ORIGINAL ARTICLE

MicroRNA regulatory networks associated with abnormal muscle repair in survivors of critical illness

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Abstract

Background   Intensive care unit (ICU)-acquired weakness is characterized by muscle atrophy and impaired contractility that may persist after ICU discharge. Dysregulated muscle repair and regeneration gene co-expression networks are present in critical illness survivors with persistent muscle wasting and weakness. We aimed to identify microRNAs (miRs) regulating the gene networks and determine their role in the self-renewal of muscle in ICU survivors.

Methods   Muscle whole-transcriptome expression was assessed with microarrays in banked quadriceps biopsies obtained at 7 days and 6 months post-ICU discharge from critically ill patients ($n = 15$) in the RECOVER programme and healthy individuals ($n = 8$). We conducted an integrated miR–messenger RNA analysis to identify miR/gene pairs associated with muscle recovery post-critical illness and evaluated their impact on myoblast proliferation and differentiation in human AB1167 and murine C2C12 cell lines *in vitro*. Select target genes were validated with quantitative PCR.

Results   Twenty-two miRs were predicted to regulate the Day 7 post-ICU muscle transcriptome vs. controls. Thirty per cent of all differentially expressed genes shared a 3'UTR regulatory sequence for miR-424-3p/5p, which was 10-fold down-regulated in patients ($P < 0.001$) and correlated with quadriceps size ($R = 0.86$, $P < 0.001$), strength ($R = 0.75$, $P = 0.007$), and physical function (Functional Independence Measures motor subscore, $R = 0.92$, $P < 0.001$) suggesting its potential role as a master regulator of early recovery of muscle mass and strength following ICU discharge. Network analysis demonstrated enrichment for cellular respiration and muscle fate commitment/development related genes. At 6 months post-ICU discharge, a 14-miR expression signature, including miRs-490-3p and -744-5p, identified patients with muscle mass recovery vs. those with sustained atrophy. Constitutive overexpression of the novel miR-490-3p significantly inhibited AB1167 and C2C12 myoblast proliferation (cell count AB1167 miR-490-3p mimic or scrambled-miR transfected myoblasts 7926 ± 4060 vs. 14 159 ± 3515 respectively, $P = 0.006$; proportion Ki67-positive nuclei AB1167 miR-490-3p mimic or scrambled-miR transfected myoblasts 0.38 ± 0.07 vs. 0.54 ± 0.06 respectively, $P < 0.001$; proliferating cell nuclear antigen expression AB1167 miR-490-3p mimic or scrambled-miR transfected myoblasts 11.48 ± 1.97 vs. 16.75 ± 1.19 respectively, $P = 0.040$). Constitutive overexpression of miR-744-5p, a known regulator of myogenesis, significantly inhibited AB1167 and C2C12 myoblast differentiation (fusion index AB1167 miR-744-5p mimic or scrambled-miR transfected myoblasts 8.31 ± 7.00% vs. 40.29 ± 9.37% respectively, $P < 0.001$; myosin heavy chain expression miR-744-5p mimic or scrambled-miR transfected myoblasts 0.92 ± 0.39 vs. 13.53 ± 5.5 respectively, $P = 0.01$).

Conclusions   Combined functional transcriptomics identified 36 miRs including miRs-424-3p/5p, -490-3p, and -744-5p as potential regulators of gene networks associated with recovery of muscle mass and strength following critical illness. MiR-490-3p is identified as a novel regulator of myogenesis.

Keywords   ICU-acquired weakness; Microarray; Gene network analysis; Muscle weakness; Muscle atrophy; Muscle regeneration

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Introduction

Intensive care unit-acquired weakness (ICUAW) develops secondary to mixed combinations of peripheral nerve dysfunction, rapid skeletal muscle proteolysis, decreased muscle protein synthesis, and impaired contractility. It is associated with prolonged mechanical ventilation, longer hospital stays, increased mortality, and major physical functional impairment.[1,2]

Muscle recovery and repair are heterogeneous. While some patients exhibit sustained weakness with permanent disability, others achieve functional recovery within 6 months (6M) post-ICU discharge, before plateauing at 1 year.[2–4] This time period is thus critical to the institution of impactful interventions. Therapeutic approaches currently focus primarily on modulating known ICUAW risk factors, such as prolonged inactivity and hyperglycaemia, with early mobility and intensive insulin therapy. Early mobility has positive effects on short-term outcomes, but efficacy in the long term remains unproven.[5] Intensive insulin therapy reduces the duration of mechanical ventilation but poses a significant hypoglycaemic risk. No treatment universally averts or corrects ICUAW.[5]

We previously reported on the mechanistic processes underscoring sustained muscle weakness in mechanically ventilated critically ill patients by serially assessing their physical functional capacity, strength, and quadriceps size and performing vastus lateralis biopsies for molecular analyses over a 6M period after ICU discharge.[3,6] Persistent weakness resulted variably from sustained muscle wasting and/or impaired contractility. Patients with sustained muscle atrophy had a decreased muscle progenitor (satellite) cell content.[3] We profiled muscle RNA content and identified groups of co-expressed genes (modules) that were differentially regulated between healthy individuals and ICUAW patients, termed ICUAW-relevant modules.[6] These modules were highly correlated with measures of muscle mass, strength, and/or physical function and contained genes known to regulate skeletal muscle regeneration. Collectively, these findings suggested a link between persistent muscle atrophy and impairment of muscle regeneration in individuals with sustained ICUAW at 6M after ICU discharge.[3,6]

Here, we advance discovery using microRNA (miR) profiling to identify novel regulator(s) of human myogenesis that may play a role in muscle wasting and weakness in survivors of ICU care. MiRs are single-stranded RNAs (18-25 nucleotides) that bind to and impact messenger RNA (mRNA) stability, inducing mRNA degradation and altering transcription. Although small, miRs are potent effectors of gene expression where a single aberrant miR can regulate entire gene networks, thereby substantially contributing to disease development and progression (e.g. cancer).[7,8] Moreover, miR-based therapies are now emerging as promising innovative treatment strategies for disease.[9–11] In skeletal muscle, nine miRs (miR-1,-133a/b, -486, -499a/b, -206, and -208a/b) are key regulators of myogenesis, well known to impact myoblast proliferation and differentiation.[12]

We hypothesized that alterations in miR expression mediate aberrant expression of muscle regenerative genes post-ICU discharge, inducing sustained muscle wasting and weakness vs recovery in critical illness survivors. To test this hypothesis, we performed paired miR and mRNA expression analysis of banked muscle biopsy samples obtained at 7 days (7D) and 6M post-ICU discharge from patients with and without sustained ICUAW and from healthy individuals. We adapted a previously published computational approach[13] to identify gene expression modules that contained mRNAs that shared binding sequences for differentially expressed miRs, and then selected specific miRs for further in vitro analysis based on their functional relevance as indicated by the degree of association with features of ICUAW (decreased muscle mass or strength, or impaired physical function). Top miRs predicted to regulate muscle atrophy and weakness associated gene expression networks in critical illness survivors were termed master miR-regulators. Gain and loss of function experiments in muscle cells were used to demonstrate a causal relationship between selected master miR-regulators and myogenesis in vitro. Importantly, such miRs might serve as therapeutic targets or agents themselves for ICUAW.

Materials and methods

Detailed material and methods are presented in the supporting information.

Patient population

The study population has been previously described.[3,6] Briefly, patients were prospectively recruited to this nested study (*n* = 27, Supporting Information, *Figure S1*) at St Michael's Hospital (SMH) and the University Health Network (UHN), Toronto, Canada from the RECOVER programme, a Canadian multicenter longitudinal study evaluating 1 year physical functional outcomes in critically ill patients undergoing prolonged (≥1 week) mechanical ventilation.[4] All patients had full physical functional capacity and were ambulatory without an aid prior to their critical illness. Vastus lateralis biopsies, skeletal muscle electrophysiologic studies (electromyography & nerve conduction studies), and clinical measures of muscle mass, strength, and physical function (Functional Independence Measures score, Medical Research Council sum score, quadriceps isometric peak torque, and computed tomography-determined quadriceps cross-sectional area) were obtained serially at 7D and 6M post-ICU discharge.[3] Fifteen and eleven patients completed the 7D and 6M post-ICU analyses, respectively. Demographics, pre-morbid status, severity, and duration of critical illness of patients completing the study [median age 53 years, interquartile range (IQR) 40–62; 47% female patients; median mechanical ventilation duration 14 days (IQR 10–29); median hospital length of stay 38 days (IQR 27–58); comorbidities (Charlson Index median score 1, IQR 0–1); critical illness severity (APACHE II median score 22; IQR 14–30)] were not different from patients who were lost to follow up, died, or withdrew and were representative of the larger RECOVER patient cohort (*n* = 103) at SMH and UHN.[3]

Adequate RNA was available from fourteen 7D and eight 6M muscle biopsies for total RNA (miR and mRNA) profiling. Banked muscle biopsy specimens from healthy, non-sedentary individuals (*n* = 8, median age 43, 50% male patients) were used for comparator purposes and previously reported.[3] 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

MicroRNA and messenger RNA profiling

MicroRNAs were profiled using miRCURY LNA miR Arrays (Exiqon, 7th generation). mRNA was profiled using IlluminaHT-12 V4 arrays as published.[6] Electronic data have been deposited (Gene Expression Omnibus: GSE78929).

