SUPPLEMENTAL METHODS

Supplemental Methods 1

Mitochondrial isolation

200 mg of liver was homogenized in a buffer containing 300 mM sucrose; 10 mM K^+-HEPES; 1 mM K^+-EGTA. The homogenate was centrifuged and filtered through two 40 µm mesh filters and a 10 µm filter. After centrifugation, the mitochondria pellet was suspended in ice-cold PBS for tail vein injection or mitochondrial respiration buffer, MiR05 [2] for respirometric analysis.

Mitochondrial respiration

High-resolution respirometry was conducted on isolated mitochondria in MiR05 [1] using a O2k-FluoRespirometer (Oroboros, Austria) to ensure that isolated mitochondria were viable and respiration competent prior to transplantation. Respiration was assessed using sequential titrations of 1) 5 mM pyruvate, 10 mM glutamate, and 2 mM malate to determine state 2 LEAK respiration supported by complex I-linked substrates. 2) 2.5 mM ADP was added to determine maximal state 3 oxidative phosphorylation (OXPHOS) through complex I [2]. 3) 10 µM Cytochrome *C* was added to assess the quality of the mitochondrial sample and the integrity of the mitochondrial outer membrane [2]. Cytochrome *C* addition did not stimulate respiration >15%, indicating that the mitochondria were intact and respiration competent.

Supplemental Methods 2

Mitochondrial respiration in MTT or PBS treated myoblasts

To confirm that mitochondria functioned normally after MTT, C2C12 cells were incubated with 50 µg/ml of isolated mitochondria for 24 hours (MTT), or the vehicle (PBS) (n=4/group). After counting, the cells were diluted to $5x10^5$ cells/ml in MiR05 and used for respirometry experiments in technical duplicates ($1x10^6$ cells per chamber). Respiration was measured prior to the addition of digitonin for cell permeabilization. The substrate-uncoupler-inhibitor-titration (SUIT) that was used to assess mitochondrial function contained: 0.1 mM malate, 500 µM octanoyl-carnitine, and 2.5 mM ADP to measure OXPHOS supported by fatty acid oxidation (FAO); 5 mM pyruvate, 10 mM glutamate, and 2 mM malate to determine OXPHOS respiration supported by complex I-linked substrates (FAO+CI OXPHOS); 10 mM succinate to measure maximal complex I and II supported OXPHOS (FAO+CI+ II OXPHOS); 10 µM Cytochrome *C* to assess the integrity of mitochondria (samples were rejected if flux increased by >15%); Stepwise titrations of 0.5 µM carbonyl cyanide 3-chlorophenylhydrazone to assess maximal electron transport system capacity supported by FAO and complex I+II substrates (FAO+CI+CII ETS); 6) 0.5 µM rotenone to inhibit complex I and assess complex II supported electron transport system capacity (CII ETS); and 7) 2.5 µM Antimycin A to inhibit complex III and acquire residual oxygen consumption (ROX) for background correction. Mitochondria respiration was normalized to the number of cells in the chamber.

Supplemental Methods 3

Mitochondrial Labeling and Imaging. Three different approaches were followed to provide a proof of concept to assess if donor mitochondria were incorporated into host muscle cells, we followed.

1. In the first *in vitro* approach, we plated C2C12 myoblasts in growth serum and grew them to 70% confluency.
2. In a second *in vitro* approach, C2C12 myoblasts were differentiated in 2% horse serum for 5 days to form myotubes for additional experiments.

For *in vitro* experiments for mitochondrial labeling

Antibodies from Invitrogen-Fisher Scientific, USA

MA3012, HSP-60-anti-Heat Shock Protein 60, IgG2a

C2C12 cells or myoblasts were incubated were incubated with 50 μg of PhAM mitochondria/ml of media for 24 h at 37°C. Mitochondria that were isolated from donor PhAM mice had a Dendra-2 (GFP-green) label which allowed us to distinguish transplanted mitochondria from the resident mitochondria in the myoblasts. Following 24 h of incubation in PhAM mitochondria, the cells were washed to remove any unincorporated mitochondria, then the myoblasts were incubated with 50 nM of MitoTracker deep red FM (ThermoFisher Scientific) for 30 minutes at 37°C to identify intrinsic and transplanted mitochondria. The cells were washed thoroughly in PBS to remove any unbound dye. The myoblasts and myotubes were then washed further in PBS to remove any unbound cells, fixed in 4% paraformaldehyde, and then imaged on a Biotek Lionheart™ FX Automated Microscope (Winooski, Vermont). Resident mitochondria stained with MitoTracker Red FM appear in the CY5 fluorescent channel with an excitation/emission 531/594 nm and donor PhAM mitochondria were indicated by their green Dendra-2 label (LED light emission/excitation at 469/525) signals. The mitochondria were not exposed to a laser signal during imaging, and therefore the donor PhAM mitochondria remained green in the transplanted cells. These findings were confirmed in experiments where C2C12 myoblasts were first incubated with Dendra-2 donor mitochondria, then the myoblasts were labeled with anti-Heat Shock Protein 60 (HSP-60, Invitrogen, USA), a mitochondria specific protein to label all mitochondria (donor+ host mitochondria) overnight at 4°C. HSP60 labeled mitochondria were imaged in the RFP channel with an emission/excitation at 531/594.

