MicroRNA-542 Promotes Mitochondrial Dysfunction and SMAD Activity and Is Elevated in Intensive Care Unit-acquired Weakness

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Abstract

Rationale: Loss of skeletal muscle mass and function is a common consequence of critical illness and a range of chronic diseases, but the mechanisms by which this occurs are unclear.

Objectives: To identify microRNAs (miRNAs) that were increased in the quadriceps of patients with muscle wasting and to determine the molecular pathways by which they contributed to muscle dysfunction.

Methods: miRNA-542-3p/5p (miR-542-3p/5p) were quantified in the quadriceps of patients with chronic obstructive pulmonary disease and intensive care unit-acquired weakness (ICUAW). The effect of miR-542-3p/5p was determined on mitochondrial function and transforming growth factor- β signaling *in vitro* and *in vivo*.

Measurements and Main Results: miR-542-3p/5p were elevated in patients with chronic obstructive pulmonary disease but more markedly in patients with ICUAW. *In vitro*, miR-542-3p suppressed the expression of the mitochondrial ribosomal protein MRPS10 and

reduced 12S ribosomal RNA (rRNA) expression, suggesting mitochondrial ribosomal stress. miR-542-5p increased nuclear phospho-SMAD2/3 and suppressed expression of SMAD7, SMURF1, and PPP2CA, proteins that inhibit or reduce SMAD2/3 phosphorylation, suggesting that miR-542-5p increased transforming growth factor- β signaling. In mice, miR-542 overexpression caused muscle wasting, and reduced mitochondrial function, 12S rRNA expression, and SMAD7 expression, consistent with the effects of the miRNAs *in vitro*. Similarly, in patients with ICUAW, the expression of 12S rRNA and of the inhibitors of SMAD2/3 phosphorylation were reduced, indicative of mitochondrial ribosomal stress and increased transforming growth factor- β signaling. In patients undergoing aortic surgery, preoperative levels of miR-542-3p/5p were positively correlated with muscle loss after surgery.

Conclusions: Elevated miR-542-3p/5p may cause muscle atrophy in intensive care unit patients through the promotion of mitochondrial dysfunction and activation of SMAD2/3 phosphorylation.

Keywords: microRNA; skeletal muscle wasting; mitochondrial function; SMAD signaling

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At a Glance Commentary

Scientific Knowledge on the

Subject: Muscle dysfunction associated with critical illness, intensive care unit-acquired weakness (ICUAW), is a common complication that has profound prognostic and economic implications. The mechanisms that result in muscle dysfunction are not fully understood, but a loss of mitochondrial function and increased atrophic signalling contribute to muscle dysfunction. MicroRNAs are critical regulators of cell phenotype, so are likely to be important contributors to muscle wasting.

What This Study Adds to the

Field: MicroRNA-542-3p (miR-542-3p) and -5p were increased in the quadriceps of patients with ICUAW. Increased miR-542-3p targets mitochondrial ribosomal proteins, reducing mitochondrial translation and leading to mitochondrial dysfunction *in vitro* and *in vivo*. Increased miR-542-5p promoted SMAD signalling and reduced the expression of SMAD7, SMURF1, and SMAD phosphatases *in vitro* and in an animal model. This study therefore identifies miR-542-3p and -5p as mediators of mitochondrial and muscle dysfunction in ICUAW.

Admission to the intensive care unit (ICU) is often accompanied by a marked loss of skeletal muscle mass and function (1) that extends weaning time (2) and leads to a reduction in quality of life and an increase in mortality (3, 4). Recovery from muscle wasting can be very slow with many patients still having reduced function 5 years after critical illness (5). Muscle dysfunction also occurs in several chronic diseases including chronic obstructive pulmonary disease (COPD) contributing to increased morbidity and mortality (6). Skeletal muscle loss occurs as a result of an imbalance between protein synthesis and breakdown, with similar signaling pathways being implicated. For example, increased growth differentiation factor (GDF)-15 and reduced insulin-like growth factor-1 have been reported in both critical illness and

COPD (7–10). In addition to occurring as a consequence of muscle loss, decreased function also results from a reduction in the muscles' capacity to generate ATP, associated with a reduction in the activity of mitochondrial protein complexes, notably complexes I, III, and IV (11–13). These complexes contain proteins encoded by mitochondrial DNA that require translation by mitochondrial ribosomes.

Although muscle mass and oxidative capacity are primarily thought to be controlled by separate processes (protein and mitochondrial turnover), they are to some extent interdependent, because increasing oxidative capacity requires protein synthesis and increasing protein synthesis requires energy. Similarly, reducing oxidative capacity requires protein breakdown and autophagy, processes that operate during muscle breakdown. It is perhaps therefore not surprising that the loss of muscle mass and oxidative capacity often occur together (14) raising the possibility that there are common regulatory mechanisms or factors. Understanding such factors is important in identifying novel therapeutic approaches to treating muscle dysfunction.

