Mitochondrial Transfer For Muscle Regeneration Following Ischemia Reperfusion Injury

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Disclosures: Authors have no relevant disclosures.

INTRODUCTION: Ischemia reperfusion injury (IRI) is a serious condition that is associated with compartment syndrome, crush injuries, peripheral vascular disease, and other orthopedic injuries. IRI leads to mitochondrial damage via increased production of reactive oxygen species, creating the necessity to regenerate the mitochondrial network for muscle regeneration [1]. Skeletal muscle regeneration is vital to avoid atrophy, muscle weakness, and permanent function impairment [2]. Fibroadipogenic progenitors (FAPs) are resident skeletal muscle stem cells that have been shown to transfer mitochondria to regenerating myofibers during healing [3]. Further, beta-adrenergic treatment of FAPs, inducing them into a beige adipose phenotype, has been shown to increase FAP mitochondrial transfer and lead to increased muscle regeneration following IRI [4]. However, the mechanisms for mitochondrial transfer from FAPs have not yet been determined. We hypothesized that Connexin 43 (Cx43), a gap junction protein encoded by the GJA1 gene, facilitates the transfer of mitochondria from FAPs to regenerating myofibers after IRI. Further, we hypothesized that Cx43 expression is upregulated in FAPs after beta-agonism to allow greater mitochondrial transfer.

METHODS: Hindlimb IRI was performed on mice as previously described [4]. Injured (right, RTA) and uninjured (left, LTA) tibialis anterior were harvested, at different timepoints following injury. Uninjured, control RTA and LTA were also harvested. Live cells from these samples were sent for single cell RNA-sequencing (scRNAseq) utilizing 10x Genomics 3' kits. The datasets were processed using Cellranger and analyzed and clustered using the R package Seurat. Differential gene expression was accepted if $p<0.05$. *In vivo,* immunofluorescence staining to assess for Cx43 protein expression was performed on samples harvested from PDGFRaCre^{ERT}/MitoTag mice 3 days, 7 days, 14 days, and 28 days post-IRI. *In vitro,* isolated wildtype murine FAPs (mFAPs) were cultured, treated for 72 hours with mirabegron, and stained for Cx43. All images were analyzed using ImageJ. All data is presented in the form of mean ± SE. Unpaired t-tests were used for statistical analysis. Significance was considered when $p<0.05$. This study was approved by IACUC.

RESULTS SECTION: scRNAseq showed higher expression of GJA1 in FAPs and endothelial cells compared to all other cell populations in the IRI groups across all timepoints (Fig 1). *In vivo* immunofluorescence staining revealed significantly increased expression of Cx43 in muscle following IRI compared to the corresponding contralateral uninjured side. (Fig 2). This unilateral increase peaks at about 3 days after injury (Cx43+ percent area coverage, RTA: 29.887 ± 2.071 vs LTA: 0.471 ± 0.089, $p<0.0001$) and decreases back to baseline after 28 days (Fig 2). There were no significant differences between RTA and LTA in uninjured mice (RTA: 0.505 ± 0.221 vs LTA: 0.625 ± 0.323, $p>0.05$) or at 28 days after IRI (RTA: 0.172 ± 0.169 vs LTA: 0.264 ± 0.0464, $p>0.05$). Further, *in vitro* beta-adrenergic treatment of FAPs showed increased Cx43 expression when compared to FAPs in standard media (Cx43+ percent area coverage per cell count, 0.018 ± 0.003 vs 0.005 ± 0.001, $p<0.05$) (Fig 2).

DISCUSSION: Cx43 mediated gap junctions have been shown to be involved in facilitating mitochondrial transfer from mesenchymal stem cells to injured lung cells [5]. However, the role of Cx43 has never been studied within skeletal muscle. In this study, we suggest that a function of Cx43 may be to facilitate mitochondrial transfer within skeletal muscle for myogenesis following IRI. We show here that following IRI, FAPs highly express GJA1 in comparison to other cells. Further, we show *in vivo* that there is a significant increase of Cx43 expression in injured muscle as soon as 3 days after IRI and returns to baseline after 28 days. As previous studies have shown FAPs to play a supportive role in muscle regeneration through mitochondrial transfer, this data suggests FAPs may be utilizing Cx43 mediated gap junctions to donate mitochondria to regenerating myofibers after IRI [3]. Lastly, we show that beta-agonism of FAPs *in vitro* increases Cx43 expression relative to standard media. Beta-agonism has been shown to both induce FAPs into a beige-adipose phenotype that increases mitochondrial transfer and improves muscle regeneration after IRI [4]. These findings suggest that beta-adrenergic treatment of FAPs increases their expression of Cx43 to allow for increased levels of mitochondrial transfer and may consequently enhance myogenesis.

SIGNIFICANCE/CLINICAL RELEVANCE: Understanding a novel mechanism of mitochondrial transfer for skeletal muscle regeneration unlocks the potential for exciting new therapeutic targets to enhance this process and improve myogenesis following IRI.

