RESEARCH ARTICLE | Translational Control of Muscle Mass

Quadriiceps miR-542-3p and -5p are elevated in COPD and reduce function by inhibiting ribosomal and protein synthesis

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INTRODUCTION

A reduction in muscle mass and strength occurs both in response to chronic disease and as part of normal human aging (9), and loss of muscle function reduces quality of life by limiting ability to perform normal daily tasks and is associated with increased mortality (7, 36). One chronic disease for which skeletal muscle wasting is a common comorbidity is chronic obstructive pulmonary disease (COPD) (19), and similarly, in this condition, wasting and reduced muscle function are associated with poorer quality of life and increased mortality (8, 35).

Muscle function is affected both by the amount of muscle, which predominantly affects strength (12), and its oxidative capacity, which predominantly affects endurance. Muscle oxidative capacity is dependent on the number and function of mitochondria in the tissue (31). In COPD patients, there is a reduction in both muscle mass (33) and mitochondrial oxidative capacity (13, 20). The loss of muscle mass arises from an imbalance in protein turnover with a relative increase in protein breakdown compared with protein synthesis, and diverse studies have reported changes in components of both degradative and synthetic pathways, but, taken together, these data do not convey a consistent conclusion suggesting that there are multiple factors that regulate mass (15).

There are also a number of studies examining changes in mitochondria in the muscle of COPD patients. Together they show both reduced mitochondrial density and mitochondrial dysfunction. The factors contributing to reduced mitochondrial density include reduced mitochondrialogenesis with lower levels of proliferator-activated receptor-γ coactivator-1α and mitochondrial transcription factor A. The molecular mechanisms...
leading to mitochondrial dysfunction are less clear. Reduced activity of electron transfer complexes that contain mitochondrial encoded proteins has been reported (37) as have changes in the respiratory control ratio (29). Consistent with these observations, a recent transcriptomic analysis of COPD muscle showed marked reduction in the expression of mitochondrial genes with weighted gene correlation network analysis identifying a mitochondrially enriched gene module as a major factor associated with pulmonary function and exercise capacity (41). Changes were also seen in mitochondrial dysfunction associated with aging and in patients with intensive care unit-acquired weakness (ICUAW) (3, 14, 37).

One mechanism by which this change in the activity of mitochondrial complexes that contain mitochondrial encoded proteins may occur is through the inhibition of mitochondrial translation. We recently showed that the microRNA (miRNA) miR-542-3p inhibited the expression of mitochondrial ribosomal proteins, reduced mitochondrial rRNA in particular the 12S rRNA leading to a reduction in the 12S:16S mitochondrial rRNA ratio, and reduced mitochondrial membrane potential (11). This miRNA also targets cytoplasmic ribosomal proteins suggesting that it would reduce maximal protein synthesis so may contribute to a loss of muscle mass. Elevation of this miRNA was observed in a small cohort of patients with severe COPD, but the association of this miRNA with muscle function in COPD patients was not analyzed further in our prior report (11). We also did not quantify the expression of mitochondrial RNAs to determine whether there was evidence for mitochondrial ribosomal stress in COPD patients.

The current study therefore was designed to investigate the expression of miR-542-3p/5p in the quadriceps of a second, larger cohort of COPD patients, compare it to physical performance, and quantify the expression of mitochondrial rRNAs. The expression of these miRNAs was determined in the muscle of healthy older individuals to identify any association with muscle dysfunction in the absence of overt disease. We also determined the effect of miR-542-3p on cytoplasmic protein synthesis.

METHODS

Subjects

All subjects gave informed consent before inclusion in the relevant study, which was approved by the appropriate ethical review bodies as detailed for each cohort. All procedures were carried out in accordance with the Helsinki Declaration.

COPD cohort. COPD subjects (n = 52) and controls (n = 16) were part of a larger study described by Natanek et al. (23). The patients had COPD according to the Global Initiative in Obstructive Lung Disease (GOLD) guidelines 2004 (30) and were enrolled from clinics at the Royal Brompton Hospital. Patients with a diagnosis of heart, renal, or liver failure, a systemic inflammatory or metabolic disorder, or a moderate/severe exacerbation (i.e., requiring antibiotics, oral steroids, or hospitalization) in the preceding 4 wk were excluded. Healthy age-matched controls (16 in total) were recruited by advertisement. All subjects gave written informed consent and the protocol was approved by the Royal Brompton and Harefield National Health Service Trust Research Ethics Committee (Studies 06/Q0404/35 and 06/Q0410/54). Measurements of lung volume, using plethysmography; carbon monoxide transfer factor, using the single breath technique (Compact-Lab, Jaeger, Germany); and postbronchodilator spirometry were performed according to American Thoracic Society/European Respiratory Society guidelines (40). Blood gas tensions were measured in arterialized capillary earlobe blood. Bioelectrical impedance was used to determine fat-free mass index (FFMI; Bodystat 1500, Bodystat, UK) as described previously (34).

