ONLINE SUPPLEMENT: MATERIALS AND METHODS

MicroRNA Regulatory Networks Associated with Abnormal Muscle Repair in Survivors of Critical Illness

Christopher Walsh MD[*1,2], Carlos Escudero MSc[*1,2], Muskan Gupta[*1,2], Pamela Plant PhD[1] Margaret J Herridge MD[3], Sunita Mathur PhD[4], Pingzhao Hu PhD[5], Judy Correa BSc[1], Sameen Ahmed BSc[1], Anne Bigot PhD[6], Claudia C dos Santos MSc MD[†1,2] and Jane Batt MD PhD[†1,2]
Submitted on behalf of the **M**uscl**E** and **N**erve **D**ysfunction (**MEND**)-**ICU** Group and **C**anadian **C**ritical **C**are **T**ranslational **B**iology **G**roup (**CCCTBG**)

1. Keenan Centre for Biomedical Research of Saint Michael's Hospital, Unity Health Toronto, Ontario, Canada
2. Institute of Medical Sciences and Department of Medicine, University of Toronto, Toronto, Ontario, Canada.
3. University Health Network, Interdepartmental Division of Critical Care, University of Toronto, Toronto, Ontario, Canada.
4. Department of Physical Therapy, University of Toronto, Toronto, Ontario, Canada
5. Department of Biochemistry and Medical Genetics, University of Manitoba, Winnipeg, Manitoba, Canada.
6. Sorbonne University, INSERM, Institute of Myology, Center of Research in Myology, 75013 Paris, France.

[*]Authors contributed equally to the manuscript, [†]Co-senior authors

TABLE OF CONTENTS

Patient Population

The patient population has been previously reported (1, 2). Briefly, patients were recruited to this nested MEND-ICU substudy (N=27, Supplementary Figure 1) from the RECOVER trial at St Michaels Hospital (SMH), Unity Health Toronto and the University Health Network (UHN), Toronto, Canada (N=103), a Canadian multicenter longitudinal study evaluating 1 year physical functional outcomes in critically ill patients undergoing prolonged (≥1 week) mechanical ventilation (3). Patients were excluded for any of the following: important current or pre-existing neurological injury that would preclude functional or cognitive testing; pre-existing neuromuscular disease; non-ambulatory prior to admission or requiring assistance to complete activities of daily living or; anticipated death or withdrawal of life sustaining treatment within 48 hours of ICU admission; known HIV, Hepatitis B or Hepatitis C infection; therapeutic anticoagulation and/or active cancer undergoing treatment. Written informed consent was obtained from all participants or their surrogate decision makers and participants were re-consented when capacity was regained. The study protocol was approved by the UHN and SMH Research Ethics Boards. All methods were performed in accordance with the relevant guidelines and regulations within the study protocol.

Demographics (age, sex), measures of the patients' baseline clinical status including their severity of critical illness (APACHE II score), and co-morbidity burden (Charlson Index), duration of mechanical ventilation and hospital length of stay were obtained.

Hospital discharge to long term care, rehabilitation facilities vs home was decided independent of the study investigators by the attending clinical team.

Measures of muscle mass, strength, and physical function, electrophysiologic (EP) testing and muscle biopsies

Outcome measures of muscle mass, strength and physical function, electrophysiologic testing and vastus lateralis percutaneous muscle biopsies were performed prospectively at Day 7 (7D) and Month 6 (6M) post-ICU discharge as previously described (1).

Briefly, muscle mass was assessed by computed tomography (CT)-determined midthigh quadriceps cross sectional area (4). Global strength was determined by the Medical Research Council Sum Score (MRCSS)(5); manual testing of bilateral shoulder abduction, elbow flexion, wrist extension, hip flexion, knee extension, and ankle dorsiflexion strength with a total score of 60 indicating "normal" strength for an individual of the same sex and age.

Quadriceps voluntary isometric peak torque was measured using a Biodex dynamometer (Biodex System 4) with the hip at 85 degrees and knee at 60 degrees of flexion, using a previously described protocol (6). Patients' absolute peak torque (in Newton-meters) is reported as "percent predicted" based on a validated predictor equation derived by Harbo et al (7) where the individual's sex, age, weight and height are taken into account

Physical functional capacity was determined by the 6 minute walk test (8) and motor component of the Functional Independence Measure questionnaire (FIM score)(9). The FIM score provides a numerical score for cognitive and motor function that has been validated and standardized across diverse patient populations and a higher total FIM score (maximum of 126) connotes better function in both domains. The FIM motor subscore is based on the degree to which an individual is able to perform their activities of daily living (eg toileting, dressing,

walking, climbing stairs, eating). A healthy individual with complete independence for all activities (no assistance or assist devices required) would achieve a maximal FIM motor subscore of 91.

Nerve conduction studies (NCS) and needle electromyography (EMG) were undertaken to assess for sensory polyneuropathy and myopathy respectively, as described (1), Briefly, peroneal motor, tibial motor and sural sensory tests were initially conducted. If these were normal, muscle EMG was then performed. If these were abnormal, nerve conduction testing was conducted on the upper extremities; median motor and sensory (antidromic) studies with F wave, ulnar motor and sensory (antidromic) studies and radial sensory (antidromic) response, were assessed. Muscle needle EMG studies were performed on the deltoid, triceps, tensor fascia lata and vastus lateralis.

Percutaneous muscle biopsy (100 – 200 mg) of the *vastus lateralis* was performed under local anaesthetic for molecular and histologic studies. For comparator purposes healthy muscle biopsy samples were obtained from previously banked specimens collected from consenting individuals (n = 8, median age 43, 50% males) as previously described (1).

