Investigating the Role of Cx43 in FAP-Mediated Mitochondrial Transfer for Muscle Regeneration after Ischemia-Reperfusion Injury

Luke Sang[1,2], Cristhian Montenegro[1,2], Justin Lau[1,2], Megan Le[1,2], Matthew Allen[1,2], Ryan Sadjadi[1,2], Miguel Lizarraga[1,2], Nesa Milan[1,2], Hiroyori Fusagawa[1,2], Yusef Samini[1,2], Hannah Chi[1,2], Xuhui Liu[1,2], Brian T. Feeley[1,2], Michael Davies[1,2]
[1]San Francisco Veterans Affairs Health Care System, [2]Department of Orthopaedic Surgery, University of California, San Francisco
Luke.Sang@ucsf.edu

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INTRODUCTION: Mitochondrial dysfunction is a hallmark of many pathophysiological processes in skeletal muscle. Mitochondrial transfer from mesenchymal stromal cells (MSCs) has been shown to occur, especially following ischemic insults in various organs, to facilitate cell survival following injury. In muscle, our lab has previously demonstrated that fibroadipogenic progenitors (FAPs), resident skeletal muscle MSCs, play a central role in mediating muscle regeneration and donate mitochondria to recipient myogenic cells following injury. However, the exact mechanism by which FAP-mediated mitochondrial transfer occurs has not been elucidated. We sought to investigate the potential role of Connexin 43 (Cx43), a gap-junction-associated protein encoded by the GJA1 gene, in facilitating FAP-mediated mitochondrial transfer to recipient cells after ischemia-reperfusion injury (IRI).

METHODS: Unilateral hindlimb IRI was performed on Prrx1-Cre/MitoTag mice of both sexes, and both the injured and uninjured tibialis anterior (TA) were harvested at different timepoints after injury. Live cells, digested from the injured TA of a subset of mice (N = 3 per timepoint), were sent for single-cell RNA-sequencing (scRNAseq). Additionally, TAs were utilized for immunofluorescence (IF) staining to assess for Cx43 expression and MitoTag signal and homogenized for Western Blotting (WB) at various points post-IRI (N = 3 per timepoint). FAPs were isolated from Prrx1-Cre/MitoTag mice with fluorescence-activated cell sorting, and C2C12 mice myoblasts underwent cytoplasm-targeted lentiviral transduction with mCherry. These FAPs and C2C12s were utilized in 24-hour co-culture experiments (N = 3 per condition). Flow cytometry was used to quantify the number of mitochondrial transfer events from FAPs to recipient C2C12s after co-culture and imaged with confocal microscopy. Co-culture conditions included: 1. Pre-treatment of FAPs with a beta-agonist (BAT-FAPs), mirabegron, which was also utilized for IF staining to assess for Cx43 expression. 2. Pre-treatment of C2C12s with CoCl2 to stimulate recipient cell hypoxia. 3. Co-culture treatment with Gap27, a Cx43-specific inhibitor. 4. Transwell co-culture. All data are presented in the form of mean ± SD. Unpaired t-tests and two-way ANOVAs with Tukey's post hoc test were utilized when appropriate. This study was approved by our IACUC.