MicroRNAs (miRNAs) are small RNAs that control the translation and degradation of sets of mRNAs, modulating biologic responses and cell phenotype by regulating the levels of key proteins in multiple biologic pathways. For example, miRNA-1 (miR-1) is elevated during muscle differentiation and regeneration (15), contributing to myogenesis both by suppressing the expression of HDAC4 (an inhibitor of several myogenic transcription factors) and by entering mitochondria and increasing mitochondrial translation (16). Consistent with this, we and others have previously reported reduced expression of miR-1 is associated with muscle wasting in COPD (17), renal failure (18), and ICU-acquired weakness (ICUAW) (7), although there is some discrepancy in the COPD literature (10). We have also identified a miRNA pattern associated with muscle mass in patients with COPD that is distinct from that in healthy individuals (19). The miRNAs identified were associated with pluripotency and regeneration, indicating that patients who lost the most muscle were unable to respond sufficiently to the physiologic stress imposed by disease. This observation suggests that some patients are more susceptible to muscle atrophy in the

presence of disease but does not identify factors associated with disease that drive the loss of muscle, nor did the miRNA pattern identified associate with the loss of oxidative capacity.

Consequently, we hypothesized that there would be changes in miRNA expression in the quadriceps of patients with COPD that promoted atrophy and a loss of oxidative capacity. In this study, we therefore reanalyzed our screen to identify miRNAs associated with COPD that were predicted to target pathways controlling protein turnover and energy balance; miR-542-3p and -5p fulfilled these criteria (mitochondrial translation and transforming growth factor [TGF]- β signaling, respectively). However analysis of the expression of these miRNAs in patients with established ICUAW showed that they were more markedly increased in those patients than in patients with COPD, consistent with the more rapid muscle atrophy that these patients undergo. We confirmed the ability of these miRNAs to target the appropriate biochemical pathways both *in vitro* and *in vivo* and showed that expression of the miRNA could cause muscle wasting *in vivo*. Finally, we analyzed the expression of these miRNAs in the response of patients to the insult of aortic surgery, which we have characterized as a human model of ICUAW (8). Some of the results of these studies have been previously reported in the form of an abstract (20–22).

Methods

Subjects

COPD cohort. Stable patients with COPD according to the Global Initiative for Chronic Obstructive Lung Disease guidelines 2004 (23) were enrolled from clinics at the Royal Brompton Hospital. Exclusion criteria are described in the online supplement. Healthy age-matched control subjects were recruited by advertisement. COPD subjects in this study form part of a larger well-phenotyped cohort described by Natanek and coworkers (14). Samples from 14 patients and 7 control subjects were used in the initial miRNA screen. The validation cohort included samples from 24 patients and 12 control subjects, none of which formed part of the screen. Demographic data for

these patients are shown in Tables E1 and E2 in the online supplement.

Physiologic measurements. Details of the methods used to assess physiologic parameters (body composition, lung function, physical performance, and strength) are given in the online supplement. Muscle biopsy was performed by percutaneous needle biopsy in the mid-thigh of the vastus lateralis, of the same leg in which strength had been tested, under local anesthesia using the Bergstrom technique (24).

ICUAW cohort. This cohort (17 patients with ICUAW and 7 control subjects) has been described previously (7) and recruitment details are given in the online supplement. The muscle biopsies were taken from the rectus femoris by the Bergstrom technique (24) or as open biopsies. There was no difference between patients and control subjects in age (62 ± 18 and 68 ± 11 yr) or in body mass index (27.8 ± 3.9 and 25.2 ± 3.8 kg/m²).

Aortic surgery cohort. Patients provided written informed consent and the study was approved by the National Research Ethics Committee (study 13/LO/0479). Patients undergoing elective cardiothoracic surgery at Royal Brompton Hospital were recruited. The principal inclusion criterion was a high-risk elective aortic surgical procedure requiring admission to the ICU. Presurgical exclusion criteria included preexisting muscular or neuromuscular disease, malignancy, and contraindication to muscle biopsy. Patients were also excluded if clinical instability precluded a biopsy after surgery. Rectus femoris cross-sectional area (RF_{CSA}) was determined by ultrasound before surgery and 7 days after surgery, as previously described (25). An open biopsy was taken after the induction of anesthesia but before the start of surgery from the rectus femoris and a second biopsy was taken using the Bergstrom technique 24 hours after surgery. Blood samples were collected before surgery and regularly during the first 7 days after surgery. Demographic data for these patients are shown in Table E3.

Quantification of RNA and Protein in Biologic Samples

Details of the analysis methods for RNA and protein have been described before and are detailed in the online supplement.

In Vitro and Animal Experiments

Targets of miR-542-3p/5p were identified by Ago-2 pull down. The effects of the miRNAs on gene and protein expression, and on mitochondrial function and TGF- β signaling were determined in LHCN-M2 cells, a human skeletal muscle cell line. Overexpression of the miRNA in mouse muscle was achieved by electroporation of an enhanced green fluorescent protein (EGFP)-miRNA construct (pCAGGS-miR-542). Experimental procedures for the *in vitro* and animal experiments are detailed in the online supplement.