Quadricess strength was determined by measuring supine isometric maximal voluntary contraction (MVC) as described previously (35) and physical performance measured as 6-min walk distance, 5 min after bronchodilator treatment [American Thoracic Society 2002 guidelines (1)]. Vastus lateralis muscle biopsies were obtained under local anesthesia by percutaneous needle biopsy of the in the mid thigh with the Bergstrom technique (2).

Hertfordshire Sarcopenia Study cohort. The study protocol was approved by the Hertfordshire Research Ethics Committee (study 07/ Q0204/68) and all participants gave written informed consent. Muscle mass and strength together with gait speed and a timed-up-and-go (TUG) test were ascertained; Hertfordshire Sarcopenia Study (HSS) methods have been previously described (25). Muscle biopsy was performed by percutaneous conchotome biopsy of the vastus lateralis under local anesthesia (24).

RNA Analysis

Quantification of rRNA. RNA was extracted using Trizol, and transcripts were reverse transcribed into cDNA using random primers using the Quantitect RT Kit (Qiagen) and quantified by real-time qRT-PCR as described previously using the primers described in Table 1 (10). rRNA expression was normalized to hypoxanthine phosphoribosyltransferase (HPRT) expression (human samples) or to the geometric mean of HPRT and β2-microglobulin (cell culture samples) in the same sample using the ΔΔCt method.

Quantification of miRNAs. miRNAs were reverse transcribed using MultiScribe Reverse Transcriptase and Megaplex RT Primers (human pool A, Version 3.0; Applied Biosystems) according to the manufacturer’s instructions. The samples were heated to 85°C for 5min to terminate the reactions then stored at −80°C. The cDNAs were preamplified using Megaplex PreAmp Primers (Applied Biosystems) for 12 cycles of 95°C for 15 s and 60°C for 4 min, and the reactions were terminated by heating to 99.9°C for 10 min. The resulting cDNA was diluted to 100 μl by addition of 0.1× TE buffer pH 8.0 (Qiagen) and stored at −80°C. Primers and probes for miR-542-3p and miR-542-5p were purchased for each test gene from Applied Biosystems, and amplification was carried out according to the manufacturer’s instructions. Each reaction was performed in duplicate, and the average Ct value was normalized to the corresponding geometric mean of U6 and RNU48 using the ΔΔCt method. RNA isolated from cells was analyzed using single RT reactions.

Table 1. Primers used in this study

| Target | Forward | Reverse |
|--------|---------|---------|
| 12S    | GCTCTGCCATGTTTTTCATTGCTG | GCTCACTTTTATTCTTTCCAGTCTG |
| 16S    | GCCATCTTACCTTTTACCTGTTT | CACCTTGGTATTCTTCTCCT |
| HPRT   | GCTCTGCTGCTGCTGCTGCTGCTG | GCTCTGCTGCTGCTGCTGCTGCTG |
| β2-Microglobulin | TGCTCTGCTGCTGCTGCTGCTG | TCTGTGCTGCTGCTGCTGCTG |

HRPT, hypoxanthine phosphoribosyltransferase.

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Cell Culture

HLHCN-M2 cells were maintained in skeletal muscle growth medium (PromoCell) supplemented with 20% FCS, as previously described (42). All experiments were performed on cells as myoblasts. RNA was extracted using Trizol; mRNA and miRNAs were quantified as described above.

miRNA transfection. Six-thousand two-hundred and fifty cells were seeded into each well of a 96-well plate. After 24 h, cells were transfected with mirVana miRNA mimics with each well treated with a mixture containing 0.5 μl of 20 μM mirVana miRNA mimic(s) and 0.5 μl Lipofectamine 2000 (ThermoFisher) prepared according to the manufacturer’s instructions.

RNA extraction. Forty-eight hours after microRNA transfection, RNA was extracted from HLHCN-M2 cells using the CellAmp Direct RNA Prep Kit (TaKaRa) according to the manufacturer’s instructions. miRNA and rRNA were analyzed as described above.

Protein Analysis

Western blotting. Western blotting for RPS23 was carried out as previously described (18) but using rabbit anti-RPS23 (Novus) at 1:100.