MiR and gene microarray pre-processing and quality control

RNA was processed, amplified, and labeled as previously described (10). High quality muscle RNA samples were labeled using the miRCURY LNA miR Hy3/Hy5 Hi-Power labeling Kit (Exiqon) and hybridized on the miRCURY LNA miR Array (Exiqon, 7th generation, containing 2042 *H. sapiens* miRs as annotated by miRBase 19, spotted in quadruplicate) according to the manufacturer's guidelines. All capture probes for control spike-in oligonucleotides produced signals in the expected range. Data quality control included high inter-array correlation (Pearson correlation coefficients >0.85) and detection of outlier arrays based on mean inter-array correlation and hierarchical clustering. All samples fulfilled data quality control criteria. Probes were processed, background corrected, and normalized using the *MmPalateMiRNA* package (11). Probes with foreground intensity values above 1.3 times their background intensity level in at least 3 samples were retained for further analysis. After background correction, between-array normalization was performed using quantile normalization. Quadruplicate probes were averaged to a single expression value for each miR, resulting in 514 high-quality, robustly expressed miRs included for further analysis.

Gene expression profiles were obtained using IlluminaHT-12 V4 microarrays (1 microarray per sample) and previously deposited in GEO under accession number GSE78929 and previously published (2). All samples fulfilled data quality control criteria. Probes listed as "No match" or "Bad" using the *illuminaHumanv4* package were removed, resulting in 34,476 high-quality probes. Robustly expressed probes were defined as those with detection *p*-value < 0.05 for at least half of the samples in the data set and standard deviation of probe expression >0.25 (11,482 probes). The probe level measurements were then converted to gene-level measurements using the *CollapseRows* function in WGCNA (12) using the "MaxMean" setting corresponding to 9869 high-quality, robustly expressed unique genes.

Master MiR-Regulator Analysis (MMRA)

MMRA is an analysis pipeline designed to detect differentially expressed (DE) miRs significantly contributing to the expression of target genes in disease subgroups (13). MMRA combines statistical tests, target prediction and unsupervised network analysis and was implemented using R code available at http://eda.polito.it/ MMRA, with the following modifications: a) Use of *limma* in place of Kolmogorov-Smirnov test in step 1, b) updated versions of target prediction databases in step 2, c) Empirical significance thresholds for enrichment analysis implemented in step 2, d) updated version of the Algorithm for the Reconstruction of Accurate Cellular Networks [ARACNe] in step 3 with the rationale for each modification (described below). All expression profiles analyzed in MMRA were adjusted for age and sex using random effects linear model in *lme4* package to account for intra-subject correlation between samples obtained longitudinally. The overall approach is pictorially delineated in Figure 1.

MMRA Step 1 - Differential expression (DE) analysis. Subgroups in this analysis consist of comparisons of Intensive Care Unit Acquired Weakness (ICUAW) Day 7 (7D) post-ICU discharge vs controls, ICUAW Month 6 (6M) post-ICU discharge versus controls and improvers versus non-improvers (as defined in the manuscript). The DE genes and miRs resulting from subgroup analysis are termed the "subgroup signature" for genes and miRs, respectively. DE of all robustly expressed miRs and genes in ICUAW 7D and 6M post-ICU and control samples was assessed in *limma* using linear models adjusted for patient age and sex and for consensus correlation between patient samples using the duplicate Correlation function (14). Moderated *F*-statistics combined *t*-statistics for all three pair-wise comparisons (contrasts) into an overall test of significance for each gene or miR was used. The decideTests function with "global" setting performed error rate control across multiple contrasts and miRs or genes, simultaneously. DE of all robustly expressed miRs and genes in ICUAW patients classified as improvers and non-improvers was assessed in limma using linear models adjusted for patient age and sex with pairwise testing using *t*-statistics.

To detect DE miRs, the pre-specified significance threshold was set at Benjamini Hochberg (BH) false detection rate (FDR) < 5% and absolute values of fold change (aFC) >1.5. Differentially expressed genes were defined as a FC > 1.0 with FDR threshold < 20% for analysis of ICUAW at 7D and 6M post-ICU versus controls, and improvers vs non-improvers. For this analysis we chose more stringent FC and FDR thresholds to detect DE miRs compared to the DE gene analysis, as most high-quality studies of miR expression changes in disease states generally report FC in the range of 1.5-4 fold in miR levels (15). In contrast, gene network analysis has demonstrated that multiple co-expressed genes, each having smaller effects (i.e. FC), function to modulate disease phenotype (16) as we have previously demonstrated for this ICUAW gene expression data (2).

Diagnostic plots of the linear model fit (miR-wise or gene-wise residual standard deviations against average log-expression) were examined and no variance trends were identified. The *limma* package was used instead of the Kolmogorov-Smirnov test for DE analysis as originally implemented in MMRA (13) as the former has been shown to be advantageous in experiments with smaller sample sizes (14).

MMRA step 2) Target enrichment analysis: Target enrichment analysis was performed for each DE miR in a given subgroup comparison analysis (ICUAW at 7D and 6M post-ICU versus controls, improvers versus non-improvers) in order to assess overlap between its putative gene

targets and the DE genes in each subgroup comparison analysis. To generate a list of putative gene targets we combined the results of four prediction databases (DBs): TargetScan V7.0 (conserved and non-conserved, 2015) (17), miRDB (2014) (18), DIANA-microT v5.0 (2013) (19), and PITA (2010) (20) using mirDIP (21) (to ensure consistent gene and miR names). To increase stringency of predicted targets, we selected from PITA the targets designated "top scores" and for the other 3 DBs we filtered out the bottom 50% of targets based on each of their prediction scores. We then included all experimentally validated miR-target pairs from TarBase V7.0 (22) to our list of putative targets. miR-target pairs were selected for enrichment analysis if present in Tarbase or if present in at least two prediction databases.