Supplemental Methods 4

For *in vivo* experiments of mitochondrial labeling

Antibody from the Hybridoma Bank, Iowa, USA
MandyS8(8H11) anti-dystrophin antibody

Secondary antibody from Fisher Scientific, USA
A32732-IgG anti-mouse Alexa Fluor 488 secondary

DAPI Staining for nuclei
P36962

To confirm that isolated mitochondria that were injected through a tail vein (i.e., MTT) were taken up by injured skeletal of mice *in vivo*. Native host mitochondria were isolated from the liver of C57BL/6 donor mice and then labeled with MitoTracker Deep Red FM (ThermoFisher Scientific) at a concentration of 150 nM for 30 minutes at 4°C. Twenty-four hours after $BaCl_2$ injection into the gastrocnemius of one limb, 50 μg of MitoTracker labeled mitochondria suspended in sterile PBS was injected in the tail vein of host injured C56BL/6 mice, and the mice were euthanized 24 h after MTT. Gastrocnemius muscles of injured and the intra-animal non-injured (PBS-sham injected) limbs were removed and frozen in isopentane. Tissue cross sections or longitudinal sections were mounted on charged glass slides (ThermoFisher Scientific). The tissue sections were incubated overnight at 4°C with an anti-dystrophin antibody (MandyS8(8H11), Hybridoma Bank, Iowa) to identify the muscle fiber sarcolemma. An IgG anti-mouse Alexa Fluor 488 secondary antibody (A32732, Fisher Scientific) was used to identify the sarcolemma (green) and images were obtained with an emission/excitation at 469/525. DAPI (P36962, Fisher Scientific) was used to identify the nuclei in the muscle fibers. MitoTracker deep red FM labeled mitochondria were identified in the far-red (Cy5) channel.

Supplemental Methods 5

Antibodies, fiber typing and fiber morphology methods

From the Developmental Studies Hybridoma Bank (Iowa City, IA USA):

Myosin heavy chain (MHC)- I, (BA-F8S; IgG2b)
MHC IIA (SC-71; IgG1)
MHC IIB (BF-F3; IgGM)

The gastrocnemius muscles of both hind limbs were frozen in 2-methylbutane cooled with liquid nitrogen. Tissue sections were cut at -20°C and with an 8 μm thickness on a Leica model CM3050S cryostat (Leica Biosystems Nussloch, Germany). The tissue sections were mounted on charged glass slides (ThermoFisher Scientific, Pittsburgh PA, USA). Control non-injured and the injured gastrocnemius tissue sections for each animal were placed on the same slide. The slides were air dried then kept at -80°C until used for immunocytochemistry. The slides containing the frozen tissue sections were allowed to come to room temperature for 30 minutes then rehydrated in PBS for 10 minutes. Non-specific binding of IgGs to the tissue sections were blocked by incubating the sections in 2.5% normal goat serum in 5% M.O.M. protein concentrate (Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature. The tissue sections were next incubated in a master mix of equal volumes of supernatant from primary antibodies that were obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA USA) to myosin heavy chain (MHC)-I, MHC IIA and MHC IIB as described previously [3, 4].

The master mix of primary antibodies was applied to each tissue section overnight at 4°C. On the following day, the slides were washed in PBS then incubated for 1 h at room temperature in a mix of secondary Alexa Fluor antibodies (ThermoFisher Scientific Pittsburgh PA, USA) that consisted of 2μl/ml of PBS of each of Alexa Fluor anti-mouse IgG2b (A21146), Alexa Fluor anti-mouse IgG1 (A21121) and Alexa Fluor anti-mouse IgM (A21426). The fluorescent images were captured with a Biotek Lionheart™ FX Automated Microscope (Winooski, Vermont, USA). Type I MHC (red) fibers were imaged with emission/excitation at 628/685 nm. MHC IIA (green) fibers were imaged with emission/excitation at 469/525 nm. Images of MHC IIB (orange) fibers were obtained with emission/excitation at 531/594 nm. DAPI staining of myonuclei (blue) were obtained with an excitation/emission of 377/447 nm.

Type I MHC (red) fibers were imaged with emission/excitation at 628/685 nm. MHC IIA (green) fibers were imaged with emission/excitation at 469/525 nm. Images of MHC IIB (orange) fibers were obtained with emission/excitation at 531/594 nm. DAPI staining of myonuclei (blue) were obtained with an excitation/emission of 377/447 nm.

For muscle fiber area and feret diameter measures, gastrocnemius muscle fiber cross sectional areas were obtained by planimetry from a minimum of 500 fluorescently labeled fibers/muscle taken from 6-8 randomly selected fields at an objective magnification of 20X. Mean fiber area was calculated using ImageJ software (National Institutes of Health, Bethesda, Maryland). Left and right (i.e., control and injured-experimental muscles) were assessed from each animal. Mean fiber areas were calculated with the standard deviation measured for each time point and each experimental group. The mean differences between the control and the experimental (injured-repairing) muscles from each mouse were obtained in both the MTT and PBS (sham) treatment groups for each time point.

Supplemental Methods 6

***qPCR Analysis*.**

RNA was isolated from control and injured muscles utilizing the Purelink RNA Mini Kit (Invitrogen) and reverse transcribed using the Maxima H Minus First Strand cDNA Synthesis Kit with dsDNase (Invitrogen/ThermoFisher Scientific). Real-time qPCR was conducted using Sybr Green Master Mix (Roche) and the Lightcycler 480 PCR (Roche) was used to amplify the transcripts. Melt curves that had multiple peaks were not included in the analysis. The relative expression levels of amplified transcripts were estimated by the comparative CT method ($2^{-\Delta\Delta CT}$) and normalized to a housekeeping gene (GAPDH). The gene transcripts that were examined included transforming growth factor beta-1 (TGFβ1), type I collagen (COL I), type III collagen (COL III), and type V collagen (COL V). As changes in non-contractile elements in muscle by MTT could occur from modifications in degradation of non-muscle proteins such as collagen, we also measured transcriptional regulation of metalloproteinases (MMP)2, MMP9, MMP13 and MMP14.