Master miR-regulator analysis

Here, we adapted and modified the Master MiR-regulator analysis, a previously published protocol (R code available at http://eda.polito.it/MMRA)[13] that combines statistical tests, target prediction, and unsupervised network analysis to identify miRs predicted to best 'drive' the differential expression of target genes in disease-specific cohorts or study subgroups. *Figure 1* shows a schematic of our analysis approach. In Step 1, we identified miRs and mRNAs that were differentially expressed over time (7D and 6M post-ICU discharge) between patient and healthy individual subgroups. In Step 2, we used miR-target enrichment analysis to identify those miRs whose known (experimentally proven and published) or predicted (based on binding sequence complementarity) mRNA targets were over-represented ('enriched') in the genes differentially expressed between subgroups. In Step 3, we used the miR:mRNA pairs to (A) build a miR:mRNA regulatory network and then (B) performed a functional enrichment analysis to identify those miRs whose network target genes were over-represented in the differentially expressed gene lists for each subgroup comparison and our previously reported ICUAW-relevant modules.[6] In Step 4, stepwise linear regression was used to filter out weak miR/gene relationships, so that only the most statistical co-regulatory miR:mRNA relationships were retained. We then determined the association between each network with measured clinical features of muscle status (i.e. muscle mass, strength, or/and physical function). The final output of the analysis delineates the gene sets differentially expressed in each ICUAW subgroup, which are robustly associated with the network miRs. The relative contribution of a miR to the regulation of each ICUAW subgroup gene signature was defined as the percentage of the differentially expressed genes in each subgroup predicted to be a target of that miR. Statistical cut-offs were defined for each step, and miRs satisfying all thresholds were deemed putative master miR-regulators. Detailed methods and statistical analyses for each step are described in the supporting information.

Functional validation of in silico predictions with muscle cell culture

Impact of select master miR-regulators on myoblast proliferation and differentiation was determined in murine C2C12[14] and/or human AB1167 skeletal muscle (generated by A. Bigot Sorbonne University, France) cell lines *in vitro*. Endogenous miR expression was determined using (TaqMan) qPCR (Applied Biosystems, ThermoFisher Scientific). For
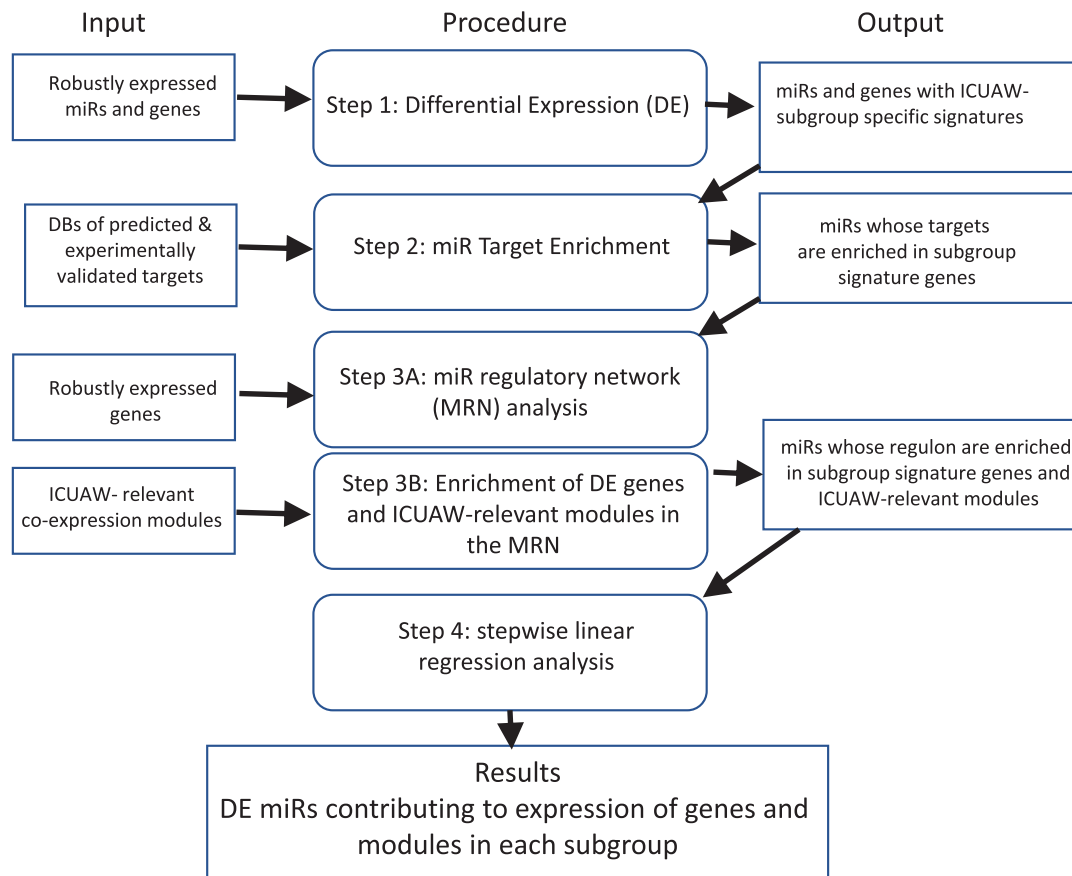
Input | Procedure | Output

Robustly expressed miRs and genes → Step 1: Differential Expression (DE) → miRs and genes with ICUAW-subgroup specific signatures

DBs of predicted & experimentally validated targets → Step 2: miR Target Enrichment → miRs whose targets are enriched in subgroup signature genes

Robustly expressed genes → Step 3A: miR regulatory network (MRN) analysis

ICUAW- relevant co-expression modules → Step 3B: Enrichment of DE genes and ICUAW-relevant modules in the MRN → miRs whose regulon are enriched in subgroup signature genes and ICUAW-relevant modules

Step 4: stepwise linear regression analysis

Results
DE miRs contributing to expression of genes and modules in each subgroup

Figure 1 Workflow of master MiR-regulator analysis (MMRA) pipeline[13] adapted based on this study's research objectives. The figure indicates the procedure performed in each of the four analytic steps (middle panels) using data required as initial input (left panels) and/or the outputs of preceding analytic steps (right panels). DBs, databases; DE, differential expression; ICUAW, ICU-acquired weakness; MiR, microRNA; MRN, miR regulatory network.

gain-of-function and loss-of-function experiments, myoblasts were transfected with either miR mimics or inhibitors (mirVana™, ThermoFisher Scientific), respectively, and scrambled miRs as negative controls. Proliferation assays (determination of ki67 nuclear localization, cell count, & proliferating cell nuclear antigen expression) and differentiation assays (determination of fusion index & myosin heavy chain expression) were performed at serial time points post-myoblast plating as detailed in the supporting information. A minimum of three replicates per experimental condition and four repeated experiments per assay were performed. Statistical analyses for cell culture experiments were undertaken using two-way analysis of variance with repeated measures. Statistical significance was assumed if $P < 0.05$.

Differential expression of miR-specific putative gene targets following miR transfection was determined using nCounter Gene Expression System and Advanced Analysis Module (Nanostring Technologies) for C2C12 cells or relative quantitative RT-PCR (Applied Biosystems) for C2C12 and AB1167 cells.

Results

Identification of putative master miR-regulators

We analysed 30 *vastus lateralis* muscle samples from 14 patients at 7D and 8 follow-up biopsies at 6M post-ICU discharge, and 8 healthy individuals. In Step 1, linear models in *limma* were used to identify differentially expressed miRs and mRNAs adjusting for age, sex, and correlation within samples from the same individual.[15] A total of 55 miRs and 3 miRs were differentially expressed at 7D and 6M post-ICU discharge respectively, compared with healthy individuals (false discovery rate $< 5\%$, fold change > 1.5; *Table* S1a,S1b). Differentially expressed genes are shown in *Table* S1d,S1e.