For each DE miR identified in a given subgroup comparison analysis in step 1**,** target enrichment analysis of the putative miR-targets in the up-regulated and down-regulated DE genes in each subgroup comparison analysis were calculated using the hypergeometric test p-values and observed/expected (O/E) ratios. The significance threshold for miRs were Bonferroni adjusted p-values < 0.2 and O/E ratios > 1.1; thresholds were selected empirically for enrichment analysis of predicted miR-target pairs.

*MMRA step 3A. Network analysis***:** Unsupervised network analysis was performed using the Algorithm for the Reconstruction of Accurate Cellular Networks (ARACNe) with Adaptive Partitioning strategy (AP) (23, 24) to infer interactions between each DE miR selected in step 2 and any of the robustly expressed genes. ARACNe uses an unbiased approach without assuming the underlying network topology (25) to construct a network based on mutual information (MI), an information theoretic measure of the mutual dependence between two variables, for each input miR and putative gene targets. The ARACNe algorithm provides advantages over simple pairwise miR-gene MI analysis as it removes indirect candidate relationships using the direct processing inequality algorithm and uses bootstrapping to improve the statistical significance of the network for limited sample sizes (26). The ARACNe algorithm performs bootstrapping with randomly selected samples and builds a consensus network of edges (miR-gene interactions) computed by estimating the statistical significance of the number of times a specific edge is detected across all bootstrap runs. For each network, 100 bootstraps were performed and the chosen MI p-value significance threshold (10^{-7}) was selected based on previously recommended threshold (26).

*MMRA step 3B Master regulator analysis***:** The consensus networks constructed around each miR from step3A were then tested for significant enrichment in DE genes for each subgroup using the master regulator analysis (MRA) algorithm (27); the statistical significance (Fisher's exact test p-value) of the overlap between the genes within each network and the DE genes of the subgroup in which the miR was identified as DE. Next, we tested the significance of the overlap between the genes within each network and the 11 ICUAW-relevant modules identified in Walsh *et al* (2). To determine a p-value threshold, a random null model was built consisting of networks built using miRs not DE in any subgroup (signal-to-noise ratio, corresponding to FC divided by within-group standard deviation, < -0.05). The threshold for the MRA p-value was estimated by comparing with the p-values obtained in our analysis with those of the null model. We performed MRA on a null model built of 30 randomly selected non-DE miRs and tested the enrichment in DE genes of all ICUAW subgroup as well as for enrichment for all ICUAW-relevant modules. The MRA p-value threshold of 10^{-4} was chosen for DE genes, and 10^{-3} for ICUAW-relevant modules, corresponding to the 90^{th} percentile of each of the null models.

MMRA step 4 Stepwise linear regression (SLR) analysis: Next, SLR was used to filter out weak miR-gene relations within each network. The SLR constructs a linear model for each gene target as the response variable and all miRs linked to the gene (by ARACNe-AP in step 3A as explanatory variables. A stepwise algorithm is used to select the best minimal set of explanatory variable within the model. The Akaike information criteria (28) was used as the stop criterion. The output of SLR analysis is then reorganized at the miR level, to identify for each miR, the DE genes in each subgroup that are linearly associated with the miR. The degree of regulation by a miR for each ICUAW subgroup signature was defined as the percentage of DE genes (up or down) in each subgroup identified by SLR (positive or negative coefficient). For the final output of MMRA pipeline, miRs satisfying all of the above thresholds were selected and deemed *master miR-regulators*.

MiR-clinical variable relationships

Spearman's correlation was calculated between continuous clinical variables and miR expression values (unadjusted for age and sex). Significant correlation to clinical variables was empirically defined as $R \geq 0.5$ and p-value < 0.05.

Gene ontology

Functional enrichment of Gene Ontology (GO) was performed using the gProfiler tool (29) in R with FDR corrected $p < 0.05$ with gene set size range 10-300, and minimum of 5 genes intersecting. The background list ("universe") for the enrichment analysis included all genes represented on the Illumina Human HT-12 v4 array with a detection *p*-value < 0.05 in at least three samples.

Murine C2C12 cell culture

C2C12 myoblasts were maintained and passaged in DMEM, supplemented with 10% FBS and 2% penicillin-streptomycin at 37ºC and 5% CO2. Cells were used for experimentation up to Passage 12, since beyond this C2C12s show a loss of proliferative and myogenic potential (30). Cells were allowed to proliferate with medium replacement every 48 hours and passaged prior to 70% confluency, or differentiated to myotubes at 70% confluency with DMEM and 2% horse serum (31).

Human AB1167 cell culture

AB1167 human skeletal myoblasts gifted by Anne Bigot and Vincent Mouly (Sorbonne University, INSERM, Institute of Myology, Center of Research in Myology FRANCE) were maintained and passaged in Skeletal Muscle Cell Basal Medium (SKCBM) (Promocell, #C-23260) and SupplementMix (Promocell, #C-39365), with 15% FBS and 1% penicillin-

streptomycin at 37ºC and 5% CO2, with media replaced every 48 hours. Cells were passaged at approx. 50 to 60% confluency. Cells were induced to differentiate at 70% confluency with DMEM + 2% horse serum + 1% P/S + 10µg/mL insulin, and media was changed every 48 hours. Cells were used for experimentation up to Passage 11, since beyond this AB1167cells show a loss of proliferative and myogenic potential.