RESULTS SECTION: Our scRNAseq analyses demonstrated that FAPs and endothelial cells showed high expression of GJA1 compared to all other populations (**Figure 1A-B**). Further, CellChat analysis showed that GJA1-positive FAPs had upregulated signaling pathways involved in myogenic differentiation (NCAM, OSM, FGF) to satellite cells (SCs) compared to FAPs not expressing GJA1 and all other cell types (**Figure 1C**). IF microscopy revealed MitoTag-positive fibers were only found at 14 days post-IRI in both the injured and uninjured TA ($p<0.001$) (**Figure 1E-N, P**). Cx43 expression in the injured TA peaks at 3 days after IRI compared to the uninjured TA (**Figure 1 Q-R**). WB demonstrated that the full-length Cx43 (43 kDa) protein and the GJA1-20k isoform are significantly expressed in the injured TA at 3 days post-IRI in comparison with the uninjured TA (**Figure 1S-V**). In comparison, the full-length Cx43 was more upregulated than the GJA1-20k isoform. Both translational products decrease by day 7 after injury. Confocal microscopy of FAP C2C12 co-culture provided visual confirmation of FAP-mediated mitochondrial transfer as seen in mCherry C2C12 cells containing GFP mitochondria from FAPs (**Figure 2G**). Flow cytometry of co-cultures showed that mirabegron treatment of FAPs significantly increases mitochondrial transfer events to both untreated ($p<0.001$) and CoCl2-treated ($p<0.001$) recipient cells (**Figure 2H**). Further, mirabegron treatment significantly increased FAP Cx43 expression in culture compared to standard media (**Figure 2I-K**). Gap27 treatment significantly decreased FAP-mediated mitochondrial transfer events ($p<0.01$), and there was little to no transfer seen when the cell populations were not in contact with each other ($p<0.001$) (**Figure 2 O-P**).

DISCUSSION: In this study, we show that FAPs highly express GJA1 after IRI and that this subset of FAPs is involved in muscle regeneration pathways. Cx43 expression in injured muscle peaks acutely after injury, and before FAP-mitochondria is seen in regenerating muscle fibers. We show evidence of FAP-mediated mitochondrial transfer with confocal microscopy and demonstrate that this process is contact-dependent. The specific blockade of Cx43 significantly decreases FAP-mediated mitochondrial transfer events. Beta-agonism increases FAP Cx43 expression and consequent mitochondrial transfer. Our findings suggest that Cx43 plays a crucial role in facilitating the transfer of mitochondria from FAPs to myogenic cells for muscle regeneration following IRI and may potentially be leveraged with beta-agonist treatment.

SIGNIFICANCE/CLINICAL RELEVANCE: Detailing a novel mechanism of mitochondrial transfer in skeletal muscle after IRI unlocks a potential new therapeutic target to improve myogenesis following injury.