Statistical Analysis

Gene expression data were log transformed to produce a normal distribution and stabilize variance. Pearson correlations were performed for correlation analysis assuming linearity (*Aabel*). Differences between groups were

calculated using Student's *t* test for normally distributed data or by Mann-Whitney *U* test for nonparametric data (*Aabel*). *In vitro* data shown were produced in three independent experiments; for gene expression data and luciferase assays treatments were performed in sextuplet within each experiment.

Results

Analyzing our original screen (19) of 14 male patients with Global Initiative for Chronic Obstructive Lung Disease 3–4 COPD and 7 age-matched healthy male control subjects to identify miRNAs associated with disease showed that 26 miRNAs were suppressed at *P* less than 0.01 (Table 1). The most statistically significantly down-regulated miRNAs were miR-144 and miR-499. Only six miRNAs

Table 1. miRNAs Differentially Expressed in the Quadriceps of Patients with COPD Compared with Control Subjects

miRNA	Chromosomal Location	Mean Fold Change COPD/Control	P Value
hsa-miR-499-5p	20q11.22	0.211	0.006
hsa-miR-144#	17q11.2	0.250	0.006
hsa-miR-367	4q25	0.307	0.005
hsa-miR-302b	4q25	0.335	0.002
hsa-miR-31#	9p21.3	0.344	0.008
hsa-miR-126#	9q34.3	0.359	0.001
hsa-miR-374a#	xq13.2	0.374	0.005
hsa-miR-20a#	13q31.3	0.377	0.005
hsa-miR-651	xp22.31	0.383	0.006
hsa-miR-145#	5q32	0.440	0.006
hsa-miR-31	9p21.3	0.449	0.001
hsa-miR-32	9q31.3	0.456	0.002
hsa-miR-302a	4q25	0.477	0.006
hsa-miR-520c-3p	19q13.42	0.514	0.006
hsa-miR-545	xq13.2	0.530	0.001
hsa-miR-190	15q22.2	0.531	0.005
hsa-miR-598	8p23.1	0.571	0.003
hsa-miR-136#	14q32.31	0.585	0.001
hsa-miR-210	11p15.5	0.604	0.004
hsa-miR-150	19q13.33	0.628	0.008
hsa-miR-576-3p	4q25	0.649	0.008
hsa-miR-340	5q35.3	0.683	0.004
hsa-miR-590-5p	7q11.23	0.702	0.004
hsa-miR-652	xq23	0.723	0.007
hsa-miR-30c	1p34.2, 6q13	0.744	0.004
hsa-miR-140-5p	16q22.1	0.786	0.008
hsa-miR-128	2q21.3, 3p21.3	1.731	0.000
hsa-miR-542-3p	xq26.3	4.073	0.000
hsa-miR-424	xq26.3	4.124	0.000
hsa-miR-450a	xq26.3	4.728	0.000
hsa-miR-424#	xq26.3	6.723	0.004
hsa-miR-542-5p	xq26.3	9.617	0.001

Definition of abbreviations: COPD = chronic obstructive pulmonary disease; miR = miRNA; miRNA = microRNA.

were increased in the muscle of patients with COPD, five of which (miR-542-5p, miR-542-3p, miR-424, miR-424*, and miR-450a) came from a single locus on the X chromosome. The most up-regulated miRNA from this cluster was miR-542-5p and the miRNA showing the most significant increase was miR-542-3p. Analyzing the expression of miR-542-3p in a larger cohort of male control subjects and patients with the same inclusion criteria ($n = 12$ control subjects and $n = 24$ patients with COPD) confirmed the elevation of miR-542-3p twofold ($P = 0.012$) (see Figure E1) indicating that the finding was robust.

We had previously shown similar changes in miR-499 in patients with ICUAW (7), so we determined whether miR-542-3p and -5p were increased in samples from the same patients and found that they were markedly more elevated than in the patients with COPD (15-fold and 50-fold, respectively) (Figures 1A and 1B) and correlated with length of hospital stay (Figures 1C and 1D). Moreover miR-542-5p was higher in those with a Sequential Organ Failure Assessment score at the time of biopsy greater than 10 than in patients with a Sequential Organ Failure Assessment score less than 10 (Figures 1E and 1F).

miR-542-3p Promotes Mitochondrial Ribosomal Stress

For these miRNAs to be important regulators of muscle phenotype in disease they must target appropriate biochemical pathways. Bioinformatic analysis using miRwalk (26) showed that miR-542-3p may target components of the mitochondrial ribosome including mitochondrial ribosomal protein (MRP) S2, S10, S18C, S25, S26, and S27, so may induce mitochondrial ribosomal stress, thereby reducing the expression of mitochondrially encoded proteins, which would be expected to reduce mitochondrial function including energy production. Similar analysis showed that miR-542-5p would target inhibitors of the TGF- β signaling pathway, which is known to mediate muscle atrophy, suggesting that it may increase TGF- β signaling. Hence miR-542-3p/5p are predicted to target pathways that affect both muscle mass and function.