Protein synthesis. Forty-eight hours after microRNA transfection, HLHCN-M2 cells were serum and leucine-starved by incubation in leucine-free DMEM. Two hours later, the medium was replaced with leucine-containing DMEM supplemented with 130 nM of IGF-1 (Cambridge Bioscience), and the cells were incubated for 45 min before addition of 100 ng/ml puromycin (Sigma) and harvested 30 min later, and protein was extracted. Three-hundred nanograms of the extracted protein were diluted in 200 μl of PBS containing 5% BSA (wt/vol; PBS-BSA) for 30 min at room temperature. The solution was replaced with 100 μl of 100 ng/ml anti-puromycin (Millipore) diluted in PBS-BSA and the samples were incubated for 1 h at room temperature. After being washed two times in PBS, 100 μl of sheep anti-mouse (GE Healthcare) was added at a 1/10,000 dilution in PBS-BSA. The samples were washed four times in PBS before 100 μl of TMB substrate (Sigma) was added, and the samples were incubated for 15 min. The reaction was stopped with 100 μl of stopping solution (Sigma), and absorbance was determined at 450 nm.

Statistical Analysis

Gene expression data from human studies were log transformed to stabilize variance and produce a normal distribution. Gene expression data from in vitro studies were normalized to the mean value for the appropriate control data set. Statistical analysis was performed in Aabel (Gigawiz). Pearson correlations were used to identify linear correlations after visual inspection of scatter plots. Differences between groups were calculated by Student’s t-test for normally distributed data and by Mann-Whitney U-test for non-parametric data. Differences between multiple groups were identified by ANOVA with post hoc testing using a Bonferroni-Dunn correction.

Bioinformatic Analysis

The quadriceps gene expression profiles of the majority of the patients included in this study have been published previously (n = 41 patients and n = 12 controls) (41). Correlation analysis was performed within the WGCNA R package using robust biweight midcorrelation to determine the correlation coefficients. Gene set enrichment analysis was then performed to identify groups of genes associated with the miRNAs comparing the correlation coefficients for genes that were significant at P < 0.02. To determine the association of the miRNAs with miRNAs for mitochondrial ribosomal proteins, the geometric mean of the target proteins individually and for all detected mitochondrial small ribosomal proteins was calculated and compared with miR-542 expression in the same sample.

RESULTS

Patient Demographics

By design and consistent with a diagnosis of COPD, the patients had poorer lung function than controls by all measures. The patient cohort included 11 patients with GOLD 1–2, 25 patients with GOLD 3, and 16 patients with GOLD 4 COPD. There was no difference in FFM1 between patients and controls, but patients had poorer exercise performance measured both as 6-min walk distance and quadriceps MVC. The patients also had lower daily activity levels than the controls. The demographic data are presented in Table 2.

Expression of miR-542-3p/5p in the Quadriceps of Patients with COPD

The expression of miR-542-3p/5p was determined in a cohort of COPD patients (both sexes and all GOLD grades) and controls. Both miRNAs were elevated in the muscle of COPD patients compared with controls (542-3p: 5.5-fold, P < 0.001 and 542-5p: 5.9-fold, P < 0.001; Fig. 1, A and B) and highly correlated with each other (r = 0.95, P < 0.001). Quadriceps expression of these miRNAs was inversely associated with lung function measured either as transfer capacity of the lung for CO percent predicted (TLCO%) (542-3p: r = −0.60, P < 0.001 and 542-5p: r = −0.62, P < 0.001 for the whole cohort; and 542-3p: r = −0.44, P = 0.001 and 542-5p: r = −0.42, P = 0.002, for the patients alone; Fig. 1, C and D) or as forced expiratory volume in 1 s in percent predicted (FEV1, %) 542-3p: r = −0.54, P < 0.001 and 542-5p:

| Table 2. Physiological characteristics of the COPD cohort |
|-----------------|-----------------|-----------------|-----------------|
|                  | Control (n = 16) | COPD (n = 52)   |
| Sex (male/female)| 7, 9            | 30, 22          |
| Age, yr          | 66 ± 8          | 66 ± 8          |
| Smoking history,*| pack-yr         | 0 (0, 10)       |
|                  |                 | (30, 60)***     |
| Weight,* kg      | 67 (61, 74)     | 66 (59, 77)     |
| BMI,* kg/m2      | 24.8 (23.5, 26.2)| 23.3 (21.4, 26.2)|
| FFMI* kg/m2      | 16.1 (15.3, 17.2) | 15.3 (14.5, 16.8)|
| FEV1,* %pred     | 107.6 (100.6, 112) | 40.7 (27.2, 48.4)*** |
| RV TLC           | 38 ± 6          | 59 ± 8***       |
| TLCO,* %pred     | 92.3 (83.0, 98.1) | 45.0 (32.1, 52.4)*** |
| 6-Min walk, m    | 621 ± 84        | 387 ± 125       |
| 6-Min walk, %pred| 126 ± 12        | 76 ± 24***      |
| PVO2,* %pred     | 99 (88, 111)    | 46 (38.58)***   |
| SGRQ*            | 2 (0.8)         | 54 (45.61)***   |
| Quadriceps MVC, kg| 34.7 ± 10.6    | 28.2 ± 8.9*     |
| Quadriceps MVC, %pred | 78 ± 19          | 63 ± 14**       |
| Locomotion time,*| 96 (84, 127)    | 41 (26, 56)***  |
| min/12 h         | 23 ± 6          | 13 ± 5***       |