Unsupervised hierarchical clustering showed miR expression profiles from patients 7D post-ICU discharge clustered together, separately from healthy individuals and independent of age, sex, severity of critical illness, and pre-morbid status (*Figure* 2A). By 6M post-ICU discharge however,
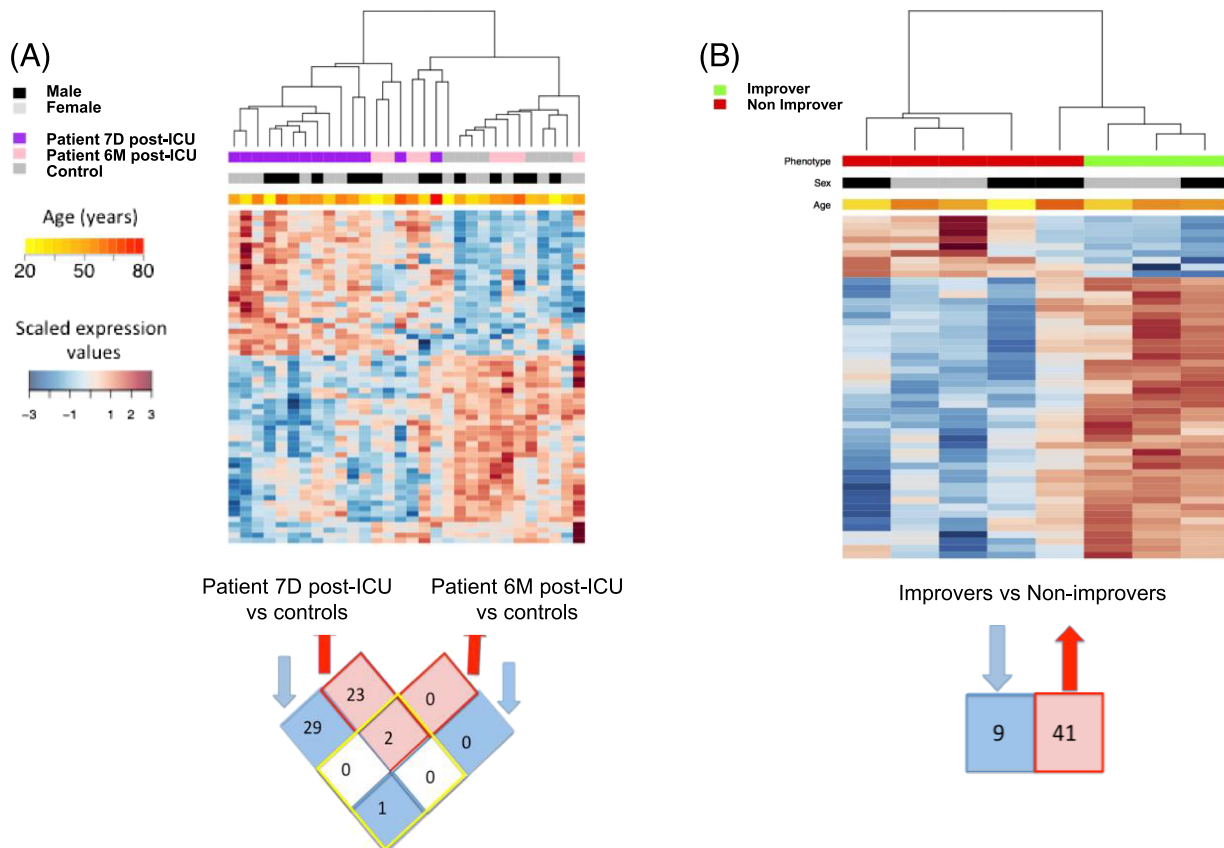
Figure 2 Expression patterns of quadriceps differentially expressed (DE) miRs in survivors of ICU care. (*A*) Heatmap of 55 miRs DE in quadriceps biopsies between patients at Day 7 (*n* = 14, 7D) and Month 6 (*n* = 8, 6M) post-ICU discharge vs. healthy individuals (*n* = 8, controls). The top bars indicate study subgroup, age, and sex (values are colour coded according to respective legends to the left of the heat map). Below heatmap is a Venn diagram of DE miRs in Patient 7D (left) and Patient 6M (right) post-ICU vs. controls. Number of overlapping genes shared between 7D and 6M are shown within the four squares within the yellow diamond. Number of probes exclusively DE in 7D (left) or 6M (right) post-ICU are shown in the four squares outside the yellow diamond. (*B*) Heatmap of 50 miRs DE between patient 'improvers' and 'non-improvers' at Month 6 post-ICU. The top bar indicates subgroups (red, non-improvers and green, improvers). Number of down-regulated and up-regulated probes are shown below heatmap. Forty-one miRs were up-regulated in the improvers vs. non-improvers, and nine were down-regulated [Differential expression was assessed at false positive discovery rate (FDR) < 0.05 and fold change > 1.5. Scaled expression values are colour coded according to the legend to the far left of the heat maps].

clustering of patient miR expression profiles was no longer evident. We speculated that this variability in miR regulation reflected heterogeneity in long-term (6M) muscle outcomes in critical illness survivors. Indeed, we previously reported that a subset of patients (*n* = 3, deemed 'improvers' here) normalized their quadriceps cross-sectional area relative to age-matched and sex-matched population-based norms with a significant increase is muscle size (>10 cm^2) at 6M post-ICU discharge, while the rest of the patients did not, experiencing sustained quadriceps wasting (*n* = 5, 'non-improvers').[3,6] MiRs were expected to be differentially regulated between improvers and non-improvers. Accordingly, 50 differentially expressed miRs at 6M post-ICU distinctly separate improvers from non-improvers (*Figure* 2B, *Table* S1c, gene targets *Table* S1f), suggesting an association between the degree of muscle mass recovery and miR expression.

We then used miR-target enrichment analysis (Step 2) to determine that 25 of the 55 miRs had mRNA targets over-represented in the differentially expressed gene signatures at 7D post-ICU vs. control, 2 of the 3 miRs were enriched for targets at 6M post-ICU vs. control, and 37 of the 50 miRs were enriched for targets in improvers vs. non-improvers (*Table* S2).

For each miR and its corresponding set of differentially expressed genes, a network composed of a single-hub miR-regulated gene targets was constructed using Algorithm for the Reconstruction of Accurate Cellular Networks,[16] which computationally assists in delineating direct transcriptional interactions. The complete set of genes putatively regulated by a miR is termed its 'regulon'.[17] The number of miR-target interactions in the regulons varied from 29 to 414 genes, and the mutual information values (theoretical measures of

the mutual dependence between each input miR and the putative gene targets) within each network ranged between 0.3 and 0.51 (*Table* S3). We used the regulons to determine which miRs were most responsible for the transcriptional profile documented in each comparison between study subgroups (Step 3): 25 of the 25 miRs were enriched at 7D post-ICU vs. healthy individuals, 0 of the 2 miRs enriched at 6M post-ICU vs. healthy individuals, and 27 of the 37 miRs for improvers vs. non-improvers were identified (*Table* S4).

MicroRNAs were further restricted using stepwise linear regression (Step 4) to determine the final master miR-regulators. Thirty-six miRs (*Table* 1) were identified, found to be significantly co-expressed with their putative network targets (*Table* S5) and with significant correlation between miR expression and clinical measures (*Table* 1) of strength, muscle size or physical function.

Twenty-two miRs (miR-424-3p/5p, -4780, -3175, -3622a-3p,-600, 23a-3p, 3136-3p, -206, -4795-5p, -502-3p, -574-3p, -29a/b-3p, -3133, -4488, -663a, -638, -5704, -4516, -551a, and -4764-3p) were identified as predicted master regulators of the Day 7 post-ICU vs. healthy control muscle transcriptome (*Table* 1, *Figure* 3A and 3C). Notably, 30% of all differentially expressed genes shared a 3'UTR regulatory sequence for miR-424-3p/5p, which was 10-fold down-regulated in patients (*Table* S1a) and highly correlated with quadriceps size ($R = 0.86$, $P < 0.001$), strength ($R = 0.75$, $P = 0.007$) and physical function ($R = 0.92$, $P < 0.001$) (*Table* 1). Seven miRs, including miR-424-3p/5p, and miRs-206, -29b-3p, -29a-3p, -23a-3p, and -502-3p were collectively identified as putative master regulators of two-thirds of the 7D skeletal muscle transcriptome (*Table* 1, *Figure* 3C). Notably, these seven miRs are well known to modulate skeletal muscle development, regeneration, hypertrophy, and/or atrophy, and all were down-regulated in 7D muscle, indicating a potential pro-myogenic response. Additionally, regulons associated with master miR-regulators at 7D were enriched for Gene Ontology functions relating to cellular respiration/mitochondrial structure or muscle fate commitment/muscle development (*Table* 1).

At 6M post-ICU discharge, a 14-miR expression signature (miR-490-3p, -744-5p, -4732-3p, -589-5p,-4739, -597, -4762-5p, -485-3p, -205-3p, -4530, -4279, -4701-5p, -642b-5p, and -3611) (*Table* 1; *Figure* 3B and 3D, left panel) identified critical illness survivors with recovery of quadriceps mass vs. those with sustained atrophy. MiR-490-3p was increased in improvers vs. non-improvers and associated with approximately 4% of differentially expressed genes in the transcriptome. The biologic relevance of the vast majority of the 14 miRs identified as master regulators of the gene signature in improvers vs. non-improvers, including miR-490-3p, is unknown.

Of the 11 ICUAW-relevant modules we previously characterized to be highly correlated with measures of muscle mass, strength, and/or physical function post-ICU discharge,[6] nine modules were significantly enriched in at least one master miR regulon (*Tables* 1 and S6).