Supplemental Methods 7

Antibodies and methods for western blots

Antibodies purchased from (ThermoFisher Scientific, USA)

Collagen I (#50-172-9648)

Collagen III (50-#173-3935) MMP 9 (#50-172-6255)

Antibody purchased from Sigma-Aldrich Chemical Co. (St. Louis MO, USA).

Collagen IV (#SAB4500369)

Approximately 40 mg of muscle was homogenized in ice cold RIPA (Thermo Fisher Scientific) containing 2mM PMSF (Sigma Aldrich, St. Louis MO, USA), 5mM EDTA (ThermoFisher Scientific, USA), and 1x Halt protease and phosphatase inhibitor cocktail (ThermoFisher Scientific USA) at 4°C. Tissue lysates were centrifuged for 15 minutes at 14,000 g at 4°C and the supernatant collected. The protein content of the tissue supernatant was measured via a colorimetric DC protein assay kit (Bio-Rad/Thermo Fisher Scientific USA). Twenty micrograms of cell lysate were loaded and separated on SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Nitrocellulose membranes were then stained for total protein utilizing No-Stain Protein Labeling Reagent (ThermoFisher Scientific/Invitrogen, USA). The membranes were blocked in 5% bovine serum albumin (BSA) in Tris-buffered saline-Tween 20 (TBST) and total protein was fluorescently quantified via the iBright FL1500 imaging system (Invitrogen/ThermoFisher Scientific USA). After blocking, membranes were incubated overnight at 4°C with primary antibodies purchased from Invitrogen/ThermoFisher Scientific USA) against Collagen I (#50-172-9648), Collagen III (50-#173-3935), and MMP 9 (#50-172-6255), and anti-Collagen IV (#SAB4500369) that was purchased from Sigma-Aldrich Chemical Co. (St. Louis MO, USA). The primary antibodies were diluted at a 1:1000 concentration in TBST. The membranes were washed and incubated with anti-rabbit or anti-mouse IgG-conjugated secondary antibodies for 1 h at room temperature. The signals were developed with Supersignal West Pico Plus ECL reagent (ThermoFisher Scientific, USA) for 5 minutes and the blots imaged and analyzed utilizing the iBright FL1500 imager and analysis software (ThermoFisher Scientific/Invitrogen, USA).

Verification of rigor of antibody specificity. The antibodies used in our experiments were obtained from vendors who provided data to support their testing, and production validation. We also confirmed that the lots of the antibodies remained constant over the duration of the study. The antibodies used in the western blot analysis were validated by testing on appropriate positive and negative controls by immunoblotting. Negative controls were used to confirm that there was no cross-labeling by the antibodies. Western blots were conducted to validate that the protein band sizes were at the appropriate and predicted position.

Supplemental Methods 8

C-Reactive Protein (CRP)
ThermoFisher Scientific, USA
EM20BX, CRP Elisa

To assess if systemic injection of autologous mitochondria resulted in a systemic inflammatory response, we assessed serum CRP in uninjured mice that received no systemic treatment (n=3), or uninjured mice that received a systemic injection of either 50 μl of PBS as a sham treatment (n=3), or 50 μg of mitochondria suspended in 50 μl of PBS as the MTT treatment group (n=4). A mouse CRP Elisa (EM20BX, ThermoFisher Scientific, USA) was conducted according to the manufacture's recommendations. Briefly, 20 μl of serum was diluted in the assay dilutant. The diluted serum was plated in triplicate in the ELISA plate supplied by the manufacture and incubated for 2.5 h at room temperature. Standards provided by the manufacture were loaded on the same plate. The plate was washed then incubated in the biotin conjugate for 1 h at room temperature then washed in PBS. The plate was then incubated in Strepatvidin-HRP for 45 min, washed and the color was developed by incubation in TMB in the dark for 30 min. A stop solution was added, and the plate was quantified by utilizing a multimode microplate reader (Synergy™ H1, Biotek, Santa Clara, CA, USA) at 450 nm. The optical density in each well was fitted to a standard curve that was obtained on the same plate.

IFN-γ
Invitrogen/ ThermoFisher Scientific, USA
MIF00, IFN-γ ELISA kit

Plasma IFN-γ was assessed in non-injured non-injected mice (n=4), and in PBS-treated (n=4), and MTT mice (n=4), using a commercial mouse IFN-γ ELISA kit (MIF00, Invitrogen/ ThermoFisher Scientific, USA). A recombinant IFN-γ positive control that was supplied by manufacturer was used to confirm ELISA function/sensitivity and to establish a standard curve for the assay. Twenty microliters of plasma were obtained for each assay and ran in triplicate on the ELISA plate for each animal. The standard curve was used to calculate IFN-γ as pg/ml. Triplicate samples were averaged for each animal.

