

High Resolution In Situ Analysis of a Hindlimb Ischemia-Reperfusion Injury Reveals Transcriptional Changes Associated with the Transfer of Mitochondria from FAPs to Injured Myofibers

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INTRODUCTION: In ischemia-reperfusion injuries (IRI), the large production of reactive oxygen species has been shown to cause skeletal muscle mitochondrial dysfunction and, consequently, impaired muscle regeneration. Our group has recently demonstrated that fibroadipogenic progenitors (FAPs), resident skeletal muscle mesenchymal stromal cells, have the capability to donate mitochondria to myogenic cells after injury to aid in recovery. However, the subcellular changes after IRI and mechanistic impact of FAP-mediated mitochondrial transfer in muscle regeneration remain poorly defined. This is the first study to leverage single-cell resolution spatial transcriptomics to temporally map subcellular transcriptional changes in skeletal muscle regeneration after IRI. Further, we sought to investigate the consequent impacts of FAP-mediated mitochondrial transfer to regenerating myofibers.

METHODS: FAP-mitochondria reporter mice were generated by crossing Prrx1-Cre and MitoTag mice. Unilateral hindlimb IRI was performed on these mice of both sexes, and the injured tibialis anterior (TA) was harvested at 3, 14, and 28 days after injury (N = 3 per timepoint) (**Figure 1A**). TA muscle from uninjured, control mice was also collected in the same fashion (N = 3). Samples were freshly frozen and then utilized for cryosectioning to produce sequential transverse 10 μm-thick muscle sections. After confirming each sample's quality and morphology testing per the manufacturer's manual, muscle sections were placed on a Xenium slide and processed with the 10x Genomics Xenium In Situ Platform for spatial transcriptomics data. Probe hybridization was performed utilizing a predesigned mouse tissue atlas panel in conjunction with a custom-designed panel for muscle regeneration and mitochondrial bioenergetics genes. The next immediate sequential section was utilized for immunofluorescence (IF) staining to assess for MitoTag signal. IF images were registered onto Xenium spatial transcriptomics output data with Fiji and Xenium Explorer. Post-IRI temporal subcellular transcript analysis and visualization were conducted with Xenium Explorer. All MitoTag-positive regions, identified only at the 14-day post-IRI timepoint, were manually outlined and analyzed against all MitoTag-negative regions with Xenium Explorer. For all analyses, each sample was analyzed separately (N = 12 total, N = 3 per timepoint). All data are presented in the form of mean ± SEM. Two-way ANOVAs with Sidak post hoc test were utilized to compare each transcript across temporal groups and MitoTag signal status. This study was approved by our institutional IACUC.

RESULTS SECTION: All samples demonstrated a high level of high-quality transcripts, Q-score ≥ 20, across our entire gene panel (90.0% ± 2.1%). We were able to visualize and identify distinct cell populations of interest based on cell marker transcripts across the control and all post-IRI timepoints (**Figure 1B**). Temporal cell marker analysis revealed a significant increase in FAP (p<0.05) and myoblast (p<0.001) transcript densities 3 days after IRI, which begins to subside by day 28 (**Figure 1C**). Additionally, there was a significant increase in Myh2 transcription at the 14-day timepoint (p<0.01) (**Figure 1C**). When profiling subcellular mitochondrial dynamics and bioenergetics, there was a corresponding spike in mitochondrial content, fission, fusion, and mitophagy-related transcripts at 14 days post-injury compared to all other timepoints (**Figure 1D**). Xenium data integration with sequential section IF histology demonstrated that regenerating muscle fibers that had received mitochondria transferred from FAPs at 14 days post-IRI had significantly higher expression of Myh2 compared to MitoTag-negative fibers (p<0.05) (**Figure 2A**). Further, these MitoTag-positive fibers had increased transcripts for mitochondrial content and fusion (p<0.001), but not for fission or mitophagy (**Figure 2B**).

DISCUSSION: To our knowledge, we show here the first application of single-cell resolution spatial transcriptomics in skeletal muscle post-IRI to temporally map subcellular transcriptional programs of regeneration and detail the transcriptional consequences of FAP-mediated mitochondrial transfer. Our temporal analysis revealed a 14-day peak in mitochondrial remodeling transcripts, accompanied by increased transcription of fast oxidative Type IIA fiber markers (Myh2), reflecting a shift in regenerating fibers toward an oxidative phenotype. By integrating MitoTag IF images, we demonstrate that muscle fibers that have received mitochondria from FAPs are associated with regenerating fast oxidative Type IIA fibers, in comparison to fibers that have not

received FAP-derived mitochondria. Further, MitoTag-positive fibers exhibit distinct transcriptional profiles, including enhanced mitochondrial content and fusion. Our findings establish a novel spatial framework linking FAP-mediated mitochondrial transfer to increased oxidative fast-twitch fiber regeneration and mitochondrial bioenergetics.