Ago-2 pull-down followed by quantitative polymerase chain reaction showed enrichment of all the MRPs tested (MRP S2, S27, S18C, and S10) in RNA isolated from miR-542-3p-transfected cells

Figure 1. MicroRNA-542-3p/5p (miR-542-3p/5p) are increased in patients with intensive care unit-acquired weakness (ICUAW) and are associated with length of ICU stay. miR-542-3p/5p were quantified in quadriceps biopsies from patients ($n = 17$) with established ICUAW and preoperative control subjects ($n = 7$). (A and B) Box-and-whisker plots showing that the expression of miR-542-3p (A) and miR-542-5p (B) are increased in patients compared with control subjects ($P < 0.001$ and $P < 0.001$, respectively; whiskers to 90% with outliers shown). (C and D) In patients with ICUAW, miR-542-3p (C) and miR-542-5p (D) expression were positively correlated with length of ICU stay ($r = 0.58$, $P = 0.014$ and $r = 0.57$, $P = 0.016$, respectively). (E and F) miR-542-5p (F) but not miR-542-3p (E) was increased in patients with a Sequential Organ Failure Assessment (SOFA) score greater than 10 on the day of biopsy compared with those with a SOFA score less than 10. AU = arbitrary units.

compared with cells transfected with control miRNA (see Figure E2A). To determine whether these interactions resulted in reduced MRP and thereby suppressed mitochondrial translation, we quantified the expression of one target (MRPS10) and one product of

mitochondrial protein synthesis (Cytb5). Protein levels of MRPS10 were reduced in response to transfection with miR-542-3p by 50%. Cytb5 protein levels were also reduced but to a much smaller extent (Figures 2A–2C). Transfection with miR-542-3p also reduced the mitochondrial

Figure 2. MicroRNA-542-3p (miR-542-3p) promotes mitochondrial ribosomal stress and inhibits mitochondrial function *in vitro*. LHCN-M2 cells were transfected with scrambled mimic (scr), miR-542-3p (542), or miR-542-3p in the presence of its antagomiR (542 + ant). Transfected miR-mimic levels were kept constant in all experiments by cotransfection with scr. Forty-eight hours later, protein or RNA was extracted or the mitochondrial membrane potential was measured as described in the METHODS section. (A–C) Levels of the miR-542-3p target, MRPS10, and the mitochondrially encoded CytB were lower in miR-542-3p-transfected cells. (D) miR-542-3p suppressed 12S and 16S rRNA compared with scrambled miRNA, and this effect was reversed by the antagomiR. (E) miR-542-3p suppressed mitochondrial membrane potential measured by JC-1 fluorescence ratio, and this effect was reversed by the antagomiR. AU = arbitrary units; MRP = mitochondrial ribosomal protein; rRNA = ribosomal RNA.

membrane potential measured using Mitotracker Red and JC1 (Figure 2E; see Figure E2B). Both effects were reversible by cotransfection with the antagomiR

(Figure 2E; see Figure E2C). There was no effect of the miRNA on the amount of mitochondrial DNA relative to nuclear DNA, indicating that the results

were not caused by a reduction in mitochondrial content.

Ribosome stress induced by reduced small subunit proteins is accompanied by a decrease in ribosomal RNA especially the small subunit RNA (27). Consistent with the induction of ribosome stress, miR-542-3p suppressed the expression of the 12S ribosomal RNA (rRNA) to a greater extent than the 16S rRNA (mitochondrial rRNAs from the small and large subunits, respectively) and these changes were reversible with an antagomiR (Figure 2D).

miR-542-5p Causes Ligand-Independent SMAD2/3 Phosphorylation and Suppresses SMAD7 *In Vitro*

Bioinformatic analysis predicted that miR-542-5p targets several components of the TGF- β family signaling pathway, including SMAD7 and SMURF1 (which inhibit the TGF- β type I receptor [28]), and the proteins that dephosphorylate and inactivate the SMAD2/3 complex (see PPP2CA in the online supplement RESULTS and Figure E3). Ago2 pull-down and quantitative polymerase chain reaction confirmed enrichment for SMURF1 and PPP2CA (see Figure E3A) but SMAD7 mRNA expression was too low to prove targeting.

The effect of miR-542-5p on TGF- β signaling was determined using a SMAD-dependent luciferase reporter (29). In the absence of exogenous ligand, miR-542-5p increased luciferase activity twofold (analysis of variance, $P < 0.001$) (Figure 3B). Furthermore, transfection of cells with a dominant inhibitory form of the TGF- β type II receptor inhibited the effects of the miRNA suggesting that a functional receptor complex was required for the response (Figure 3C). After phosphorylation, SMAD2/3 translocates to the nucleus and, consistent with the increase in basal SMAD-dependent luciferase activity, immunofluorescence assay showed increased nuclear pSMAD2/3 in cells transfected with miR-542-5p compared with scrambled miRNA (analysis of variance, $P < 0.001$) (Figures 3D and 3E; see Figure E4). Western blotting confirmed increased pSMAD2/3 in the presence of miR-542-5p (see Figure E4).