MiRs-490-3p and -744-5p are negative regulators of myoblast proliferation and differentiation, respectively

Because the biologic relevance in muscle of the miRs associated with the transcriptome of improvers vs. non-improvers is unknown, we previously reported muscle satellite cell content correlates with recovery of muscle mass in ICUAW patients[3] and miRs can be developed as therapeutic agents, and we sought to determine the impact of select master miR-regulators on muscle regeneration/myogenesis using murine C2C12 and human AB1167 skeletal muscle cell lines. We selected miRs-490-3p and -744-5p, both increased in improvers vs. non-improvers, as they robustly correlated with clinical measures of quadriceps size ($R = 0.85$, $P = 0.008$ and $R = 0.66$, $P = 0.008$ respectively; *Table* 1) and human and murine orthologues have high sequence homology enabling the use of common Taqman probes for functional genomics experiments in both species.

We found miRs-490-3p and -744-5p expressed in proliferating myoblasts (*Figure* S2). MiR-744-5p expression remained relatively constant in differentiated myotubes, but the expression of miR 490-3p decreased significantly with differentiation. Given both miRs were differentially expressed during myogenesis *in vitro*, we next performed gain and loss of function experiments, transfecting miRs-744-5p and -490-3p mimics or inhibitors respectively into C2C12 and/or AB1167 myoblasts and assessed their impact on myoblast proliferation (determined by cell count, Ki67 nuclear localization, and proliferating cell nuclear antigen expression) and differentiation (determined by myotube fusion index and myosin heavy chain expression). We found miR-490-3p negatively regulates myoblast proliferation of both AB1167 and C2C12 myoblasts (*Figure* 3D, right panel; *Figure* 4A,C,D; *Figures* S3 to S6) but did not alter myoblast differentiation to mature myotubes in either cell line (*Figures* 5A–C, S7, and S8). In contrast, we found miR-744-5p is a negative regulator of AB1167 and C2C12 myoblast differentiation (*Figures* 3D [right panel], 5A–C, S7, and S8). However, its impact on proliferation was not consistent between the cell lines. MiR-744-5p inhibited AB1167 myoblast proliferation but had no impact on C2C12 myoblast proliferation (*Figures* 4B–D and S3–S6).

Putative miR-490-3p target expression in C2C12 and AB1167 muscle cells

During preparation of this manuscript, miR-744-5p was reported to promote bovine myoblast proliferation and inhibit differentiation, and its cellular signalling was described.[18]

Table 1 Identification of putative ICUAW master miR-regulators

MiR	MiR association with ICUAW	Gene targets association with ICUAW	SLR-estimated association (%)	Association with phenotypes, R (P)* Strength	Mass	Function	ICUAW modules regulated	GO terms (FDR P value)
hsa-mir-424-3p	Down in 7D	Up in 7D	12.2	0.72 (1.32e-02)	0.68 (2.15e-02)	0.89 (2.12e-04)	M1, M2, M3, M4, M17	Response to muscle activity (1.67E-02)
hsa-mir-424-5p	Down in 7D	Up in 7D	10.4	0.75 (7.36e-03)	0.86 (7.72e-04)	0.92 (5.66e-05)	M1, M2, M3, M4, M7	NA
hsa-mir-4780	Down in 7D	Up in 7D	10.2	0.75 (7.36e-03)	0.65 (2.99e-02)	0.76 (6.30e-03)	M1, M2, M3, M4	Cellular respiration (3.01E-03)
hsa-mir-424-5p	Down in 7D	Down in 7D	7.2	0.75 (7.36e-03)	0.86 (7.72e-04)	0.92 (5.66e-05)	M1, M2, M3, M4, M7	NA
hsa-mir-3175	Down in 7D	Up in 7D	6.8	0.83 (1.57e-03)	0.75 (7.39e-03)	0.88 (3.05e-04)	M1, M2, M3, M4	Mitochondrial matrix (9.02E-07)
hsa-mir-3622a-3p	Up in 7D	Down in 7D	6.7	−0.65 (2.95e-02)	−0.77 (5.34e-03)	−0.89 (2.12e-04)	M1, M2, M3, M4	Mitochondrial inner membrane (2.17E-09)
hsa-mir-600	Up in 7D	Up in 7D	5.8	−0.77 (5.92e-03)	−0.74 (9.95e-03)	−0.78 (4.49e-03)	M1, M3, M4, M13	Mitochondrial matrix (1.29E-20)
hsa-mir-3175	Down in 7D	Down in 7D	5.5	0.83 (1.57e-03)	0.75 (7.39e-03)	0.88 (3.05e-04)	M1, M2, M3, M4	Mitochondrial matrix (9.02E-07)
hsa-mir-23a-3p	Down in 7D	Up in 7D	5.4	0.82 (2.13e-03)	0.86 (7.72e-04)	0.8 (3.10e-03)	M1, M2, M3	Muscle cell fate commitment (4.08E-02)
hsa-mir-3136-3p	Up in 7D	Up in 7D	5.4	−0.58 (5.99e-02)	−0.56 (7.14e-02)	−0.6 (5.09e-02)	M1, M2, M3, M4	Cellular respiration (6.14E-11)
hsa-mir-206	Down in 7D	Up in 7D	5.3	0.8 (2.82e-03)	0.83 (1.64e-03)	0.81 (2.53e-03)	M2, M11	Actin cytoskeleton (4.81E-02)
hsa-mir-4795-5p	Up in 7D	Up in 7D	4.7	−0.72 (1.32e-02)	−0.51 (1.08e-01)	−0.78 (4.49e-03)	M1, M2, M3, M4	Cofactor biosynthetic process (2.64E-05)
hsa-mir-502-3p	Down in 7D	Up in 7D	4.7	0.75 (8.17e-03)	0.79 (4.11e-03)	0.67 (2.55e-02)	M1, M2, M3	Cofactor biosynthetic process (1.76E-02)
hsa-mir-574-3p	Up in 7D	Up in 7D	4.6	−0.89 (2.12e-04)	−0.79 (3.75e-03)	−0.75 (7.39e-03)	M1, M2, M3, M4	Cellular respiration (1.35E-07)
hsa-mir-29b-3p	Down in 7D	Up in 7D	3.6	0.44 (1.79e-01)	0.53 (9.33e-02)	0.65 (2.99e-02)	M1, M2, M3, M4, M7	Telomere cap complex (8.75E-04)
hsa-mir-29a-3p	Down in 7D	Down in7D	3.5	0.56 (7.08e-02)	0.62 (4.04e-02)	0.61 (4.44e-02)	M1, M2, M3, M4, M7	NA
hsa-mir-502-3p	Down in 7D	Down in 7D	3.4	0.75 (8.17e-03)	0.79 (4.11e-03)	0.67 (2.55e-02)	M1, M2, M3	Cofactor biosynthetic process (1.76E-02)
hsa-mir-29a-3p	Down in 7D	Down in 7D	3.1	0.56 (7.08e-02)	0.62 (4.04e-02)	0.61 (4.44e-02)	M1, M2, M3, M4, M7	NA
hsa-mir-3133	Up in 7D	Up in 7D	3.1	−0.54 (8.74e-02)	−0.61 (4.65e-02)	−0.5 (1.19e-01)	M2, M4	Cellular respiration (1.50E-02)
hsa-mir-29b-3p	Down in 7D	Down in 7D	3	0.44 (1.79e-01)	0.53 (9.33e-02)	0.65 (2.99e-02)	M1, M2, M3, M4, M7	Telomere cap complex (8.75E-04)
hsa-mir-4488	Down in 7D	Up in 7D	2.7	0.83 (1.57e-03)	0.91 (9.22e-05)	0.75 (7.39e-03)	M1, M3, M6, M11	Collagen-containing extracellular matrix (1.99E-02)
hsa-mir-663a	Down in 7D	Down in 7D	2.7	0.8 (2.82e-03)	0.86 (7.72e-04)	0.8 (3.10e-03)	M1, M2, M3	Translational initiation (7.30E-05)
hsa-mir-638	Down in 7D	Down in 7D	2.5	0.8 (2.82e-03)	0.87 (4.98e-04)	0.68 (2.03e-02)	M1, M2, M3, M4	Mitochondrial respiratory chain (8.57E-03)
hsa-mir-4732-3p	Up in improver	Up in improver	2.4	0.76 (2.74e-02)	0.74 (3.75e-02)	0.67 (6.91e-02)	M1	NA
hsa-mir-4732-3p	Up in improver	Down in improver	2.1	0.76 (2.74e-02)	0.74 (3.75e-02)	0.67 (6.91e-02)	M1	NA
hsa-mir-5704	Up in 7D	Up in 7D	2	−0.48 (1.34e-01)	−0.32 (3.43e-01)	−0.29 (3.90e-01)	M1, M3, M4	Cellular respiration (3.06E-09)
hsa-mir-4516	Down in 7D	Up in 7D	1.9	0.8 (2.82e-03)	0.83 (1.46e-03)	0.69 (1.80e-02)	M1, M2, M3, M4	Cytoskeletal Adaptor activity (4.42E-02)
hsa-mir-490-3p	Up in improver	Up in improver	1.9	0.79 (1.94e-02)	0.85 (7.54e-03)	0.77 (2.59e-02)	NA	NA
hsa-mir-551a	Up in 7D	Down in 7D	1.7	−0.59 (5.65e-02)	−0.76 (6.30e-03)	−0.68 (2.15e-02)	M2, M4	Spindle microtubule (1.17E-02)

