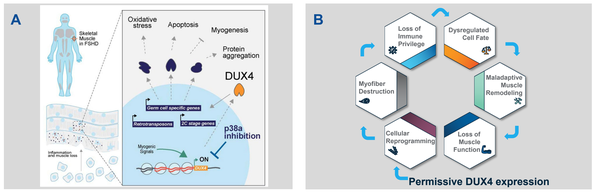
Abstract

- Facioscapulohumeral muscular dystrophy (FSHD), one of the most common forms of muscular dystrophy, is a rare monogenic disease that currently has no effective treatment. It is caused by deletions of the macrosatellite D4Z4 repeats at sub-telomeric region of chromosome 4q35. Deletions to repeat numbers less than 10 within this region cause chromatin de-repression resulting in stochastic and aberrant expression of the embryonic transcription factor DUX4 in skeletal muscle. The expression of DUX4 is myotoxic and results in muscle weakness in facial muscles and around the shoulder, eventually progressing to lower extremity and truncal weakness. While the root cause of FSHD has been elucidated, the molecular and cellular microenvironmental underpinnings that drive the loss of muscle tissue and function are not well understood.
- Here we describe the first characterization of human FSHD skeletal muscle biopsies using single nuclear RNA-sequencing (snRNAseq). Our goal was to understand the dynamic FSHD microenvironment at higher resolution. Analysis of these snRNA-seq profiles suggested an increase in relative populations of infiltrating immune cells and expansion of resident progenitor cell populations in FSHD versus healthy biopsies. In addition to expanded intercellular heterogeneity within the FSHD microenvironment, we also saw a significant intracellular heterogeneity suggestive of altered cell states within diseased muscle. Characterization of these FSHD muscle biopsies will help us to understand the processes contributing to FSHD pathogenesis that will hopefully lead to new target/therapeutic hypotheses.

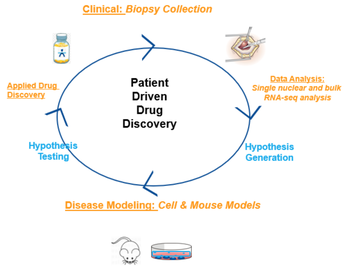
1. Background



(A) In FSHD, loss of repression of the D4Z4 locus leads to expression of DUX4 in skeletal muscle. p38a has been shown to regulate DUX4 expression in FSHD myotubes

(B) Aberrant expression of DUX4 leads to a disease cycle characterized by discrete hallmarks of disease driven by altered cellular communication within dystrophic tissue.

2. Approach



1. Collection of muscle biopsies from FSHD patients
2. Bulk RNA-seq of muscle biopsies
3. Selection of muscle biopsies with predicted cellular heterogeneity (based on bulk RNA-seq de-convolution) in progenitor and immune cell populations for snRNA-seq
4. Characterization and analysis of snRNA-seq profiles to understand changes in cell type proportion and state between FSHD and healthy biopsies
5. Characterization of differences in cell-to-cell interactions between skeletal muscle cell types in FSHD vs healthy muscle. Prediction of ligand-receptor interactions that drive these cell-to-cell interactions.

3. Experimental Design/Methods

A. FSHD needle biopsies from human skeletal muscle

Phenotype	Rationale for selection based on previous analyses	# of nuclei	Median genes/nuclei
FSHD	Predicted cellular heterogeneity	5237	1589
FSHD	High levels of Dux4/Dux4 co-expressed genes	8016	2119
FSHD	High levels of Dux4/Dux4 co-expressed genes	4422	2146
FSHD	High levels of Dux4/Dux4 co-expressed genes	8677	2150
Healthy		16334	1932
Healthy		7224	1894
Healthy	Publicly available single cell profiling of skeletal muscle (Rubenstein et al 2020) (2)	2860	574.5

B. Single Nuclear Analysis

Initial Processing: We used Seurat v3(i) to process single nuclear RNA-seq data. Cells with more than 200 genes and less than 5000 genes were retained in the analysis. Additionally, we filtered out cells with more than 5% mitochondrial genes. In order to merge FSHD and healthy biopsies we used the CCA algorithm as implemented in the Seurat v3 package.

Cell Type Annotation: For each biopsy, we clustered the snRNA-seq profiles and identified differentially expressed genes between clusters using a non-parametric Wilcoxon rank sum test. We then used an FDR cut off 5% and ranked genes in terms of log2FC of differential expression. We looked to see if any of these highly differentially expressed genes (marker genes) were known cell-type specific markers. We also overlaid known gene signatures of skeletal muscle cell types from (2). Finally, we performed gene enrichment analysis (GO; Reactome pathway enrichment) on the lists of marker genes for clusters we couldn't annotate with certainty. We named these clusters by their enrichment terms (ie: "ECM associated"). Any remaining unannotated clusters were labeled as undefined and were not prioritized for downstream analysis.

Pseudotime trajectories: In order to understand differences between healthy and disease FAP cells we first took all healthy cells from (2) and co-clustered them with disease FAP cells from each biopsy using the CCA algorithm to perform batch correction. Based on these clustering results, we then identified healthy cells that were most similar to the disease FAPs. Finally, we subsetted this group of cells, representative of disease and healthy FAPs, and used monocle v2 (ii) to infer a pseudotime trajectory based on the 2000 most variable genes in the data (healthy and diseases FAPs). For data interpretation purposes, we rooted the tree with the healthy cells. We performed this analysis on each FSHD biopsy. In order to identify differentially expressed genes between disease FAPs and healthy FAPs, we identified FSHD FAP cells at the end of each pseudotime trajectory and compared them to all healthy FAP cells using a non-parametric Wilcoxon rank sum test.