MiR mimic and inhibitor transfection

C2C12 myoblasts at passage 10 were plated in Greiner CELLSTAR® 12-well plates at a density of 15,000 cells/well and grown in 1mL standard media (DMEM -10% FBS, 2% pen/strep) at 37ºC and 5% CO2. At 24 hours, C2C12 myoblasts were synchronized with 2mM Thymidine (Sigma-Aldrich, #T1895-1G) in DMEM for 24 hours. Cells were subsequently released in standard media for 1 hour, after which 10µM miR mimics were transfected using Lipofectamine RNAiMAX Reagent in Opti-MEM Reduced Serum Media (Invitrogen), as per the manufacturers instructions. Cells were transfected with 10pmol/well of mirVana miR mimics (Thermo Fisher Scientific); mmu-miR-490-3p (Catalog # 4460466) or mmu-miR-744-5p (#4464066) or mirVana negative control #1 (#4464060), a random sequence mimic demonstrated not to influence cell activity or affect miR function. Mock transfection (RNAiMAX reagent without mimic), and untransfected C2C12 myoblasts served as additional negative controls. Transfection media was aspirated 24 hours post transfection and replaced with standard C2C12 media for proliferation assays. At 48 hours media was aspirated and replaced with DMEM and 2% horse serum for differentiation assays.

AB1167 myoblasts at passage 8 were plated in Greiner CELLSTAR® 6-well plates at a density of 35,000 cells/well and grown in standard media (1.8mL SKCBM and supplement mix + 15% FBS + 1% pen/strep) at 37ºC and 5% CO2. At 24 hours AB1167 myoblasts were synchronized with SKCBM + 2mM Thymidine (Sigma-Aldrich, #T1895-1G) for 24 hours. Cells were released and transfected with 10µM miR mimics or inhibitors using Lipofectamine RNAiMAX Reagent in Opti-MEM Reduced Serum Media as per the manufacturers instructions. Cells were transfected with 10pmol/well of one of mirVana miRNA mimics or inhibitors(Thermo Fisher Scientific): hsa-miR-490-3p (Catalog # 4464066, MC10560), hsa-miR-490-3p inhibitor (Catalog # 4464084, MH10560), hsa-miR-744-5p (Catalog #4464066, MC13027), hsa-miR-744-5p inhibitor (Catalog # 4464084, MH13027) or mirVana negative control #1 (#4464060). Untransfected myoblasts served as additional negative controls. At 24h post-transfection media was aspirated and replaced with standard AB1167 media, and maintained in standard media for proliferation assays. At 48 hours standard media was changed to DMEM + 2% horse serum + 1% P/S + 10µg/mL insulin 48h post-transfection, and replaced every 48 hours, for differentiation assays.

C2C12 and AB1167 RNA isolation

Total RNA (>18bp) was isolated from C2C12 and AB1167 cultures using the miRNeasy Kit (Qiagen, Venlo, Netherlands) for miRNA and mRNA applications and DNAse (Thermo Fisher, #EN0521) treated, according to the manufacturer's instructions. RNA quality and quantity (total

and miRNA) was determined using the Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA) and Qubit 4 fluorometer (Thermo-Fisher Scientific). Only RNA of RIN greater than 8.5 was used for analyses.

C2C12 and AB1167 protein isolation

AB1167 and C2C12 myoblasts or myotubes, as required, were washed in PBS on ice. Lysis buffer (150mM NaCl, 1.5mM $MgCl_2$, 50mM HEPES, 10% Glycerol, 1% Triton-X) with protease inhibitors (Complete -mini EDTA-free- protease inhibitor cocktail, Milipore, Sigma) was added, cells were scraped with a rubber policeman, lysates were homogenized by vortexing for 1 minute and incubated on ice for 10 minutes. Whole cell lysates were stored at -80 ºC.

MiR qPCR – C2C12 cells

Endogenous expression of miR-490-3p and miR-744-5p was determined in proliferating and differentiated C2C12 myoblasts and myocytes respectively using Applied Biosystems Taqman microRNA Assays (Cat# 4427975; ID#001037- miR490 & ID#002324-miR 744, Thermofisher Scientific). miRNA-specific cDNA synthesis was completed with Taqman miRNA Reverse Transcription Kit (#4366596, Thermofisher Scientific), which utilizes a multiscribe reverse transcriptase (RT). 1.5ng of miRNA-enriched cDNA was reverse transcribed per sample in 15µl reactions, for a final concentration of 100pg/µl. For Taqman qPCR reactions, 200pg cDNA was added in 10µl volume reactions per well in 384-well plates with the miRNA-specific PCR MGB probes and Taqman Advanced Master Mix. No-RT controls and no-template controls were included to ensure signal accuracy and specificity (32). Samples were run in triplicate. The Taqman qPCR reaction was performed with the QuantStudio7 Flex Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA), beginning with a 20 second enzyme activation at 95ºC, followed by 40 cycles of 1 second denature step at 95ºC and 20 second annealing/extension step at 60ºC.

miR expression was quantified by calculating the fold change in expression of each miR between timepoints using the 2-ΔΔCt method and the Gaussian propagation of error to determine fold change + Standard Deviation (SD). miR expression was normalized to the geometric mean of two housekeeping small RNA – RNU6b and snoRNA234. RNU6b and snoRNAs234 have been established as stable reference RNAs in multiple murine cell lines in proliferative conditions.

Droplet digital PCR (ddPCR) – C2C12 and AB1167 cells

ddPCR was used to assess endogenous expression of miRs-490-3p and 744-5p in AB1167 proliferating myoblasts and differentiated myocytes and to confirm C2C12 and AB1167 transfection. cDNA was generated from total RNA for each miR as previously delineated. 20µl reaction mixtures per well consisting of 11µl ddPCR Supermix (Bio-Rad), 1µl 20x miR-specific Taqman Primer/Probe mix, cDNA (0.15 ng or 5.3ng for C2C12 and AB1167 respectively) and remaining (up to 20 ul) volume Nuclease-free water were loaded in a 96-well plate (#12001925

Bio-Rad) in the AutoDG Droplet Generator (Biorad). Following droplet generation, the plate was sealed with a foil heat seal (#181-4040, Bio-Rad) using the PX1 PCR Plate Sealer (#181-4000, Bio-Rad) and loaded in the PCR thermocycler (C1000, Biorad). PCR reaction was initiated with a 95ºC enzyme activation step for 5 minutes, followed 40 amplification cycles (94ºC for 30 seconds and 60ºC for 1 minute per cycle), a final extension step for 10 minutes and subsequently left at 4ºC. Post-cycling droplet detection and quantification performed with the QX200 Droplet Reader (Biorad), and data was processed and analyzed using Quantasoft (v.1.7.4) software (33).