To determine which predicted targets of miR-542-5p were suppressed, the mRNAs

Figure 3. MicroRNA-542-5p (miR-542-5p) increases basal SMAD2/3 activity. (A) LHCN-M2 myoblasts were transfected with miR-542-5p mimic (5p) or scrambled control (scr) and then with CAGA₁₂ luciferase reporter and pRLTK 24 hours later as described in the METHODS section. After 24 hours, the cells were treated with vehicle or 5 ng/ml transforming growth factor (TGF)- β for 2 hours. miR-542-5p increased CAGA₁₂ promoter activity ($P=0.002$, Student's t test) in the absence of TGF- β but did not further increase TGF- β -stimulated CAGA₁₂ promoter activity in the presence of 5 ng/ml TGF- β ($n=6$ samples repeated in two independent experiments). (B) LHCN-M2 myoblasts were transfected with miR-542-5p mimic or scrambled control and then with CAGA₁₂ luciferase reporter, pRLTK, and either pcDNA3 (Cont) or a dominant inhibitory form of the TGF- β type II receptor (DNR) in pcDNA3. Twenty-four hours later, the cells were stimulated with 5 ng/ml TGF- β for 6 hours. Expression of the DNR inhibited both TGF- β and miR-542-5p-induced increases in luciferase activity ($n=6$ samples repeated in three independent experiments). (C and D) LHCN-M2 myoblasts were transfected with miR-542-5p mimic or scrambled control, cultured for 48 hours, and stained for pSMAD2/3 and then treated with TGF- β for 2 hours. (C) Box-and-whisker plot of mean fluorescence in the nuclei from cells transfected with miR-mimic or scrambled control (three independent experiments). (D, left) DAPI staining for nuclei; (D, right) pSMAD2/3 staining only. Color images and a merged view are shown in Figure E4. P values are given for individual Student's t tests between data sets with a Bonferroni corrected P value for significance = 0.0083. AU = arbitrary units; DAPI = 4',6-diamidino-2-phenylindole.

for SMAD7, SMURF1, and PPP2CA were quantified by polymerase chain reaction. miR-542-5p suppressed the expression of all components (Figure 4A). SMAD7 expression was not readily detectable by

Western blotting in LHCN-M2 cells, but was detectable by immunoprecipitation, which demonstrated that miR-542-5p suppressed the expression of SMAD7 (Figures 4B and 4C) suggesting

that SMAD7 is targeted by the miRNA. However, because we could not confirm an interaction between the miRNA and SMAD7 mRNA this reduction may be indirect.

Figure 4. MicroRNA-542-5p (miR-542-5p) suppresses the expression of inhibitors of the transforming growth factor- β signaling pathway. (A) LHCN-M2 myoblasts were transfected with miR-542-5p mimic (5p) or scrambled control (scr), and 48 hours later, RNA was isolated. mRNA for SMURF1, PPP2CA (a catalytic component of PP2A), and SMAD7 were determined. All mRNAs were reduced in miR-542-5p-transfected cells compared with those transfected with scrambled control subjects. Data are from three experiments of $n = 6$ per experiment. SMAD7 was immunoprecipitated from LHCN-M2 myoblasts that were left untransfected or were transfected with miR-mimic or scrambled control and analyzed by Western blotting. Box-and-whisker plot shows mean signal from $n = 4$ independent transfections. AU = arbitrary units.

Overexpression of miR-542 Promotes Mitochondrial Dysfunction, Suppresses SMAD7, and Causes Atrophy *In Vivo*

To determine whether the effects of miR-542-3p and -5p observed *in vitro* could also occur *in vivo* and to determine whether it could cause atrophy, miR-542 was overexpressed in the tibialis anterior of mice by electroporation with positively transfected fibers identified by coexpression of EGFP and compared with expression of EGFP alone. Three days after electroporation, 37% of the fibers expressed detectable EGFP (38% EGFP-only leg and 36% miR-542 leg) (see Figure E5). miR-542-3p levels were 10-fold higher than in the control leg ($P = 0.04$ paired Student's *t* test). The miR-542-expressing muscle was smaller than the contralateral muscle expressing EGFP alone (Figure 5A). The average diameter of transfected, EGFP-expressing fibers in the

miR-542-transfected leg were smaller than the untransfected fibers in the same leg (Figure 5B; see Figure E5A). There was no difference in size between the transfected and untransfected fibers in the muscle of the control-transfected leg, or between the untransfected fibers in the two muscles. Quantification of mitochondrial complex I activity (the first component of the electron transport chain) showed a marked reduction in the muscles expressing the miRNA compared with those expressing the EGFP alone (Figure 5C). Similarly, JC-1 staining of the mitochondria showed a reduction in mitochondrial membrane potential (Figure 5D).