Predicted cell to cell interactions: For each biopsy, we took our cell type annotations and used the CellPhoneDB(iii) database of ligand-receptor interactions to computationally predict significant cell: cell interactions, as well as ligand-receptor interactions between these cells.

Software References

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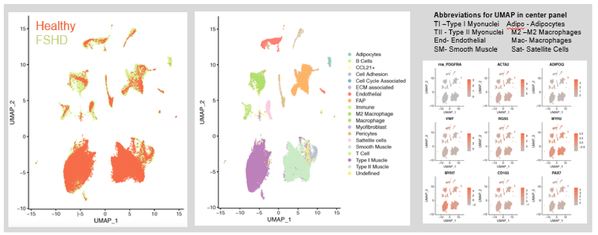
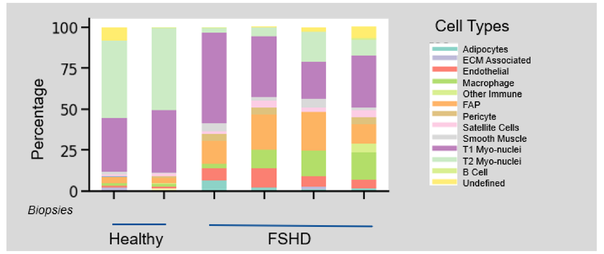
(iii) Efremova, M., Vento-Tormo, M., Teichmann, S.A. *et al*. CellPhoneDB: inferring cell–cell communication from combined expression of multi-subunit ligand–receptor complexes. *Nat Protoc* **15,** 1484–1506 (2020). https://doi.org/10.1038/s41596-020-0292-x

4. Results

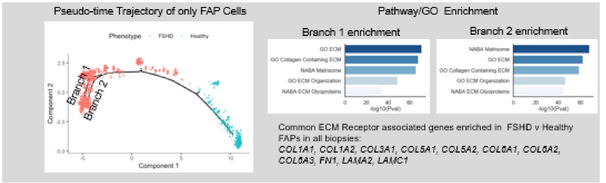
A. FSHD snRNA-seq profiles



B. Relative Cell Type Composition



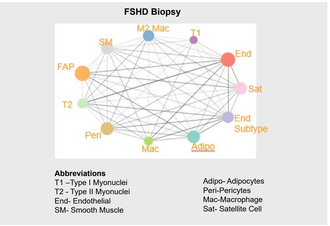
C. FAP cell state in healthy and disease biopsies



A) UMAP representation of four FSHD skeletal muscle biopsies combined with two healthy skeletal muscle biopsies show the presence of all predicted resident and non-resident skeletal muscle cell types. Expression of cell-type specific/canonical cell type markers overlaid on the UMAP representation captures the presence of major skeletal muscle cell types. Values represent scaled count values for each gene. **(B)** Intra-biopsy cell type heterogeneity is represented in stacked bar plots of relative abundances. Y axis is percentage of total cells captured; first two bars represent healthy biopsies 1 & 2; the rest of the bars are FSHD Biopsies from distinct patients (A-D). We observe an increase in endothelial cells, FAPs, endothelial cells, and macrophages in FSHD vs healthy biopsies. **(C)** Pseudotime trajectory of FAP cells suggest that FSHD FAPs are phenotypically different from healthy and are characterized by expression of ECM-related genes.

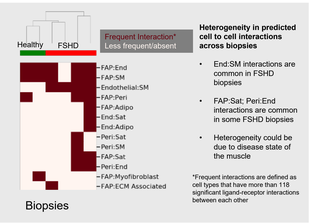
5. Discussion

A. Representative cell:cell interactome of FSHD muscle



(A) We know that certain molecules play a role in maintaining cell to cell interactions in skeletal muscle (3). We can use these interactions as positive controls when assessing predicted cell to cell interactions within healthy and FSHD biopsies. Differences in relative cell type abundance between dystrophic and healthy muscle can affect these interactions, and contribute to the presence of infiltrating fibroblasts and adipocytes in muscle, as well as to unwanted ECM remodeling

B. Common cell to cell interactions in skeletal muscle



(B) One hypothesis is that receptor-ligand interactions, which are present in cell-to-cell interactions that are common in FSHD but not healthy muscle, could be contributing to a disease phenotype. In this heatmap, we show the most common, predicted, cell-to-cell interactions in FSHD and healthy biopsies, and highlight which biopsies they are highly common in. We notice that interactions between endothelial and smooth muscle cells are common in all FSHD but not healthy biopsies, as well as several cell-to-cell interactions that are common in several but not all FSHD biopsies. By using our single nuclear data to predict cell to cell interactions and receptor-ligand interactions between those cells, we can start to understand what interactions may be associated with a disease phenotype.

6. Conclusion

We report a first-in-class single nuclear profiling of the dystrophic FSHD microenvironment which is characterized by an expansion of FAP, macrophage, and endothelial cell populations, as well as a decrease in myonuclei in FSHD muscle compared to healthy muscle biopsies. Further analysis to characterize cell: cell interactions via ligand: receptor pairs expressed in these 'expanded' cell populations in FSHD will allow us to understand how dysregulation of healthy skeletal muscle composition and state may alter the FSHD micro-environment leading to dystrophic pathophysiology.

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