Ki67 proliferation index and myoblast cell count

To determine C2C12 myoblast proliferation and cell count, cells were fixed and immunostained for Ki67 (a nuclear protein and cellular marker of proliferation) and DAPI nuclear stain at 24, 48 and 72 hours post transfection. Following aspiration of media, cells were washed with phosphate buffered saline (PBS), fixed in-well using 4% paraformaldehyde in PBS for 30 minutes, glycine-inactivated using 0.375g Glycine in 50mL PBS for 10 minutes, permeabilized using 0.1% Triton X-100 for 20 minutes, and then blocked with 3% Bovine Serum Albumin (BSA) in PBS for 1 hour. Cells were immunostained for Ki67 (ab15580; Abcam, Cambridge, UK) at a 1:250 dilution, detected with Alexa Fluor 555 conjugated secondary antibody (A21428; Invitrogen Carlsbad, CA). Nuclei were stained using DAPI at 1:12,000 dilution.

Bright field microscopy images of the cells were taken at the selected timepoints for visual confirmation of optimal cell growth and estimates of cell density (NIKON Eclipse TS100, Tokyo, Japan). Immunofluorescent imaging of fixed cells was conducted using the ImageXpress® Micro Acquisition System (Molecular Devices, San Jose, CA) to quantify cells with Ki67-positive nuclei as a percentage of total myoblasts (34). The ImageExpress Micro was set to capture 42 images at 10X magnification per well, in a 7x6 grid format fitting the entire well area. Each individual image site was 899.23 x 671.84μm in size. DAPI-stained nuclei were detected as positive if fluorescence intensity was 1500 graylevels above local background. The threshold for Ki67-positive cells was set as determined by using a control well of confluent myotubes that do not express Ki67.

To determine AB1167 myoblast proliferation and cell count, cells were fixed and immunostained for Ki67 and DAPI nuclear stain as detailed above for C2C12 myoblasts, with the following exceptions. Serial timepoint included 0 (baseline), 24, 72 and 120 hours post-transfection. The ImageXpress Micro Acquisition System was set to capture 25 images at 10X magnification per well, in a 5x5 grid format fitting the entire well area. Using a macro code on Fiji, threshold values were set for DAPI and Ki67 stained nuclei which was then applied to all images in an experiment.

For C2C12 and AB1167 cells data logged included total cell count as indicated by DAPI nuclear stain, and Ki67 positive cell count indicating proliferative cells, which was then used to calculate the proportion of proliferative myoblasts as cells positive for both DAPI and Ki67 divided by total number of cells, per image.

Myosin Heavy Chain (MHC) immunohistochemistry and Fusion Index

To assess differentiation and myotube fusion, C2C12 and AB1167 cells were fixed as delineated above at serial timepoints post-transfection and immunostained for MHC (marker of mature myocytes, anti-MF20, ab 2147781, 1:50, Developmental Studies Hybridoma Bank, University of Iowa) and detected with Alexa Fluor 555-conjugated secondary antibody (A21422, 1:1000, Invitrogen). Nuclei were stained with DAPI (1:12,000). Immunofluorescent imaging of fixed cells was conducted using the ImageXpress® Micro Acquisition System (Molecular Devices, San Jose, CA). The ImageXpress Micro was set to capture 25 images at 10X magnification per well, in a 5x5 grid format fitting the entire well area. The fusion index was determined as the percentage of nuclei in fused myotubes (MHC positive cells with 2 or more nuclei) relative to the total number of nuclei, per image (35). For the fusion index, nuclei from 50-150 myotubes were counted from each experimental condition/experiment.

Western Blotting

Proliferating cell nuclear antigen (PCNA) is a marker of proliferating cells, with increased protein expression correlating with the cellular proliferation. C2C12 whole cell protein lysates derived at serial time points post-transfection were thawed on ice and quantified using the Pierce 660nm Protein Assay Reagent (#22660, Thermo Fisher). 30ug protein lysates were separated via SDS-PAGE, transferred to nitrocellulose membrane, immunoblotted for PCNA (Santa Cruz Biotechnology, sc-56, 1:200) or α-actinin (Cell Signaling Technology, #3134s, 1:1000) as a loading control and detected with HRP-linked secondary antibody (Thermo Fisher, #65-6120, 1:2000). Western Blots were imaged using the Gel Doc EZ Imager (Bio-Rad Laboratories, Berkeley, CA). All imaged gels were quantified using ImageLab Studios software (Bio-Rad).

AB1167 whole cell protein lysates were quantified as above. 25μg of lysates were separated via 4-20% Mini-PROTEAN TGX precast protein gels (Bio-Rad, #4561094), transferred to Nitrocellulose membrane, immunoblotted simultaneously for PCNA and α-actinin (as above) and myosin heavy chain (MHC; MF-20 Developmental Studies Hybridoma Bank, 0.5μg/mL). IRDye 800CW goat anti-rabbit (Li-Cor, #926-32211) and IRDye 680RD goat anti-mouse (Li-Cor, #926-68070) fluorescent secondary antibodies, 1:4000 dilution, were used for multi-colour detection. Western Blots were imaged using the Li-Cor Odyssey infrared imager. All imaged gels were quantified using ImageStudio software (Li-Cor).