Because the electroporation does not transfect all of the tissue, we enriched the samples by laser capture microdissection to quantify the expression of rRNAs in transfected fibers. This analysis showed a significant reduction in 12S rRNA in fibers expressing EGFP and miR-542 compared

with those expressing EGFP alone that was greater than any reduction in 16S rRNA consistent with mitochondrial ribosomal stress (Figure 5E). Quantification of SMAD7 protein also showed a marked down-regulation of the protein in the miR-542-expressing leg (Figure 5F; see Figure E5).

Patients with ICUAW Show Reduced Expression of 12S rRNA and Inhibitors of TGF- β Signaling

Mitochondrial rRNAs and SMAD7, SMURF1, and PPP2CA were analyzed in patients with ICUAW to determine whether a similar pattern of changes occurred. Both the 12S and 16S rRNAs were reduced but the reduction in 12S rRNA was larger resulting in a significant reduction in the 12S:16S ratio (Figures 6A–6C) consistent with the *in vitro* and *in vivo* findings. Furthermore the expression of SMURF1 and PPP2CA were reduced at the mRNA level (Figure 7A), and both mRNA and protein levels of SMAD7 were reduced (Figures 7B and 7C). Our previous analysis of the ICUAW cohort samples analyzed here showed that patients with established ICUAW have higher nuclear pSMAD2/3 and increased expression of Cyr-61, a TGF- β -responsive gene (7). These data are consistent with mitochondrial ribosome stress and increased TGF- β signaling in the patients.

miR-542-3p/5p Is Associated with Muscle Loss after Cardiopulmonary Bypass Surgery

Half of patients admitted to the ICU after aortic surgery lose more than 10% of their RF_{CSA} over the subsequent 7 days (8). We therefore quantified change in muscle size over 7 days in a separate cohort of 40 patients in whom we also took muscle biopsies immediately before surgery (see Table E3). Nineteen of these patients lost more than 10% RF_{CSA} after surgery. Presurgery expression of miR-542-3p and -5p were weakly associated with presurgery left ventricular ejection fraction (LVEF%) (Figures 8A and 8B).

The preoperative expression of miR-542-3p and -5p was higher in patients who lost more than 10% RF_{CSA} in the 7 days after surgery than in those who lost less than 10% RF_{CSA} (Figures 8C and 8D). Furthermore, preoperative expression of these miRNAs correlated with the amount of muscle lost (Figures 8E and 8F) and

Figure 5. Expression of microRNA-542 (miR-542) causes muscle wasting, mitochondrial dysfunction, and suppression of SMAD7 in mice. The tibialis anterior (TA) muscles of mice ($n = 15$) were electroporated with an expression vector for EGFP alone (pCAGGS) or with an expression vector for EGFP and miR-542 (pCAGGS-542) as described in the Methods section in three batches of five mice. Three days later, the mice were killed and the muscle dissected out and weighed. (A) The weight of the TA normalized to body weight of the muscle expressing miR-542 was reduced compared with that expressing the EGFP alone ($P = 0.016$, $n = 15$ mice, paired Student's t test). (B) Fiber diameter was determined for transfected fibers and nontransfected fibers in each leg from the two sets of mice in which the muscles were embedded for sectioning of mice ($n = 10$). Fibers expressing EGFP and miR-542 were smaller than all other fibers ($P < 0.001$, analysis of variance) and were smaller than those expressing EGFP alone ($P < 0.001$, Student's t test). There was no difference in size of the transfected fibers and the untransfected fibers in the pCAGGS electroporated muscles. Cytoplasmic and mitochondrial extracts were made from the second batch of animals and laser capture microdissection was performed on the third batch of muscle samples. (C and D) Expression of miR-542 caused a reduction in both complex I activity ($n = 4$ per group) (C) and mitochondrial membrane potential (assayed by JC-1 fluorescence, $n = 5$ per group) (D) determined in mitochondria isolated from the TA. (E) The expression of the 12S rRNA, measured in RNA isolated from laser capture microdissected tissue, was markedly reduced in muscle fibers expressing miR-542. (F) Immunoprecipitation of SMAD7 from cytoplasmic extracts showed a reduction in SMAD7 expression in muscles expressing miR-542 compared with those expressing EGFP alone ($P = 0.004$). ANOVA = analysis of variance; AU = arbitrary units; OD = optical density; rRNA = ribosomal RNA.

length of hospital stay (see Figures E6A and E6B). Age and preoperative LVEF% were also associated with muscle loss after surgery (see Figure E7). However, in multiple regression analysis using LVEF%, age, and either miR-542-3p or miR-542-5p expression before surgery, only the miRNAs were retained as having a significant association.