(Continues)

Table 1 (continued)

MiR	MiR association with ICUAW	Gene targets association with ICUAW	SLR-estimated association (%)	Association with phenotypes, R (P)*			ICUAW modules regulated	GO terms (FDR P value)
				Strength	Mass	Function		
hsa-mir-589-5p	Down in improver	Down in improver	1.7	−0.74 (3.72e-02)	−0.72 (4.27e-02)	−0.49 (2.15e-01)	M4, M13	Cellular respiration (2.44E-05)
hsa-mir-4764-3p	Up in 7D	Down in 7D	1.5	−0.44 (1.72e-01)	−0.25 (4.56e-01)	−0.16 (6.42e-01)	NA	Cellular respiration (8.82E-10)
hsa-mir-4739	Up in improver	Down in improver	1.5	0.85 (8.17e-03)	0.88 (4.42e-03)	0.77 (2.59e-02)	M1, M3, M4	NA
hsa-mir-597	Down in improver	Down in improver	1.5	−0.79 (1.94e-02)	−0.67 (6.78e-02)	−0.57 (1.39e-01)	M4	Cellular respiration (3.01E-05)
hsa-mir-4762-5p	Up in improver	Down in improver	1.4	0.74 (3.72e-02)	0.6 (1.19e-01)	0.3 (4.77e-01)	NA	NA
hsa-mir-490-3p	Up in improver	Down in improver	1.4	0.79 (1.94e-02)	0.85 (7.54e-03)	0.77 (2.59e-02)	NA	Spindle microtubule (1.17E-02)
hsa-mir-485-3p	Up in improver	Up in improver	1.3	0.08 (8.47e-01)	−0.13 (7.65e-01)	−0.79 (2.02e-02)	NA	Axon cytoplasm (3.61E-02)
hsa-mir-638	Down in 7D	Up in 7D	1.2	0.8 (2.82e-03)	0.87 (4.98e-04)	0.68 (2.03e-02)	NA	Mitochondrial respiratory chain (8.57E-03)
hsa-mir-205-3p	Up in improver	Down in improver	1.2	0.46 (2.47e-01)	0.32 (4.44e-01)	0.16 (7.09e-01)	M4	Regulation of IL-8 secretion (3.77E-03)
hsa-mir-4530	Up in improver	Down in improver	1.2	0.71 (4.88e-02)	0.49 (2.13e-01)	0.33 (4.17e-01)	M1, M2, M3, M4	Skeletal muscle organ development (1.24E-05)
hsa-mir-744-5p	Up in improver	Down in improver	1.2	0.87 (4.66e-03)	0.66 (7.52e-03)	0.51 (1.94e-01)	M6	Mitochondrial ribosome (4.51E-02)
hsa-mir-4279	Up in improver	Down in improver	1.1	0.68 (6.25e-02)	0.76 (2.83e-02)	0.59 (1.23e-01)	NA	Myofibril (2.49E-02)
hsa-mir-4701-5p	Down in improver	Down in improver	1.1	−0.74 (3.72e-02)	−0.72 (4.27e-02)	−0.49 (2.15e-01)	M4	NA
hsa-mir-642b-5p	Up in improver	Down in improver	1.1	0.68 (6.25e-02)	0.76 (2.83e-02)	0.59 (1.23e-01)	NA	NA
hsa-mir-3611	Up in improver	Up in improver	1	0.33 (4.29e-01)	0.25 (5.44e-01)	−0.60 (8.89e-01)	M4	NA

Master MiR-regulator analysis,[13] a data-driven analytic pipeline, was used to identify miRs that best 'explained' the gene signatures and ICUAW-relevant modules we observed in patients at 7 days post-ICU discharge (7D) vs. healthy individuals, and in patients who normalized their quadriceps cross-sectional area compared with age-matched and sex-matched population-based norms (improvers) vs. those with sustained muscle atrophy (non-improvers) at 6 months post-ICU discharge. The degree of regulation by a miR for each subgroup signature was defined as the percentage of differentially expressed genes (up or down) in each subgroup identified by stepwise linear regression (SLR, positive or negative coefficient). Module phenotype Spearman's correlations (R, *unadjusted P values) performed for muscle strength (based on the Medical Research Council sum score at 7 days post-discharge, and % predicted quadriceps isometric peak torque 6 months post-discharge), muscle mass (quadriceps cross-sectional area), and physical function [motor subscore of the Functional Independence Measures (FIM) score]. Regulons controlled by master miR-regulators were enriched for Gene Ontology (GO) functions (FDR-adjusted P values). ICUAW-relevant modules identified by gene co-expression analysis we previously characterized to be highly correlated with clinical measures of muscle outcome post-ICU discharge[6] were significantly enriched in at least one master miR regulon.
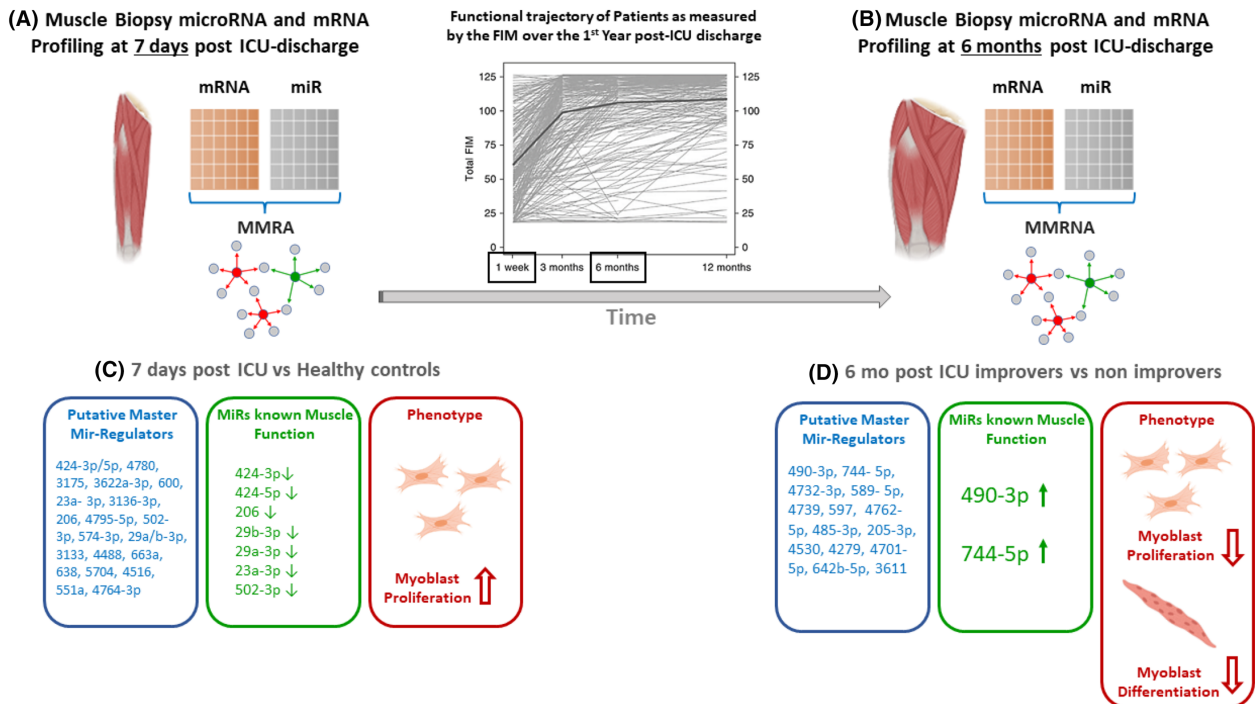
Figure 3 Master MiR-regulator analysis (MMRA) identifies miRs predicted to best 'drive' the differential expression of putative target genes in study subgroups. Muscle biopsies from patients enrolled in the RECOVER programme[4] were used for miR and mRNA expression profiling and MMRA analysis. Graph (top middle panel) showing the functional trajectory of patients enrolled in RECOVER, as measured by the Functional Independence Measure (FIM), over the first-year post-ICU discharge is reproduced[4] to underscore changes in functional measures over time. MMRA analysis (miR:mRNA integration) identified 22 miRs (*A,C*) and 14 miRs (*B,D*) as putative master regulators of the Day 7 post-ICU vs. healthy control and 'improver' vs. 'non-improver' 6 months post-ICU muscle transcriptome, respectively. Seven miRs down-regulated at Day 7 (C, middle panel) are known to negatively impact myoblast proliferation or muscle hypertrophy,[29–36] indicating a potential pro-myogenic phenotype in all patients immediately post-ICU discharge. In the 6 month improver vs. non-improver transcriptome, muscle function of the majority of miRs is unknown (*D*, left panel). miRs-490-3p and -744-5p were up-regulated in improver relative to non-improvers (*D*, middle panel); *in vitro* gain and loss of function experiments showed miR-490-3p to be a negative regulator of myoblast proliferation and miR-744-5p to be a negative regulator of differentiation (*D*, right panel). Their increased expression in improvers potentially represents a normal muscle baseline and the relative decrease of miR-490-3p and -744-5p in the non-improvers may enable continued efforts at muscle regrowth.