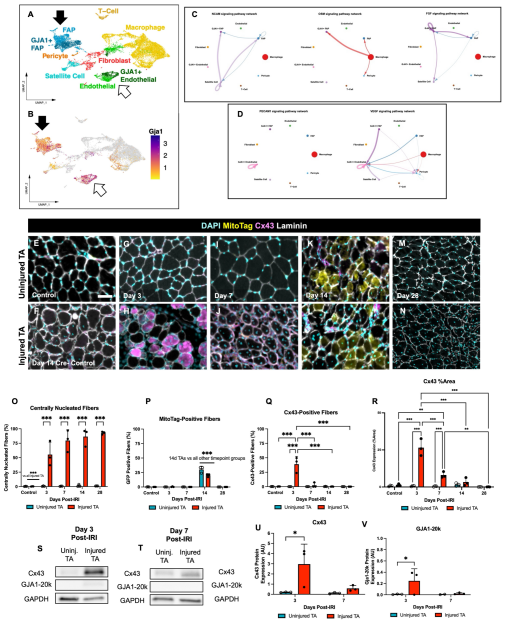

Figure 1. Muscle Ischemia-Reperfusion Injury (IRI) Single-Cell RNA Sequencing, Immunofluorescent (IF) Histology, and Western Blotting (WB). (A) Uniform Manifold Approximation and Projection (UMAP) plot of tibialis anterior (TA) muscle cells combined from control, 3-day, and 14-day post-IRI samples, clustered by cell type. (B) UMAP plot demonstrating the expression of GJA1 across different cell types. Increased expression of GJA1 is seen primarily in fibroadipogenic progenitors (FAPs) and endothelial cells. CellChat signaling analysis of injured muscle 3-day post-IRI demonstrating that (C) GJA1-positive FAPs have upregulated signaling pathways associated with myogenic differentiation. (D) Meanwhile, GJA1-positive endothelial cells have upregulated signaling in pathways associated with angiogenesis and endothelial cell migration. The thickness of the lines correlates with the strength of signaling, and arrows demonstrate the direction of cell-cell communication. (E-N) Representative uninjured TA and injured TA muscle sections from Prrx1-Cre/MitoTag mice post-IRI imaged by IF microscopy for Cx43, MitoTag, Laminin, and DAPI. Scale bar = 50μm. (O) Percentage of centrally nucleated fibers across different timepoints post-IRI, which demonstrated a continued rise in the injured TA at 28 days post-injury. (P) Percentage of MitoTag-positive fibers, representing mitochondrial transfer from FAPs. Fibers with positive signal were found only at 14 days after IRI in both uninjured and injured TA. Cx43 expression measured as (Q) positive fibers and (R) percent area, peaking at 3 days post-IRI in the injured TA. (S-V) Representative WB images and quantification of GJA1 isoform expression in whole uninjured and injured TA muscle at 3 and 7 days post-IRI. *p<0.05**p<0.01***p<0.001

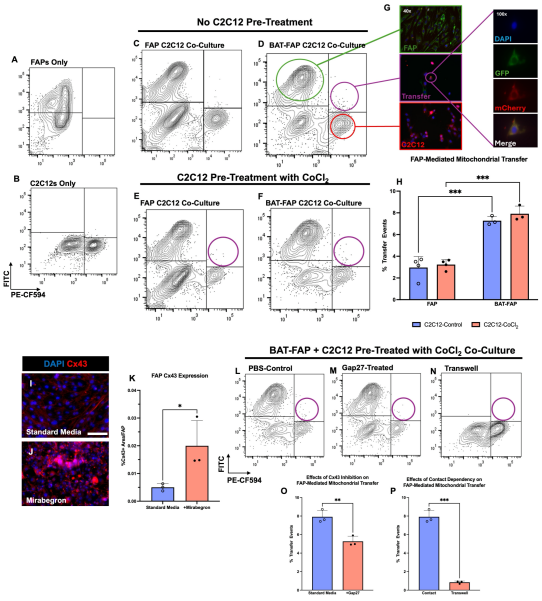

Figure 2. In Vitro Quantification of Fibroadipogenic Progenitor (FAP)-Mediated Mitochondrial Transfer and the Effects of FAP Mirabegron Pre-Treatment. (A-B) Representative flow cytometry plots of single-color, mono-culture FAPs and C2C12s to establish the gating strategy controls. Representative flow cytometry plots of 24-hour (C) FAP C2C12 co-culture for quantification of mitochondrial transfer events, circled in purple (double-positive GFP and mCherry cells). (D) Separate groups of FAPs were also pre-treated with mirabegron for 72 hours to induce beige fat differentiation (FAP-BAT) before 24-hour co-culture with C2C12s. (E) Additionally, groups of C2C12 cells were pre-treated with $CoCl_2$ to stimulate hypoxia before co-culture with FAPs or (F) BAT-FAPs. (G) Confocal visualization of FAP-mediated mitochondrial transfer events to C2C12, demonstrating mCherry C2C12 cells containing GFP mitochondria from FAPs. (H) Analysis of mitochondrial transfer events from all co-culture conditions, demonstrating that the highest rates of transfer occurred from BAT-FAPs. Representative images from immunofluorescent microscopy for Cx43 and DAPI in FAPs grown in (I) standard medium and (J) 72-hour Mirabegron treatment with (K) quantification. Scale bar = 100μm. FAPs were pre-treated with mirabegron for 72 hours and co-cultured with C2C12 cells pre-treated with $CoCl_2$. Either (L) PBS, as a control, or (M) Gap27, a Cx43-specific channel inhibitor, was added to their media during co-culture. (N) A separate group was also co-cultured using a transwell to separate the cells, instead of direct contact culturing, which was done in all other experimental conditions. (O-P) Analysis of FAP-mediated mitochondrial transfer events across experimental conditions revealed a significant decrease in transfer events with Cx43-specific inhibition and little to no transfer if FAPs and C2C12s were not in contact with each other. **p<0.01***p<0.001