SIGNIFICANCE/CLINICAL RELEVANCE: The novel application of single-cell resolution spatial transcriptomics in skeletal muscle after IRI and integration with FAP-mediated mitochondrial transfer models provides further understanding of this process that could be leveraged to improve muscle regeneration.

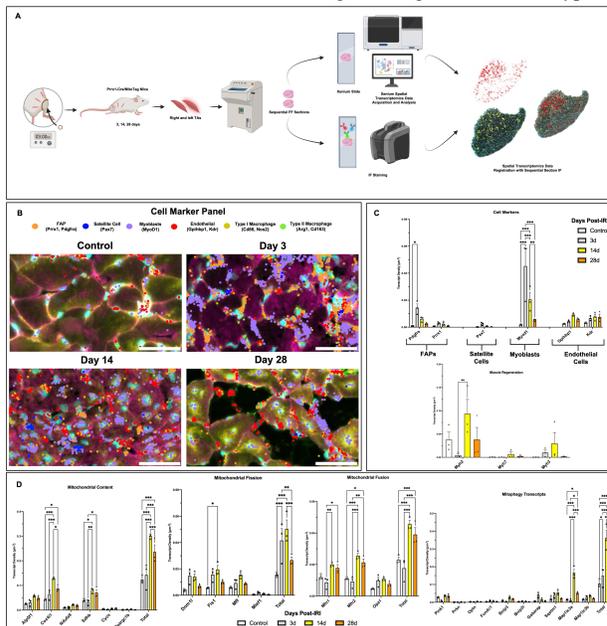


Figure 1. Single-Cell Resolution In Situ Spatial Profiling of Skeletal Muscle Ischemia-Reperfusion Injury (IRI) Demonstrates Subcellular Transcriptional Changes Over the Course of Muscle Injury and Regeneration. (A) Experimental methodology diagram of unilateral hindlimb IRI applied to fibroadipogenic progenitor (FAP) mitochondria-reporter mice (Prrx1-Cre/MitoTag). Injured tibialis anterior (TA) muscle was harvested at multiple timepoints post-IRI and sequential sections were utilized for spatial transcriptomics data collection with the 10x Genomics Xenium In Situ Platform and immunofluorescence (IF) staining. Spatial transcriptomics data were registered with IF images for downstream analysis of muscle fibers that received mitochondria from FAPs (MitoTag). (B) Representative single-cell resolution spatial transcriptomics images of processed samples that identify different cell populations across multiple timepoints post-IRI. Scale bar = 50μm. (C) Quantification of cell-specific transcript markers across IRI timepoints. Quantification of oxidative muscle fiber and regeneration transcript markers across IRI timepoints. (D) Quantification of mitochondrial dynamics changes across IRI timepoints, demonstrating peak alterations in mitochondrial content, fission, fusion, and mitophagy transcripts at 14 days after injury. *p<0.05 **p<0.01 ***p<0.001

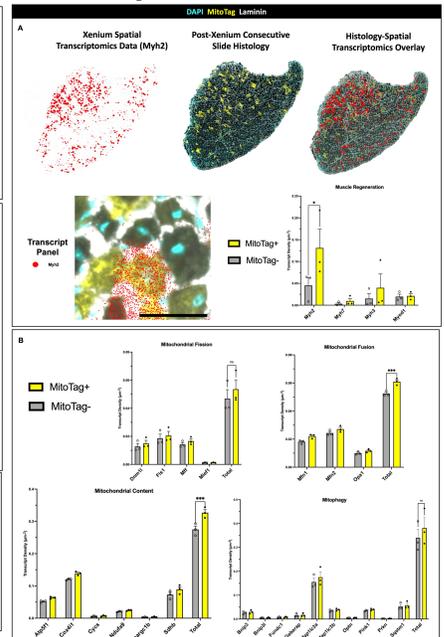


Figure 2. Mitochondria Derived From Fibroadipogenic Progenitors (FAPs) Are Spatially Associated With Regenerating Type IIA Fibers at 14 Days After Ischemia-Reperfusion Injury (IRI). (A) 14-day post-IRI representative pattern of Myh2 and IF stain image of the immediate sequential sections post-Xenium sample. Spatial transcriptomics data were integrated onto IF images. Quantification of MitoTag+ and MitoTag- regions revealed a significant increase in Myh2 expression in fibers that received mitochondria from FAPs. Scale bar = 50μm. (B) Quantification of mitochondrial dynamics transcripts across MitoTag+ and MitoTag- regions. *p<0.05 **p<0.01 ***p<0.001