30ug of a single stock cell protein lysate generated from proliferating C2C12 myoblasts and C2C12 myotubes was included on all blots to normalize results across experiments.

Cell culture experiments statistical analyses

For proliferation assays (Ki67 expression, cell count, PCNA Western Blotting) and differentiation assays (Fusion Index, MHC Western Blotting) comparisons between multiple groups were conducted using two-way ANOVA with repeated measures, followed by Dunnett's post hoc analyses to determine differences between experimental miR and the negative control scrambled miR. For all statistical tests, significant rejection of the null hypothesis was accepted as $P<0.05$.

mRNA qPCR

Expression of select genes following miR transfection in C2C12 and AB1167 myoblasts was undertaken with quantitative Real-time Polymerase Chain Reaction (qRT-PCR). Primers were published (36-40) or designed on NCBI primer blast. Primer working concentrations were optimized and validated by ensuring single amplicons of the predicted size using acrylamide gel electrophoresis. Standard curves were generated for all primers. Primers with $R^2 > 0.99$ were selected to ensure adequate primer efficiencies (>90%). Finalized primer sequences listed below [published indicated by asterisk;(36-40)].

qRT-PCR was conducted to quantify target mRNA expression in transfected cells. 500ng mRNA/sample was reverse transcribed to generate cDNA using Superscript III First-Strand Synthesis Supermix (Invitrogen, #18080400) which included Superscript III Reverse Transcriptase (a modified M-MLV Reverse Transcriptase) and random hexamer primers as per the manufacturer's instructions. A total of 1.6ng to 5ng cDNA (dependent on the mRNA target) was loaded per well on a 384-Well Clear Optical Reaction Plate in a final volume of 10µL qRT-PCR reaction mixture containing Power SYBR Green PCR Master Mix and optimized concentrations of the mRNA specific forward and reverse primers. No-RT controls and no-template controls were included to ensure signal accuracy and specificity. qPCR reactions were completed using the QuantStudio7 Flex Real-Time PCR System with the following cycling parameters: 10 minutes at 95℃, followed by 40 PCR cycles of 15s at 95℃ and 1 minute at 60℃.

qPCR data was processed and analyzed using QuantStudio Real-Time PCR software, the relative quantification 2ΔΔCT method was used for analysis(41). HPRT and GAPDH were used as housekeeping genes. Log2 fold changes in the gene of interest for miR-490-3p transfected cells were determined relative to scrambled miR transfected, and a one-tailed t-test vs a hypothetical mean (0 no change) was conducted. Significance was assumed if $p<0.05$.

Murine qRT-PCR Primer Sequences

Gene	***Forward Primer***	***Reverse Primer***
*ANTXR1**	ATGGCCCACAGTAGATGCC	GAAGTTGATACAGCGTCCGG
*FBXO9**	GGAGCGGCTACATCGAAGAG	TGAGTCTGACTGGTCTCAAGC
*SAPS2**	ATGACATCTTGCAGGAGTGTAAG	TGTGTTTGGATATTTGAAGCGGA
*HMGA2**	AACCTGTGAGCCCTCTCCTAAG	GCCGTTTTTCTCCAATGGTC
*CDK1**	GACATCTGGAGTATAGGGACC	CTTCGTTGTTAGGAGTGCC

Human qRT-PCR Primer Sequences

Gene	***Forward Primer***	***Reverse Primer***

ANTXR1	CACCCCCTGCCGAGGAGAGT	GGCGTCTACCGTTGGCCACTTT
CDK1	GACATTTGGAGTATAGGCACC	CTTCATTATTGGGAGTGCC
Mef2D	CTGAGGACCATTTAGATCTGA	ACCACTGGGGTGGTGAGCGA
Myf5	CCCCACGTCTACCCCTCAGGAAT	AGGTCAGCTCCTCTGACACCCAG
Ctnnb1	GCAGCTGCTGTTTTGTTCCGAATGT	CTGGTCAGCTCAACTGAAAGCCGT
MyoD	GCCCAGCGGTGGGTATTCCG	GGGGGTGGGAGCGTCCTAGAT
Capn3	CGTCTGGAAGAGACCTCCGGTGA	CTGCCGGAGGTGCTGAGTGAG
HMGA2	TGGAGAAAAACGGCCAAGAGGCA	CGAACGTTGGCGCCCCCTA
ZNT7	CCGGACAAGCGCTGGGGATT	GGGCCGGGTCTACTCCCCTC
NCAM	TTTGGGTGGTTTTACGTGGACTGCT	AAGGGCGACGCGCTCCTTTC
HPRT	GGACAGGACTGAACGTCTTGC	GAGCACACAGAGGGCTACATTG
GAPDH	GAAGGTGAAGGTCGGAGTCA	GAAGATGGTTGATGGGATTTC

Nanostring Analysis

We used the Nanostring nCounter Analysis System (Seattle, WA, USA) to evaluate gene expression following C2C12 miR transfection. We used a Nanostring codeset comprising target-specific IDT capture and reporter probes (Integrated DNA Technologies) that directly target and hybridize to 101 unique genes. We slightly modified the Nanostring Skeletal Myogenesis and Myopathies PlexSet Preselected Pathway Panel which contains 96 genes, including 6 housekeepers established as having stable expression during myogenic proliferation and differentiation. We removed 25 of the 96 preselected Myogenesis Panel genes from the Nanostring codeset as they were either not expressed in skeletal myoblasts, or considered unrelated to muscle proliferation, differentiation, regeneration, and growth processes studied in this project. To the remaining myogenesis-related genes, 25 gene targets that were experimentally validated targets of master miR-regulators in other systems, or putative targets of interest and that were not annotated on our Illumina mRNA chips, were added.