REFERENCES

1. Komlodi T, Sobotka O, Krumschnabel G, Bezuidenhout N, Hiller E, Doerrier C, et al. Comparison of Mitochondrial Incubation Media for Measurement of Respiration and Hydrogen Peroxide Production. Methods Mol Biol. 2018;1782:137-55.
2. Gnaiger E. Mitochondrial Pathways and Respiratory Control: An Introduction to OXPHOS Analysis. Bioenergetics Communications 2020A. 2020;5:122.
3. Brooks MJ, Hajira A, Mohamed JS, Alway SE. Voluntary wheel running increases satellite cell abundance and improves recovery from disuse in gastrocnemius muscles from mice. J Appl Physiol (1985). 2018;124:1616-28.
4. Myers MJ, Shepherd DL, Durr AJ, Stanton DS, Mohamed JS, Hollander JM, et al. The role of SIRT1 in skeletal muscle function and repair of older mice. J Cachexia Sarcopenia Muscle. 2019;929-49.

Supplemental Figures

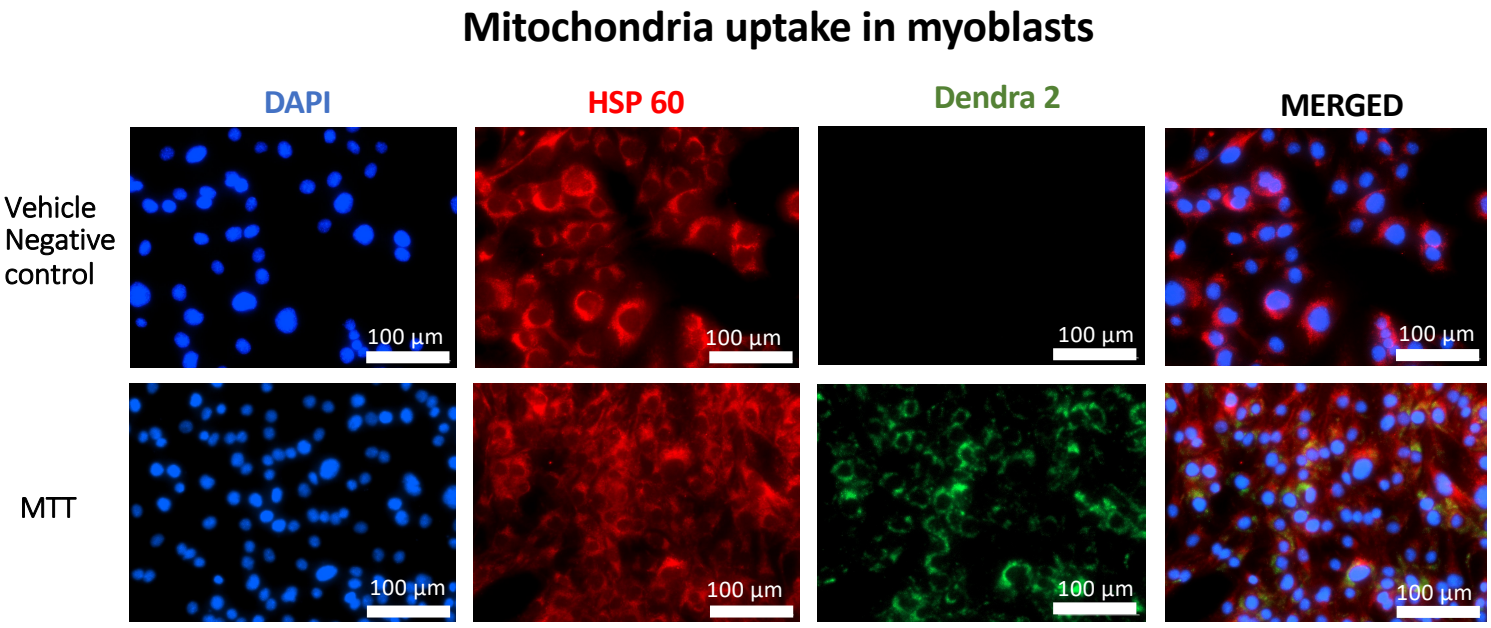
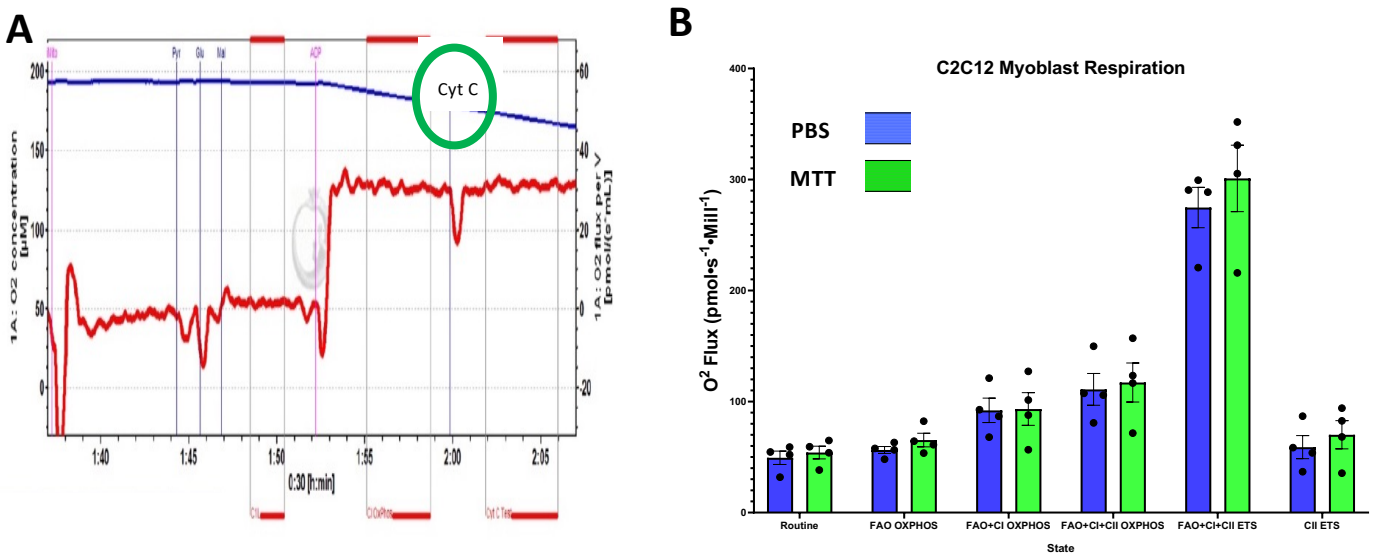