Discussion

ICUAW has a significant impact on patient mortality and morbidity, as well as on the economics of critical illness. A range of different factors (including oxidative stress, sepsis, and growth factor dysregulation [30]) mediate ICUAW by interfering with intracellular processes. Our data show that quadriceps expression of two miRNAs, miR-542-3p/5p, which are predicted to target processes essential for muscle maintenance, is markedly elevated in patients with established ICUAW suggesting that these miRNAs contribute to the wasting process. The *in vitro* studies confirm that miR-542-3p/5p regulate appropriate pathways (mitochondrial function and SMAD2/3 signaling) in a manner that would contribute to wasting. Furthermore, overexpression of miR-542, to a similar fold change as that seen in patients with ICUAW, caused muscle wasting and mitochondrial dysfunction in mice. Finally we show that in patients admitted to the ICU after aortic surgery, the level of expression of miR-542-3p/5p at the time of surgery is associated with muscle wasting in the 7 days after surgery. Consequently, we have demonstrated that the expression of specific miRNAs is associated with wasting in humans, identified mechanisms by which they may promote wasting, and demonstrated that they can cause wasting in mice.

miR-542-3p and Mitochondrial Dysfunction

Reduced mitochondrial number and mitochondrial dysfunction have been previously demonstrated in the quadriceps of patients in the ICU (12). Indeed, mitochondrial dysfunction has been suggested as a mechanism leading to the metabolic failure associated with ICUAW (31). One feature of the reported mitochondrial dysfunction in ICUAW is a reduction in the activity of electron transfer

Figure 6. Expression of mitochondrial ribosomal RNAs (rRNAs) is suppressed in patients with intensive care unit-acquired weakness (ICUAW). 12S rRNA and 16S rRNA were quantified in RNA samples from the quadriceps of patients with ICUAW. Both 12S rRNA and 16S rRNA were lower in patients with ICUAW compared with control subjects (A and B). The reduction in 12S rRNA was larger than in 16S rRNA. Consequently, the 12S:16S ratio was suppressed in patients with ICUAW compared with control subjects, indicative of mitochondrial ribosome stress (C). AU = arbitrary units.

complexes that require mitochondrially translated proteins (notably complex I, III, and IV [11, 12]). Reduced activity of these complexes as a consequence of mitochondrial ribosomal stress is also found in individuals born with mutations in MRPS16 and MRPS22 (32, 33). The identification here of a miRNA that

promotes mitochondrial ribosomal stress (as identified by the reduction in MRPS10, 12S rRNA, and mitochondrial membrane potential), together with the demonstration of a reduction in the 12S to 16S rRNA ratio in patients with established ICUAW suggest two novel findings: that mitochondrial ribosomal stress contributes to metabolic

failure and wasting in ICUAW; and that elevated miR-542-3p is a contributor to this process.

At first glance, there is a discrepancy between our data and that of Fredriksson and coworkers (34), who showed no decrease in mitochondrial protein synthesis in patients with ICUAW. However, in that study they measured protein synthesis from whole mitochondria. Most proteins in mitochondria are encoded by nuclear genes, translated in the cytoplasm and imported into mitochondria with only 13 proteins translated by mitochondrial ribosomes. Consequently, although their experiment demonstrates no change in the synthesis of mitochondrial proteins it does not measure the rate of translation within mitochondria of key components of the complexes involved in oxidative phosphorylation because the bulk of the proteins assayed will have been synthesized in the cytoplasm.

Figure 7. Expression of inhibitors of the transforming growth factor- β signaling pathway are suppressed in intensive care unit-acquired weakness (ICUAW). Expression of PPP2CA, SMURF1, and SMAD7 mRNAs was quantified in quadriceps biopsies from patients with ICUAW (I) and control (C) subjects. All of these inhibitors of the transforming growth factor- β signaling pathway were suppressed in patients compared with control subjects (A). SMAD7 was immunoprecipitated from quadriceps biopsies from patients with established ICUAW or from control subjects (B), and the intensity of the signals was quantified (C). There was a significant reduction in SMAD7 in the quadriceps of the patients with ICUAW. AU = arbitrary units; miRNA = microRNA.

miR-542-5p and TGF- β Signaling

Myostatin is an important regulator of muscle mass that promotes phosphorylation of SMAD2/3. Inhibiting the myostatin pathway and thereby suppressing SMAD2/3 phosphorylation has been suggested as a means of treating ICUAW (www.clinicaltrials.gov reference NCT01321320). However, circulating levels of myostatin did not increase in patients admitted to the ICU after aortic surgery (8). Our studies suggest that increased expression of miR-542-5p contributes to muscle wasting by promoting SMAD2/3 phosphorylation, a down-stream effect of myostatin binding to TGF- β receptors, thereby potentially

Figure 8. Levels of microRNA-542-3p (miR-542-3p) and -5p in the muscle of patients before aortic surgery are associated with postsurgical muscle loss. miR-542-3p and -5p expression was determined in rectus femoris biopsies taken from patients at the time of surgery. (A and B) Both miRNAs were associated with presurgery cardiac function, left ventricular ejection fraction (LVEF%) (miR-542-3p vs. LVEF%: $r = -0.43$, $P = 0.005$) (miR-542-5p vs. LVEF%: $r = -0.34$, $P = 0.031$). (C–F) miR-542-3p and miR-542-5p were higher in patients who lost more than 10% of the rectus femoris cross-sectional area (RF_{CSA}) as determined by ultrasound (C and D) ($P = 0.018$ and $P = 0.023$, respectively) and correlated with the amount of muscle lost over the 7 days (E and F) ($r = 0.43$, $P = 0.006$ and $r = 0.52$, $P < 0.001$, respectively). Open circles show patients who lost more than 10% RF_{CSA} , solid circles show patients who lost less than 10% RF_{CSA} . AU = arbitrary units.