The nCounter Advanced Analysis Module was used for statistical analysis to identify differentially expressed genes in miR-490-5p mimic transfected cells relative to scrambled transfected (negative control) at 48 and 96 hours post-transfection. Advanced Analysis uses multivariate linear regression to investigate differential gene expression in response to multiple covariates simultaneously. Multiple testing was corrected for using the Benjamini-Yekutieli false

discovery rate (FDR) method. Results are expressed as log2fold change in gene expression. Significant difference was considered $p<0.05$.

References

1. Dos Santos C, Hussain SN, Mathur S, Picard M, Herridge M, Correa J, Bain A, Guo Y, Advani A, Advani SL, Tomlinson G, Katzberg H, Streutker CJ, Cameron JI, Schols A, Gosker HR, Batt J, Group MI, Investigators RP, Canadian Critical Care Translational Biology G. Mechanisms of Chronic Muscle Wasting and Dysfunction after an Intensive Care Unit Stay. A Pilot Study. *Am J Respir Crit Care Med* 2016; 194: 821-830.
2. Walsh CJ, Batt J, Herridge MS, Mathur S, Bader GD, Hu P, Dos Santos CC. Transcriptomic analysis reveals abnormal muscle repair and remodeling in survivors of critical illness with sustained weakness. *Sci Rep* 2016; 6: 29334.
3. Herridge MS, Chu LM, Matte A, Tomlinson G, Chan L, Thomas C, Friedrich JO, Mehta S, Lamontagne F, Levasseur M, Ferguson ND, Adhikari NK, Rudkowski JC, Meggison H, Skrobik Y, Flannery J, Bayley M, Batt J, Santos CD, Abbey SE, Tan A, Lo V, Mathur S, Parotto M, Morris D, Flockhart L, Fan E, Lee CM, Wilcox ME, Ayas N, Choong K, Fowler R, Scales DC, Sinuff T, Cuthbertson BH, Rose L, Robles P, Burns S, Cypel M, Singer L, Chaparro C, Chow CW, Keshavjee S, Brochard L, Hebert P, Slutsky AS, Marshall JC, Cook D, Cameron JI, Investigators RP, Canadian Critical Care Trials G. The RECOVER Program: Disability Risk Groups and 1-Year Outcome after 7 or More Days of Mechanical Ventilation. *Am J Respir Crit Care Med* 2016; 194: 831-844.
4. Marquis K, Debigare R, Lacasse Y, LeBlanc P, Jobin J, Carrier G, Maltais F. Midthigh muscle cross-sectional area is a better predictor of mortality than body mass index in patients with chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2002; 166: 809-813.
5. Kleyweg RP, van der Meche FG, Schmitz PI. Interobserver agreement in the assessment of muscle strength and functional abilities in Guillain-Barre syndrome. *Muscle Nerve* 1991; 14: 1103-1109.
6. Mathur SM, L.; Hernandez, P. Test-Retest Reliability of Isometric and Isokinetic Torque in Patients With Chronic Obstructive Pulmonary Disease. *Physiotherapy Canada* 2004; 56: 94-101.
7. Harbo T, Brincks J, Andersen H. Maximal isokinetic and isometric muscle strength of major muscle groups related to age, body mass, height, and sex in 178 healthy subjects. *Eur J Appl Physiol* 2012; 112: 267-275.
8. Enright PL, Sherrill DL. Reference equations for the six-minute walk in healthy adults. *Am J Respir Crit Care Med* 1998; 158: 1384-1387.
9. Linacre JM, Heinemann AW, Wright BD, Granger CV, Hamilton BB. The structure and stability of the Functional Independence Measure. *Arch Phys Med Rehabil* 1994; 75: 127-132.

10. dos Santos CC, Murthy S, Hu P, Shan Y, Haitsma JJ, Mei SH, Stewart DJ, Liles WC. Network analysis of transcriptional responses induced by mesenchymal stem cell treatment of experimental sepsis. *The American journal of pathology* 2012; 181: 1681-1692.
11. Brock GN, Mukhopadhyay P, Pihur V, Webb C, Greene RM, Pisano MM. MmPalateMiRNA, an R package compendium illustrating analysis of miRNA microarray data. *Source Code Biol Med* 2013; 8: 1.
12. Langfelder P, Horvath S. WGCNA: an R package for weighted correlation network analysis. *BMC bioinformatics* 2008; 9: 559.
13. Cantini L, Isella C, Petti C, Picco G, Chiola S, Ficarra E, Caselle M, Medico E. MicroRNA-mRNA interactions underlying colorectal cancer molecular subtypes. *Nat Commun* 2015; 6: 8878.
14. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, Smyth GK. limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res* 2015; 43: e47.
15. Witwer KW, Halushka MK. Toward the promise of microRNAs - Enhancing reproducibility and rigor in microRNA research. *RNA biology* 2016; 13: 1103-1116.
16. Rotival M, Petretto E. Leveraging gene co-expression networks to pinpoint the regulation of complex traits and disease, with a focus on cardiovascular traits. *Brief Funct Genomics* 2014; 13: 66-78.
17. Agarwal V, Bell GW, Nam JW, Bartel DP. Predicting effective microRNA target sites in mammalian mRNAs. *Elife* 2015; 4.
18. Wang X. Improving microRNA target prediction by modeling with unambiguously identified microRNA-target pairs from CLIP-ligation studies. *Bioinformatics* 2016; 32: 1316-1322.
19. Reczko M, Maragkakis M, Alexiou P, Grosse I, Hatzigeorgiou AG. Functional microRNA targets in protein coding sequences. *Bioinformatics* 2012; 28: 771-776.
20. Kertesz M, Iovino N, Unnerstall U, Gaul U, Segal E. The role of site accessibility in microRNA target recognition. *Nature genetics* 2007; 39: 1278-1284.
21. Shirdel EA, Xie W, Mak TW, Jurisica I. NAViGaTing the micronome--using multiple microRNA prediction databases to identify signalling pathway-associated microRNAs. *PloS one* 2011; 6: e17429.
22. Vlachos IS, Paraskevopoulou MD, Karagkouni D, Georgakilas G, Vergoulis T, Kanellos I, Anastasopoulos IL, Maniou S, Karathanou K, Kalfakakou D, Fevgas A, Dalamagas T, Hatzigeorgiou AG. DIANA-TarBase v7.0: indexing more than half a million experimentally supported miRNA:mRNA interactions. *Nucleic acids research* 2015; 43: D153-159.
23. Lachmann A, Giorgi FM, Lopez G, Califano A. ARACNe-AP: gene network reverse engineering through adaptive partitioning inference of mutual information. *Bioinformatics* 2016; 32: 2233-2235.
24. Margolin AA, Nemenman I, Basso K, Wiggins C, Stolovitzky G, Dalla Favera R, Califano A. ARACNE: an algorithm for the reconstruction of gene regulatory networks in a mammalian cellular context. *BMC bioinformatics* 2006; 7 Suppl 1: S7.
25. Wehrspaun CC, Haerty W, Ponting CP. Microglia recapitulate a hematopoietic master regulator network in the aging human frontal cortex. *Neurobiol Aging* 2015; 36: 2443 e2449-2443 e2420.