Mitochondria uptake in myoblasts
DAPI
HSP 60
Dendra 2
MERGED
Vehicle
Negative
control
MTT
100 µm

Figure S1

Respiration of C2C12 myoblasts following incubation with mitochondria



Example of respiration in isolated donor mitochondria

Figure S2

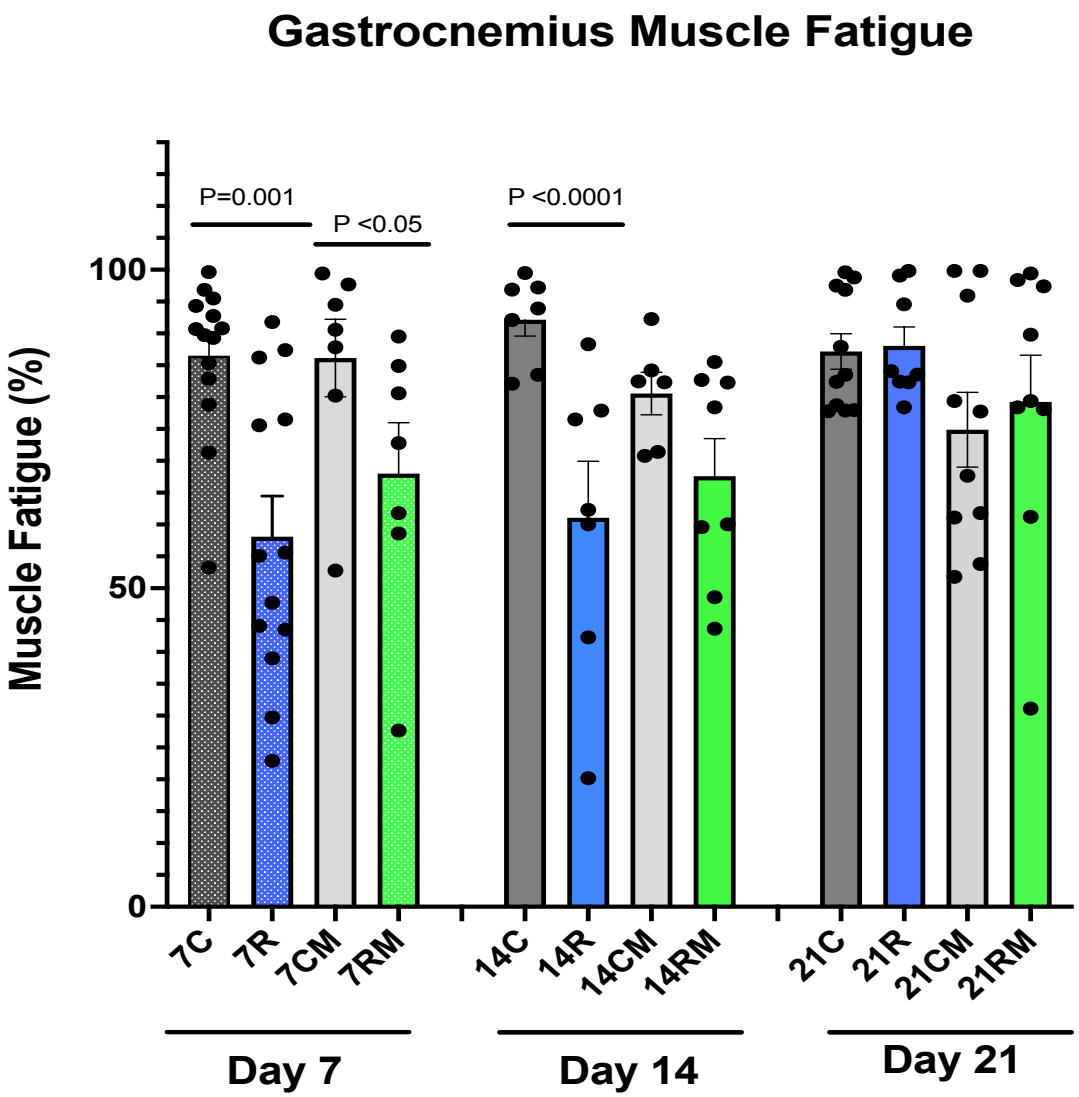
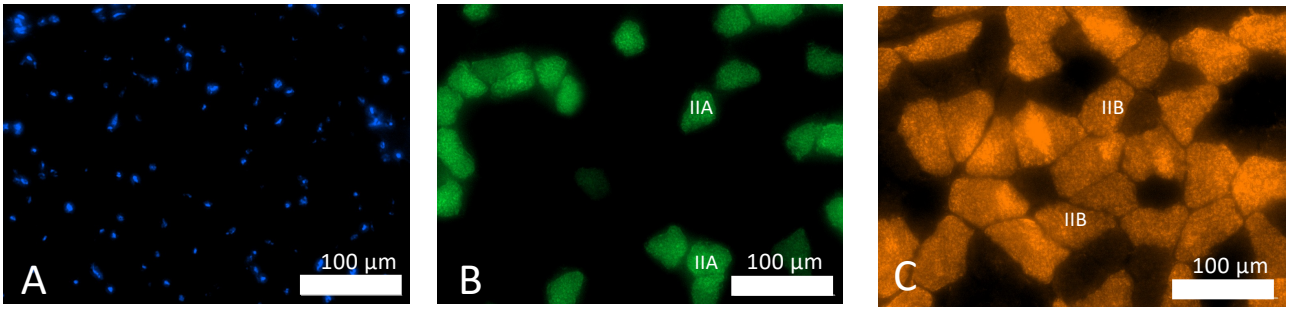
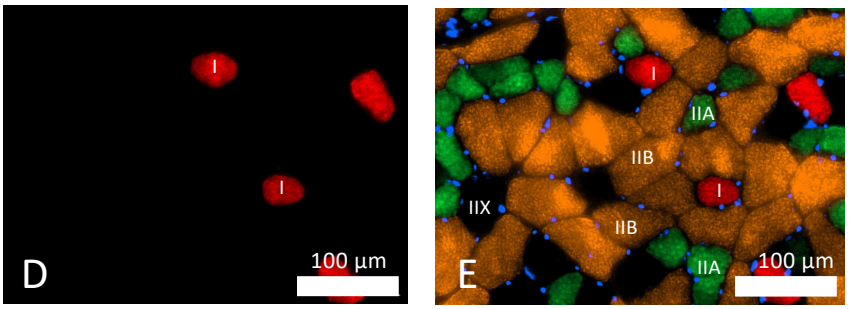