We therefore focused on miR-490-3p as the novel regulator of myogenesis identified here. To begin to delineate the mechanism of its anti-proliferative effect, we used (relative) qPCR and the Nanostring Skeletal Myogenesis and Myopathies Plexset pathway panel to determine the impact of miR-490-3p constitutive expression on AB1167 and C2C12 myoblast gene expression. Genes assessed included select putative miR-490-3p targets identified by the master miR-regulator analysis, known miR-490-3p targets in other biologic systems, and genes well known to modulate myogenesis and present on the Plexset (*Table* 2).

We found cyclin-dependent kinase 1 (CDK1), a known miR-490-3p target,[19] significantly decreased in transfected proliferating C2C12 and AB1167 myoblasts. In contrast, the expression of HMGA2 (high mobility group A2), another known miR-490-3p target,[20] was not altered in transfected AB1167 cells, and was increased in C2C12 myoblasts. Of the putative miR-490-3p targets identified in the master miR-regulator analysis on quadriceps biopsies, Capn3 (calpain 3), ASXL1 (ASXL transcriptional regulator 1), NCAM-1 (neural

cell adhesion molecule), and SMARCA4 (SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily A, member 4) were differentially expressed in miR-490-3p mimic transfected C2C12 and/or AB1167 myoblasts, but FBX09 (F box protein 9), ANTRX1 (anthrax toxin receptor 1) and SAPS2 (saps domain family, member 2) expression were not impacted. Myogenic regulatory proteins Myf5 (myogenic factor 5), MyoD1 (myogenic differentiation 1), and Mef2D (myocyte-specific enhancer factor 2D) were down-regulated in mir-490-3p transfected proliferating and/or differentiating C2C12 and AB1167 myoblasts.

Discussion

We have previously shown that muscle weakness in critical illness survivors is a complex composite of defects in contractility and failure to regain muscle mass.[3] Here, we used a multi-omics approach to exploit the canonical relationship
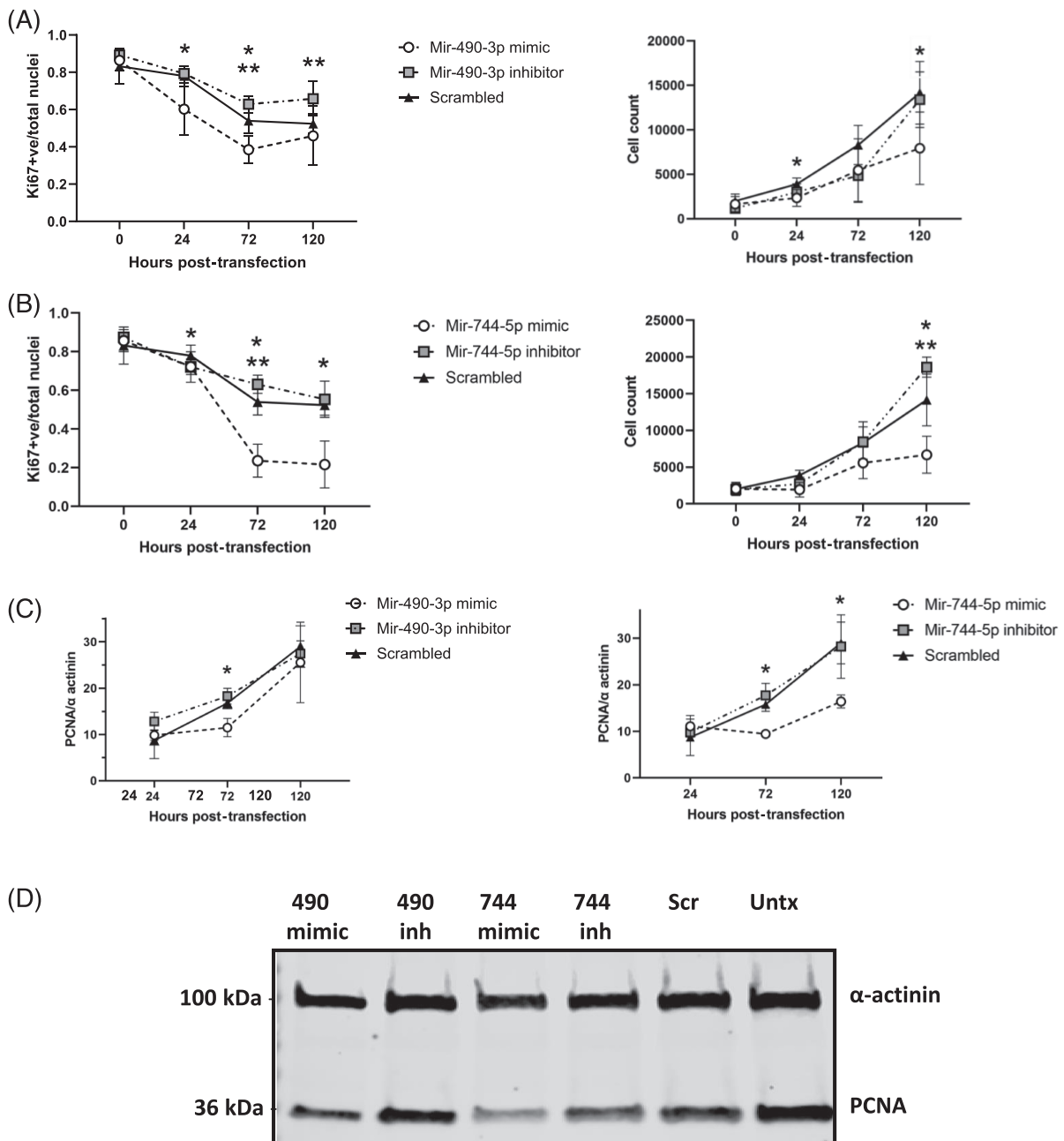
Figure 4 MiRs-490-3p and -744-5p are negative regulators of AB1167 human myoblast proliferation. AB1167 proportion of Ki67 (proliferation marker) positive nuclei (left panels) and myoblast cell count (right panels) at 24, 72, and 120 h post-transfection with (*A*) miR-490-3p mimic or inhibitor or (*B*) miR-744-5p mimic or inhibitor and a negative (scrambled) control. Representative confocal images of Ki67 immunostaining are shown in *Figure* S4. (*C*) Quantification of western blots of transfected myoblast protein lysate for proliferating cell nuclear antigen (PCNA) normalized to α-actinin loading control. (*D*) Representative western blot is shown (72 h post-transfection). MiR-490-3p and miR-744-5p mimics significantly decreased myoblast proliferation (*$P < 0.05$), while miR-490-3p and miR-744-5p inhibitors significantly increased myoblast proliferation (**$P < 0.05$), relative to scramble transfected negative control ($n = 4$ experiments/assay, cells plated in triplicate/experimental condition, data are mean ± SD, Scr = scrambled miR, Untx = untransfected, inh = inhibitor, 490 = miR-490-3p, 744 = miR-744-5p).