REFERENCES: [1] Jassem et al. Kidney International, 2004.; [2] Wilson et al. J Appl Physiol, 1985.; [3] Davies et al. Am J Sports Med, 2022.; [4] Zhang et al. JOR, 2020.; [5] Islam et al. Nat Med, 2012.

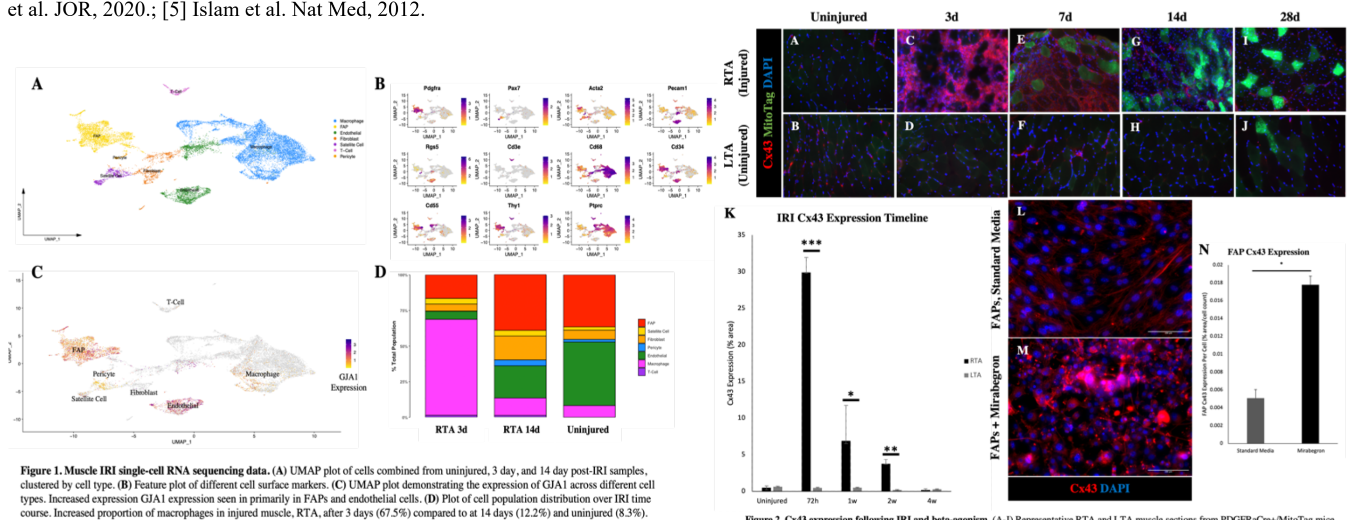

Figure 1. Muscle IRI single-cell RNA sequencing data. (A) UMAP plot of cells combined from uninjured, 3 day, and 14 day post-IRI samples, clustered by cell type. **(B)** Feature plot of different cell surface markers. **(C)** UMAP plot demonstrating the expression of GJA1 across different cell types. Increased expression GJA1 expression seen in primarily in FAPs and endothelial cells. **(D)** Plot of cell population distribution over IRI time course. Increased proportion of macrophages in injured muscle, RTA, after 3 days (67.5%) compared to at 14 days (12.2%) and uninjured (8.3%).

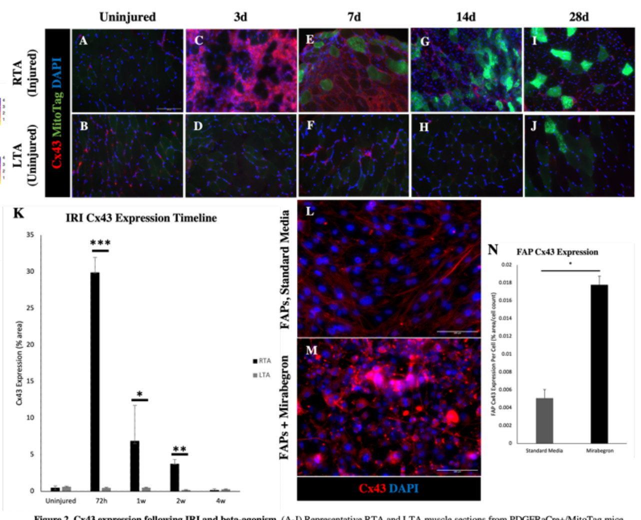

Figure 2. Cx43 expression following IRI and beta-agonism. (A-J) Representative RTA and LTA muscle sections from PDGFRaCre+/MitoTag mice post-IRI imaged by immunofluorescent microscopy for Cx43, MitoTag, and DAPI. (K) *In vivo* Cx43 expression calculated as percent area of signal. Representative images from immunofluorescent microscopy for Cx43 and DAPI in FAPs grown in (L) standard medium and (M) 72 hour beta-adrenergic treatment. (N) *In vitro* FAP cellular Cx43 expression per cell calculated as percent area of signal per cell count. *p<0.05**p<0.001***p<0.0001