Gastrocnemius Muscle Fatigue
Muscle Fatigue (%)
100
50
0
P=0.001
P <0.05
P <0.0001
7C
7R
7CM
7RM
14C
14R
14CM
14RM
21C
21R
21CM
21RM
Day 7
Day 14
Day 21

Figure S3

Immunocytochemistry of Myosin Heavy Chains to Determine Fiber Type



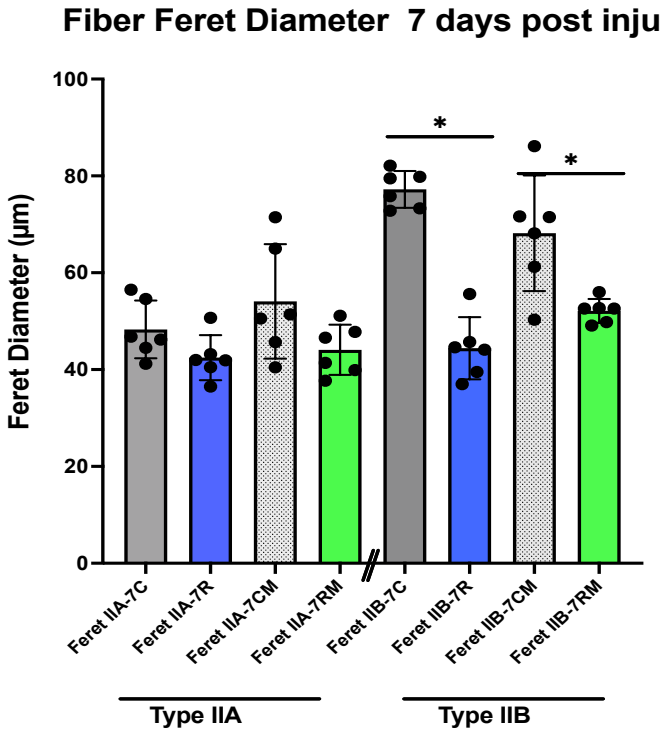
DAPI (NUCLEI) GFP (MHC IIA) CY5 (MHC IIB)