bypassing the need for significantly elevated myostatin. The effect of the dominant inhibitory receptor, together with inability of miR-542-5p to increase luciferase activity in the presence of 5 ng/ml TGF- β , indicate

that miR-542-5p is not acting via a separate pathway because the effects are not additive.

Our data indicate that miR-542-5p increases pSMAD2/3 by suppressing the

inhibitory components of the TGF- β signaling pathway with a reduction in the expression of the SMAD7 and SMURF1, inhibitors of TGF- β type I receptors, and of the phosphatases that limit TGF- β signaling by dephosphorylating and inactivating SMADs. Our observation that the increase in luciferase activity in the absence of exogenous ligand was inhibited by the dominant inhibitory receptor suggests that it occurs as a result of either low-level receptor kinase activity in the absence of ligand or a sensitization of the signal to the low levels of TGF- β receptor ligands present in the serum used to culture the cells or produced by the cells themselves.

Our previous analysis of the samples from the ICUAW cohort analyzed here showed that patients with established ICUAW have higher nuclear pSMAD2/3 and increased expression of Cyr-61, a TGF- β -responsive gene (7). Here we show that these patients also have increased miR-542-5p, alongside reduced expression of SMAD7, SMURF1, and PPP2CA, suggesting that these factors contribute to the observed increases in TGF- β signaling.

miR-542-5p is not the only miRNA from this locus to target components of the TGF- β signaling pathway. For example, miRNAs from the miR-424-503 polycistron reduce SMAD7 and promote TGF- β signaling (35). Furthermore, because these miRNAs are also regulated by TGF- β signaling it is possible that they form part of a positive feedback loop (36). It seems likely therefore that increased expression of these miRNAs also contributes to elevated TGF- β signaling and atrophy in ICUAW.

miR-542 and Muscle Atrophy

The combined effects of miR-542 on mitochondrial function and TGF- β signaling suggest that it contributes to muscle wasting and dysfunction. We confirmed the potential of these miRNAs to promote wasting by overexpression in the muscle of mice. This overexpression caused marked muscle loss within 3 days that was accompanied by mitochondrial dysfunction and reduced SMAD7 expression, demonstrating the potential of miR-542 to drive atrophy. This loss of muscle is larger and more rapid than that achieved by overexpression of myostatin or GDF-15 using the same promoter as used here (9, 37).

Clinical Implications

The data presented here identify miR-542-3p/5p as potential therapeutic targets in patients with ICUAW. Therapy could take the form of antagomiRs for miR-542-3p/5p or the suppression of the mechanisms that drive the expression of the miRNAs. The data also show that some aspects of the propensity to waste are established in patients before they become critically ill because patients with the highest preoperative miR-542 levels wasted more than those with the lowest, raising the possibility that a therapy could be given to vulnerable patients before major surgery. In addition, preoperative miR-542 levels could be used to stratify patients in high or lower risk of perioperative and postoperative muscle atrophy so that high-risk patients could have intensive nutritional and rehabilitation input. Because we identified miR-542 as being elevated in COPD and because the expression of this miRNA was associated with LVEF in our aortic surgery patients, it

is possible that this miRNA locus contributes to muscle wasting in a wider range of conditions but that its effects are greatest with the insults imposed as a result of critical illness. Contributing to both chronic and acute wasting is not without precedent because many pathways including GDF-15 and insulin-like growth factor-1 have been associated with both chronic and acute wasting (8–10). Furthermore, the pathways leading to muscle loss are common with a suppression of protein synthesis relative to degradation and mitochondrial dysfunction being end components.

Critique of the Study

The study presents a cross-sectional analysis demonstrating elevation of miR-542-3p/5p in ICUAW and a longitudinal study showing an association of these miRNAs with muscle loss after surgery. We cannot therefore directly demonstrate that elevation of the miRNA causes either the reduction in oxidative capacity or promotes the loss of

muscle mass in humans. However, the demonstration that the miRNAs cause appropriate biochemical changes *in vitro*, muscle wasting in mice, and the demonstration of appropriate phenotypic manifestations in humans strengthens our conclusions.

Conclusions

Our data indicate that expression of miR-542-3p/5p is elevated in patients with ICUAW. These miRNAs target the synthesis of MRPs and of inhibitors of TGF- β signaling. In mice their overexpression promotes mitochondrial dysfunction and muscle wasting. Elevation of these miRNAs in the muscle of patients with ICUAW is a very plausible mechanism contributing to the loss of oxidative capacity and muscle wasting observed in this condition. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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