26. Margolin AA, Wang K, Lim WK, Kustagi M, Nemenman I, Califano A. Reverse engineering cellular networks. *Nature protocols* 2006; 1: 662-671.
27. Carro MS, Lim WK, Alvarez MJ, Bollo RJ, Zhao X, Snyder EY, Sulman EP, Anne SL, Doetsch F, Colman H, Lasorella A, Aldape K, Califano A, Iavarone A. The transcriptional network for mesenchymal transformation of brain tumours. *Nature* 2010; 463: 318-325.
28. Zhang Z. Variable selection with stepwise and best subset approaches. *Annals of translational medicine* 2016; 4.
29. Reimand J, Arak T, Vilo J. g:Profiler--a web server for functional interpretation of gene lists (2011 update). *Nucleic acids research* 2011; 39: W307-315.
30. Sharples AP, Al-Shanti N, Lewis MP, Stewart CE. Reduction of myoblast differentiation following multiple population doublings in mouse C2 C12 cells: a model to investigate ageing? *Journal of cellular biochemistry* 2011; 112: 3773-3785.
31. Burattini S, Ferri P, Battistelli M, Curci R, Luchetti F, Falcieri E. C2C12 murine myoblasts as a model of skeletal muscle development: morpho-functional characterization. *European journal of histochemistry : EJH* 2004; 48: 223-233.
32. Chen C, Ridzon DA, Broomer AJ, Zhou Z, Lee DH, Nguyen JT, Barbisin M, Xu NL, Mahuvakar VR, Andersen MR, Lao KQ, Livak KJ, Guegler KJ. Real-time quantification of microRNAs by stem–loop RT–PCR. *Nucleic Acids Research* 2005; 33: e179-e179.
33. Taylor SC, Laperriere G, Germain H. Droplet Digital PCR versus qPCR for gene expression analysis with low abundant targets: from variable nonsense to publication quality data. *Scientific Reports* 2017; 7: 2409.
34. Lovric J, Mano M, Zentilin L, Eulalio A, Zacchigna S, Giacca M. Terminal Differentiation of Cardiac and Skeletal Myocytes Induces Permissivity to AAV Transduction by Relieving Inhibition Imposed by DNA Damage Response Proteins. *Molecular Therapy* 2012; 20: 2087-2097.
35. Liu Z, Wang C, Liu X, Kuang S. Shisa2 regulates the fusion of muscle progenitors. *Stem Cell Res* 2018; 31: 31-41.
36. Andersen NJ, Boguslawski EA, Naidu AS, Szot C, Bromberg-White JL, Kits K, Kuk CY, Holton LE, St Croix B, Chambers CM, Duesbery NS. Anthrax Toxin Receptor 1 Is Essential for Arteriogenesis in a Mouse Model of Hindlimb Ischemia. *PLoS One* 2016; 11: e0146586.
37. Lee KW, Kwak SH, Ahn BY, Lee HM, Jung HS, Cho YM, Park YJ, Chung SS, Park KS. F-box only protein 9 is required for adipocyte differentiation. *Biochem Biophys Res Commun* 2013; 435: 239-243.
38. Marquez J, Kohli M, Arteta B, Chang S, Li WB, Goldblatt M, Vidal-Vanaclocha F. Identification of hepatic microvascular adhesion-related genes of human colon cancer cells using random homozygous gene perturbation. *Int J Cancer* 2013; 133: 2113-2122.
39. Chen PY, Qin L, Barnes C, Charisse K, Yi T, Zhang X, Ali R, Medina PP, Yu J, Slack FJ, Anderson DG, Kotelianski V, Wang F, Tellides G, Simons M. FGF regulates TGF-beta signaling and endothelial-to-mesenchymal transition via control of let-7 miRNA expression. *Cell Rep* 2012; 2: 1684-1696.
40. Pandit SK, Westendorp B, Nantasanti S, van Liere E, Tooten PC, Cornelissen PW, Toussaint MJ, Lamers WH, de Bruin A. E2F8 is essential for polyploidization in mammalian cells. *Nat Cell Biol* 2012; 14: 1181-1191.

41. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001; 25: 402-408.