RFP (MHC I) Nuclei/MHC IIA/MHC IIB/ MHC I

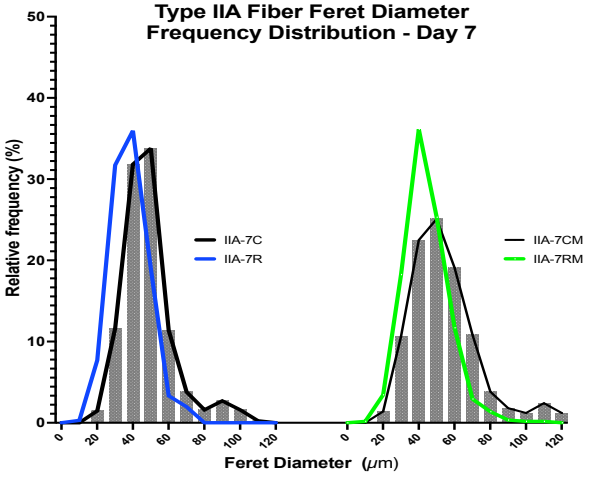
Figure S4

A



Feret Diameter

B



C

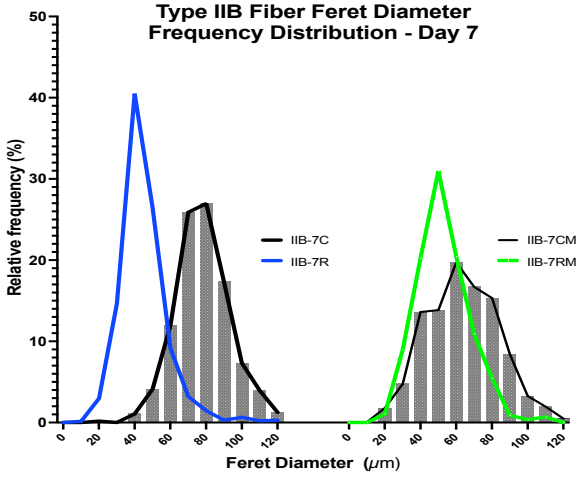
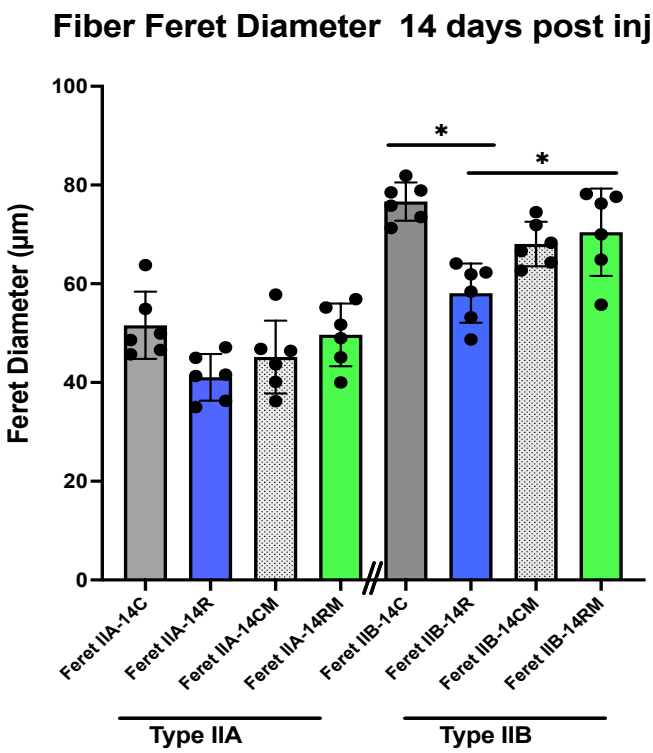


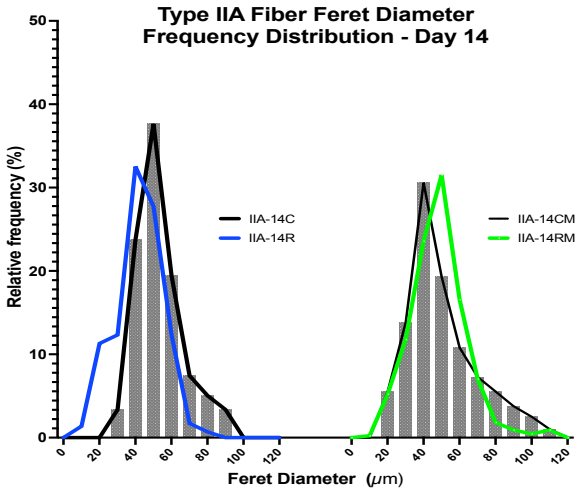
Figure S5,A-C

D



Feret Diameter

E



F

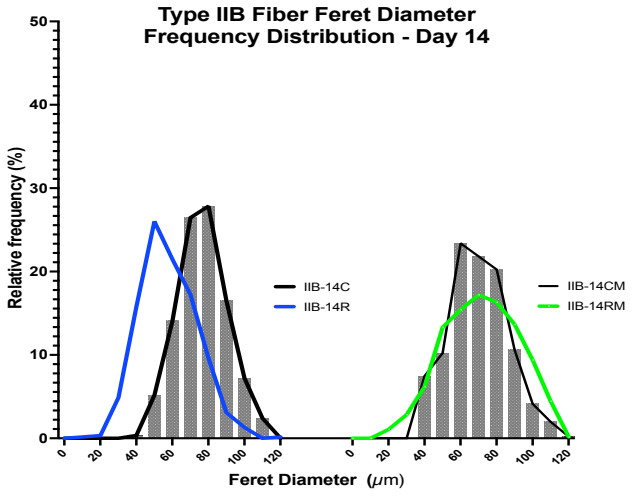
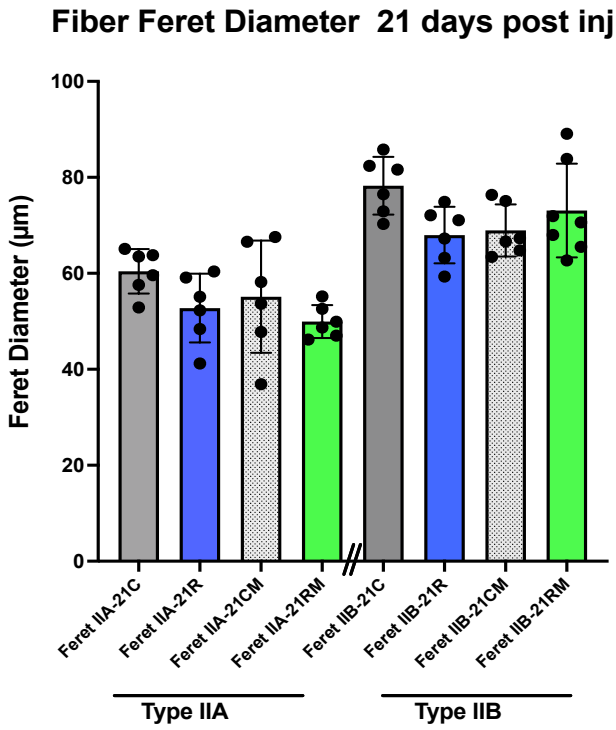