between miRs and their putative mRNA targets to demonstrate that transcriptional regulation in muscles from individuals who recover muscle mass 6M post-ICU discharge is distinct from those who have persistent muscle atrophy. Moreover, we delineate miR-dependent regulons associated with long-term ICUAW sustained 6M after ICU discharge and predicted to impact clinically relevant features of muscle cellular dysfunction such as impaired myoblast proliferation and differentiation. Our data in patients and muscle cells suggests a small number of miRs drive the pathophysiology of
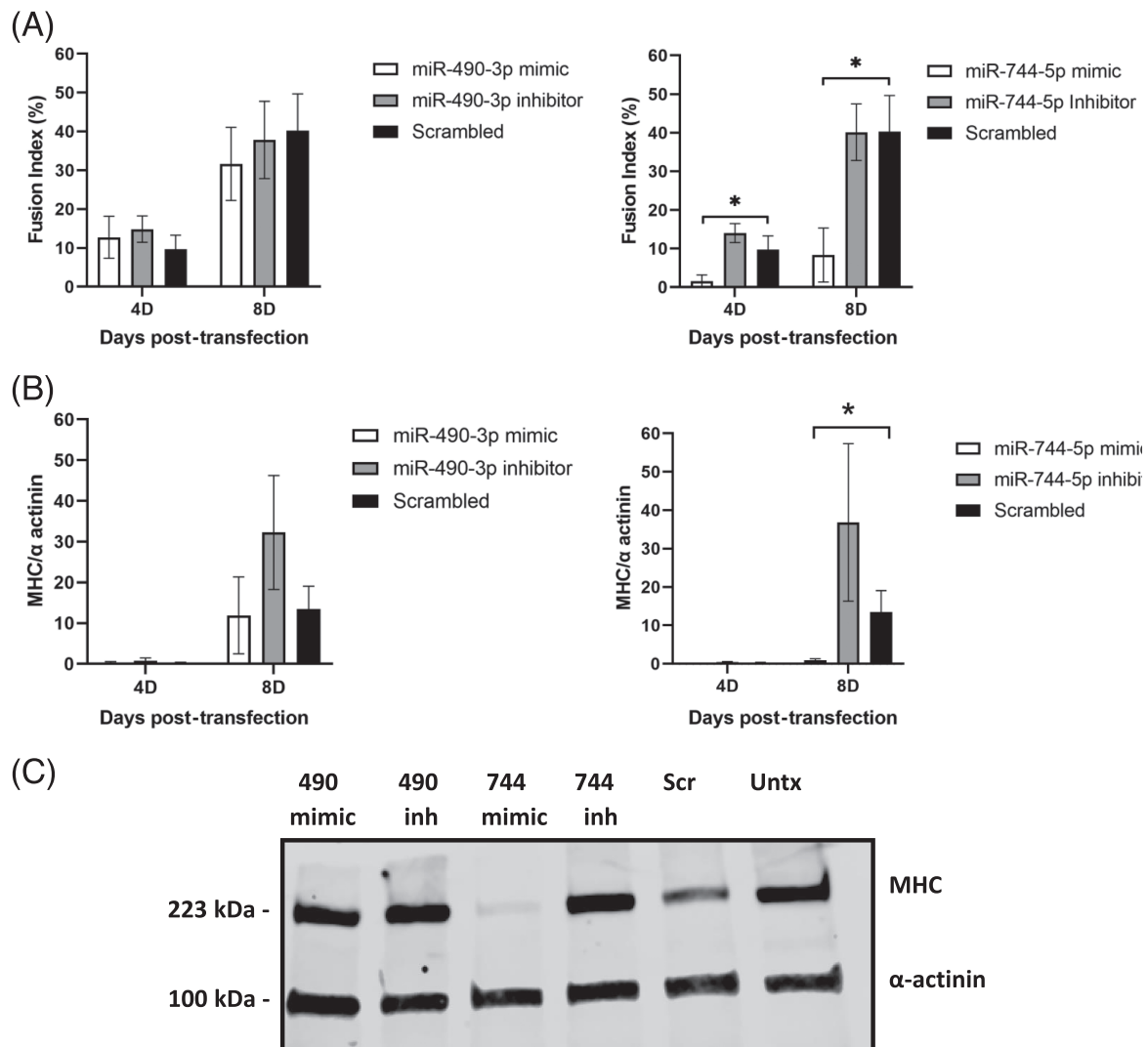
Figure 5 MiR-744-5p is a negative regulator of AB1167 human myoblast differentiation. (*A*) Fusion indices and (*B*) quantification of western blots for myosin heavy chain (MHC) normalized to α-actinin loading control for AB1167 myoblasts transfected with miR-490-3p mimic or inhibitor (left panels) and miR-744-5p mimic or inhibitor (right panels) at 4 days (4D) and 8 days (8D) post-transfection. (*C*) Representative western blot of cellular lysate 8D post-transfection is shown. Representative confocal images of MHC immunostaining are shown in *Figure* S7. MiR-744-5p mimic inhibited differentiation as indicated by decreased fusion index and MHC expression (*p < 0.05), while miR-744-5p inhibition increased MHC expression (**P < 0.05). MiR-490-3p did not significantly impact differentiation [n = 4 experiments/assay, cells plated in triplicate/experimental condition, data are mean ± SD, fusion index = percentage of nuclei in fused myotubes (MHC positive cells with two or more nuclei) relative to the total number of nuclei, per image, Scr = scrambled miR, Untx = untransfected, inh = inhibitor, 490 = miR-490-3p, 744 = miR-744-5p].

ICUAW vs. muscle recovery following ICU care. miR-424-3p/5p is predicted to account for 30% of the transcriptional changes associated with muscle wasting and weakness in survivors 7D post-ICU discharge. Combined *in silico* and cell culture analyses demonstrate miRs-490-3p (novel) and -744-5p (known) are regulators of myogenesis that potentially impact long-term reconstitution of muscle mass following critical illness.

The master MiR-regulator analysis strategy integrates statistics and unsupervised network theory to identify miR:mRNA regulatory networks containing a set of genes putatively regulated by a miR, termed its regulon, offering two levels of evidence in support for our findings. We found significant overlap between genes within regulons and the majority of ICUAW-related modules, the groups of co-expressed genes separating critical illness survivors from healthy individuals that we previously identified.[6] The majority of master miR-regulators predicted to drive the transcriptome at 7D post-ICU discharge targeted multiple overlapping ICUAW-related modules. Such combinatorial regulation is characteristic of the global architecture of mammalian miR regulatory networks,[21] that is, for one miR to regulate multiple targets thereby impacting global cellular phenomena. This has important clinical relevance because

Table 2 MiR-490-3p putative target gene expression in C2C12 and AB1167 transfected myoblasts

Cells	Method	Gene	Log2 fold change	Standard error	*P* value
C2C12	Plexset 48 h	ASXL1	−0.55	0.18	**0.014***
C2C12	Plexset 48 h	Mapk-14	−0.39	0.14	**0.024***
C2C12	Plexset 48 h	SMARCA4	0.44	0.17	**0.028***
C2C12	Plexset 48 h	Myf5	−0.79	0.31	**0.032***
C2C12	Plexset 48 h	MyoD	−0.67	0.28	**0.039***
C2C12	Plexset 48 h	Ctnnb1	0.33	0.14	**0.040***
C2C12	Plexset 48 h	Capn3	0.98	0.27	**0.005***
C2C12	Plexset 96 h	NCAM1	−0.57	0.20	**0.017***
C2C12	Plexset 96 h	Mef2d	−0.61	0.26	**0.044***
C2C12	qRT-PCR 48 h	CDK1	−0.66	0.09	**0.018**
C2C12	qRT-PCR 48 h	HMGA2	1.35	0.08	**0.004**
C2C12	qRT-PCR 48 h	ANTXR1	0.02	0.17	0.92
C2C12	qRT-PCR 48 h	FBX09	0.32	0.11	0.092
C2C12	qRT-PCR 48 h	SAPS2	0.17	0.51	0.77
AB1167	qRT-PCR 72 h	CDK1	−1.51	0.40	**0.049**
AB1167	qRT-PCR 72 h	HMGA2	0.06	1.48	0.972
AB1167	qRT-PCR 72 h	Myf5	−0.27	0.06	**0.045**
AB1167	qRT-PCR 72 h	MyoD	−1.29	0.30	**0.050**
AB1167	qRT-PCR 72 h	Ctnnb1	0.23	0.15	0.369
AB1167	qRT-PCR 72 h	ANTXR1	0.66	0.58	0.371
AB1167	qRT-PCR 72 h	Mef2d	−0.05	0.05	0.468
AB1167	qRT-PCR 72 h	NCAM1	1.21	1.72	0.552
AB1167	qRT-PCR 72 h	Capn3	1.03	0.37	**0.050**
AB1167	qRT-PCR 8D	Mef2d	−1.02	0.29	**0.017**
AB1167	qRT-PCR 8D	NCAM1	0.112	0.20	0.607
AB1167	qRT-PCR 8D	MyoD	−1.77	0.37	**0.005**

mRNA was harvested from miR-490-3p or scrambled control transfected C2C12 myoblasts at 48 h (proliferation) and 4 days (differentiation) and AB1167 myoblasts at 72 h (proliferation) and 8 days (differentiation) post-transfection. Gene expression in miR-490-3p transfected cells was determined relative to scrambled control with Nanostring nCounter technology (Skeletal Myogenesis and Myopathies Plexset pathway panel) and/or qPCR ($2^{\Delta\Delta}$CT relative quantification with HPRT and GAPDH as housekeeper genes). Genes quantified by qPCR were select genes identified as putative mir-490-3p targets in the master miR-regulator analysis of improvers vs non-improvers (NCAM1, ASXL1, SMARCA4, Capn3, FBx09, ANTRX1, and SAPS2), known miR-490-3p targets reported in the literature in other systems (CDK1 and HMGA2), or genes expressed on the Nanostring Plexset (*N* = 3 to 4 experiments/assay, cells were transfected in triplicate/experimental condition; significant *P* value < 0.05 in bold; *P value corrected for multiple testing).
ANTRX1, anthrax toxin receptor 1; ASXL1; ASXL transcriptional regulator 1; Capn3, calpain 3; CDK1, cyclin-dependent kinase 1; Ctnnb1, catenin beta 1; FBx09, F box protein 9; HMGA2, high mobility group A2; MAPK-14, mitogen-activated protein kinase 14; Mef2D, myocyte-specific enhancer factor 2D; Myf5, myogenic factor 5; myoD1, myogenic differentiation 1; NCAM1, neural cell adhesion molecule; qRT-PCR, real-time quantitative PCR; SAPS2, SAPS domain family, member 2; SMARCA4, SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily a, member 4.
*P value corrected for multiple testing.

miR signatures obtained from small focal muscle biopsies may be used in the future to guide further research, detect patients at risk for sustained muscle dysfunction, and/or to monitor response to therapy (e.g. exercise). Moreover, miR-modulatory strategies (e.g. mimics and inhibitors) may be used to prevent and/or to treat acute and/or persistent muscle dysfunction. This approach is currently being investigated for the treatment of monogenic muscle disorders, and in polygenic disorders, miR-based therapeutics are already in clinical trials.[11,22–24] While future studies will be required to determine the diagnostic and prognostic utility of miR-regulon profiles for acute and sustained ICUAW, here we provide, despite our small sample size, robust data demonstrating a potentially causative link between entirely novel miR-regulons and muscle cell dysfunction relevant to patients with sustained post-ICU muscle wasting.