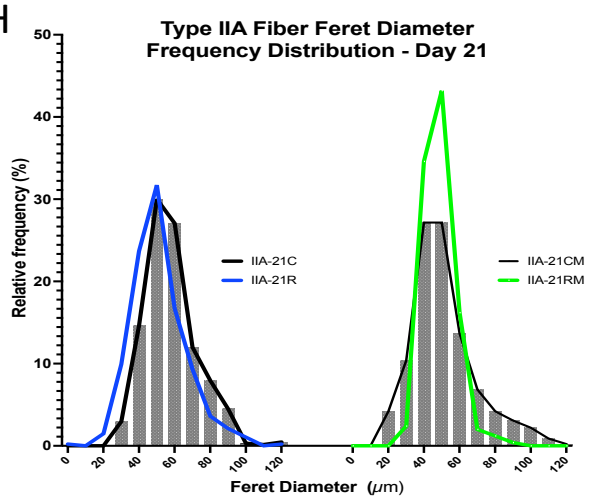
Figure S5,D-F

G



Feret Diameter

H



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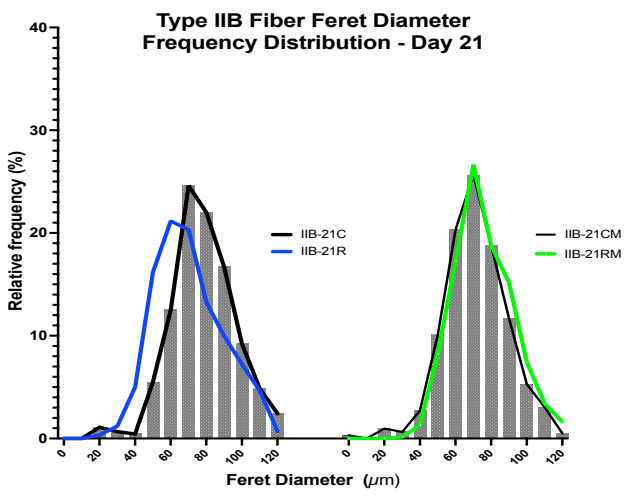

Figure S5,G-I

Sirius Red Staining for Collagen/Non-Contractile tissue

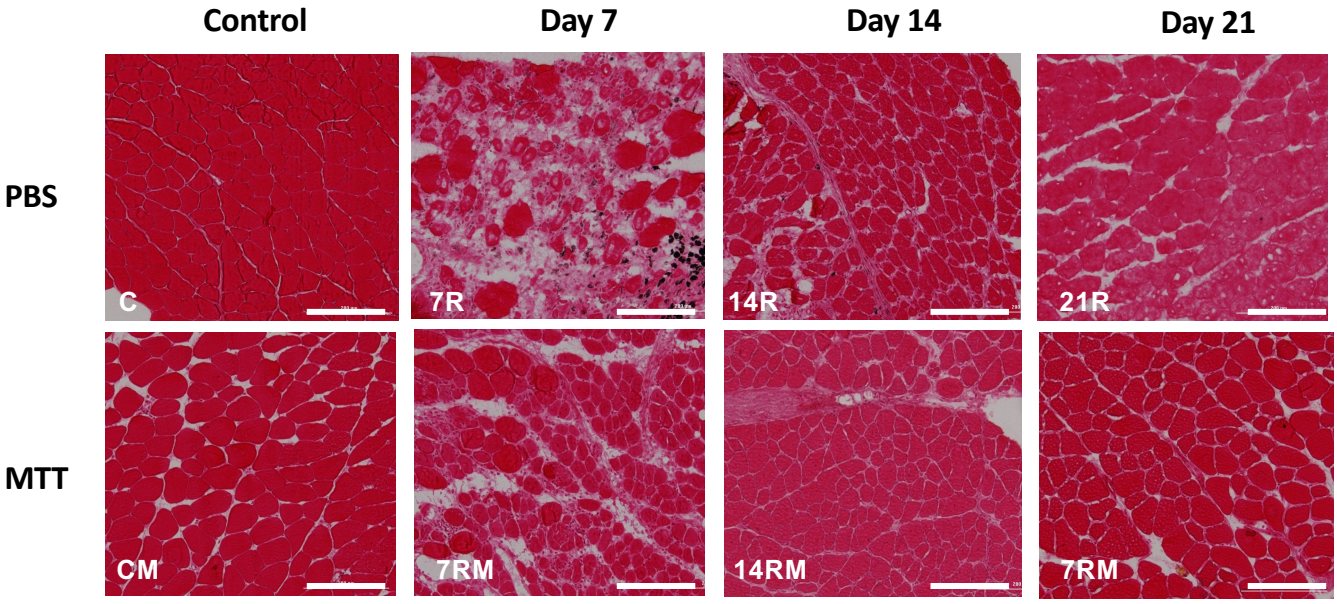

Figure S6