Concordant with our findings from ICUAW-related modules,[6] a number of regulons contained genes involved in cellular respiration and skeletal muscle commitment/develop-ment, suggesting that the master miR-regulators may have critical roles in bioenergy homeostasis and dysfunctional muscle regeneration. Studies have reported decreased muscle mitochondrial content, bioenergetic dysfunction and depressed ATP synthesis during critical illness,[25–27] and decreased mitochondrial content persists at 7D post-ICU discharge.[3] In keeping with the premise of altered muscle bioenergy status, we found here, master miR-regulators associated with target gene networks related to cellular respiration and mitochondrial function at 7D post-ICU.

The seven miRs (-424-3p/5p, -206, -29b-3p, -29a-3p, -23a-3p, and -502-3p), predicted to collectively drive two-thirds of the transcriptome at 7D post-ICU, are well known to modulate myogenesis and muscle growth and all were down-regulated in critical illness survivors vs. healthy individuals. The murine homologue of miR-424 (miR-322), miR-206, and miR-29 all block cell cycle progression during myogenesis and promote myoblast differentiation.[28–31] Loss of miR-23a delays myoblast differentiation and maintains myoblasts with

proliferative potential.[32] In the early stages of critical illness recovery (7D), wasted muscle begins mass reconstitution, albeit to varying degrees and ultimately with variable success. We suggest down-regulation of these miRs in patients releases inhibition of cell cycle progression and allow muscle to undertake hyperplastic (regenerative) growth.

Down-regulation of both miR-424 and miR-29b may also enable hypertrophic growth at 7D. MiR-424-5p inhibits rRNA and thus protein synthesis, it is increased in conditions associated with muscle wasting[33] and overexpression of its homologue mir-322 induces muscle atrophy in mice (49). MiR-29b overexpression promotes muscle atrophy while its inhibition attenuates atrophy induced by denervation and immobilization.[34] Interestingly, miR-206 plays a complex role regulating both repression and stimulation of muscle hypertrophy.[35,36] Further studies will be needed to elucidate how this might regulate muscle growth in survivors with ICUAW.

While these master miR-regulators generate a muscle growth favourable environment at 7D presumably enabling an early attempted reparative response following critical illness resolution, this is not maintained to 6M when patients have separated into non-improvers and improvers, that is, those with persistent muscle wasting vs. those who regain and normalize muscle mass. Although miRs are mitotically and meiotically heritable, it remains unclear if failure to maintain this muscle pro-proliferative, pro-hypertrophic signature is heritable or a product of post-critical illness epigenetic remodelling in muscle.

At 6M, muscle mass increase was associated with a distinct 14 master miR-regulator signature. Although the function of the majority of these miRs in skeletal muscle remains unknown, we showed miR-490-3p, which was up-regulated in improvers vs. non-improvers, to be a novel negative regulator of myoblast proliferation and myogenesis. In malignancy, miR-490-3p inhibits cellular proliferation and protects against cancer progression by targeting CDK1.[19] We found miR-490-3p overexpression similarly decreased myoblast CDK1 transcript levels, suggesting it may be a common target by which miR-490-3p mediates its anti-proliferative effects. While in osteosarcoma cells, miR-490-3p targets HMGA2 (high mobility group A2) to induce G1 arrest,[20] we did not observe a similar decrease in HMGA2 expression in the muscle cells.

Of the miR-490-3p putative target genes identified in the quadriceps biopsies by the master miR-regulator analysis, Capn3 is interesting as it is a skeletal muscle-specific protease mutated in limb-girdle muscle dystrophy 2A whose deficiency enhances myoblast proliferation,[37,38] and it was increased in AB1167 and C2C12 myoblasts following miR-490-3p transfection. Capn3 also mediates repressed transcriptional activity of the myogenic regulatory factor MyoD, which is normally highly expressed in proliferating myoblasts and which was down-regulated in miR-490-3p transfected myoblasts. Mir-490-3p overexpression similarly down-regulated myocyte en-

hancer factor MEF2D. The MEF2A isoform is a positive regulator of primary myoblast proliferation *in vitro*,[39] although others have demonstrated the individual loss of MEF2 A, B, or D, does not impact satellite cell proliferation *in vivo*.[40] Myf5 is one of the most prominent myogenic regulatory factors expressed in proliferating myoblasts and was down-regulated by miR-490-3p transfection. In contrast to the changes in expression levels of these aforementioned miR 490-3p known and potential targets, all of which are in keeping with decreased myoblast proliferation, miR-490-3p overexpression also induced increased transcript expression of pro-proliferative SMARCA4[42] and CTNNB1[43] and down-regulation of MAPK-14,[44] an inhibitor of muscle progenitor proliferation. Future study is necessary to delineate downstream signalling engaged by miR-490-3p to mediate its anti-myogenic effects.

The increased expression of miR-490-3p in patients who have recovered muscle mass may serve to prevent dysregulated growth, consistent with its role as a tumour suppressor. Improvement in muscle mass and strength begins to plateau at 6M post-ICU discharge, and increased miR-490-3p expression at this time-point may represent an observed 'brake' in muscle mass reconstitution. Alternatively, the increased expression of miR-490-3p in improvers vs. non-improvers may represent a normal baseline level of muscle expression, and the relative decrease of miR-490-3p in the non-improvers may enable continued efforts at hyperplastic muscle regrowth.

MiR-744-5p was recently reported to be a novel regulator of myogenesis, stimulating proliferation and inhibiting differentiation of bovine myoblasts.[18] While we also found miR-744-5p delayed myogenic differentiation of myoblasts, we in contrast found it inhibited proliferation of AB1167 myoblasts and had no effect on C2C12 cells. Thus, the impact of miR-744-5p on proliferation appears to be specific to the cell culture system studied. We found miR-744-5p up-regulated in patients who reconstitute their muscle mass at 6M vs. those with sustained wasting. We speculate the anti-differentiation effect of miR-744-5p may represent a normal baseline of expression with decreased expression noted in non-improvers, permitting ongoing attempts at muscle regrowth.

Our study has several limitations. While we provide direct mechanistic evidence *in vitro* that miRs-490-3p and -744-5p are negative regulators of myogenesis (proliferation and differentiation respectively), we show only association with clinical measures of muscle recovery post-ICU. It remains possible the differences in expression observed between improvers vs. non-improvers represent normal variance in the population. Alternatively, the miR:mRNA signatures at 6M may simply be reflective of muscle that is unable to mount a reparative response, as opposed to playing a causative role. Although the patient cohort assessed here was representative of the larger study population, our analysis was limited by the small number of patients. Given the nature and

severity of their illness, we experienced patient attrition due to medical complications precluding, or refusal of muscle biopsy, ICU repatriation, study withdrawal, and death. We may be underpowered for some comparisons, and results may not be applicable to the larger population, which will need to be evaluated in other ICU cohorts. The focus on the transcriptome in the humans is also limiting the scope of understanding the biology in full as transcript levels do not necessarily reflect protein expression and cannot delineate protein functional status.

In summary, we have demonstrated that a small group of master miR-regulators predicted to impact myogenesis and muscle bioenergy utilization and establish the differentially expressed gene modules may drive the sustained muscle wasting and weakness vs. muscle recovery in critical illness survivors. A reparative proliferative and hypertrophic response appears evident early post-ICU discharge in all patients based on the miR signatures, which is no longer evident long term in those with sustained muscle wasting vs. those who successfully reconstitute muscle mass. Given that miR mimics and miR inhibitors hold potential to be developed as therapeutic agents, these findings may enable future potential clinical intervention.

Acknowledgements

Conflict of interest

The authors declare that they have no conflict of interest.

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Online supplementary material

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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