Values are means ± SE or median (interquartile range). BMI, body mass index; FEV1, forced expiratory volume in 1 s; RV TLC, ratio of the reserve volume to total lung capacity; TLCO, transfer capacity of the lung for CO; SGRQ, St George’s respiratory questionnaire; MVC, maximal voluntary contraction. *P < 0.05, **P < 0.01, ***P < 0.001. Significance determined by Student’s t-test except for variables denoted *, where P values were calculated by Mann Whitney U-test.
miR-542 IN THE CONTROL OF MITOCHONDRIAL AND CYTOPLASMIC PROTEIN SYNTHESIS

Fig. 1. Expression of the miR-542-3p and miR542-5p is increased in the quadriceps of chronic obstructive pulmonary disease (COPD) patients and associated with disease severity. miR-542-3p and miR-542-5p were quantified in muscle biopsies from patients with COPD (n = 52) and controls (n = 16). A and B: miR-542-3p (A) was elevated 5.5-fold (P < 0.001) and miR-542-5p (B) was elevated 5.9-fold (P < 0.001). AU, arbitrary units. C–F: comparison of the expression of both microRNAs (miRNAs) with measurements of lung function transfer factor for CO percent predicted (TLCO%; C and D) and forced expiratory volume in 1 s in percent predicted (FEV1%; E and F) showed that expression of these miRNAs in the quadriceps was associated with disease severity. miRNA expression was normalized to the expression of U6 and RNU48 in the same samples. Data were analyzed by t-test and Pearson correlations shown are for the entire cohort. Patients are shown as closed circles and controls as empty circles.

r = −0.59, P < 0.001 for the whole cohort; and 542-3p: r = −0.38, P = 0.006 and 542-5p: r = −0.29, P = 0.035, for the patients only; Fig. 1, E and F). Furthermore, the miRNAs were inversely proportional to exercise performance measured as 6-min walk distance expressed, as percent predicted, in both the whole cohort (542-3p: r = −0.58, P < 0.001 and 542-5p: r = −0.59, P < 0.001; Fig. 2, A and B) and in the patients alone (542-3p: r = −0.037, P = 0.006 and 542-5p: r = −0.35, P = 0.012). Although both miRNAs were weakly inversely associated with strength in the patients and controls considered together (measured as MVC% predicted, 542-3p: r = −0.37, P = 0.002 and 542-5p: r = −0.43, P < 0.001; Fig. 2, C and D) and FFMI (542-3p: r = −0.27, P = 0.028 and 542-5p: r = −0.26, P = 0.034; Fig. 2, E and F), neither miRNA was associated with strength in the patients alone (542-3p: r = −0.22, P = 0.109 and 542-5p: r = −0.27, P = 0.054) or FFMI (542-3p: r = −0.177, P = 0.210 and 542-5p: r = −0.172, P = 0.224). The associations did not differ significantly between men and women (data not shown).

To further determine whether the expression of these miRNAs was also associated with muscle function in healthy seniors, we also measured the expression of the miRNAs in samples from the HSS (demographics in Table 3). In normal healthy older men from the HSS, both miRNAs were associated with poorer physical performance as indicated by 3-min walk time and time to up and go 6-min TUG test (Fig. 3, A–D). Although neither miRNA was associated with FFMI in the HSS participants, both were elevated in those defined as having sarcopenia (Fig. 3, E and F).
Do COPD Patients Show Evidence of Mitochondrial Ribosomal Stress?

In patients with established ICUAW the marked elevation of miR-542-3p was associated with a significant reduction in 12S rRNA levels compared with 16S rRNA levels indicative of mitochondrial ribosomal stress (11). To determine if increased miR-542-3p in the quadriceps of COPD patients was associated with evidence of mitochondrial ribosomal stress in COPD, we quantified the expression of 12S and 16S rRNA in the patients with COPD (*n*/H11005 40) and controls (*n*/H11005 14) for which we had appropriate cDNAs. There was a reduction in both the 12S and 16S rRNAs that was larger in the 12S rRNA resulting in a significant reduction in the 12S:16S ratio (Fig. 4).

miR-542 Expression Is Associated with Mitochondrial Gene Expression and P53 Activity in Patients

We have previously shown that overexpression of miR-542 leads to a reduction in the 12S:16S ratio in cells in culture and in the muscle of mice (11). This observation suggests that increased miR-542 in the muscle of patients with COPD may contribute to the loss of mitochondrial function in COPD.
muscle. To determine whether miR-542 was associated with mitochondrial gene expression, the recently published array data (41) from samples included in this study was compared with the miRNA expression reported here. Biweight midcorrelation coefficients were calculated for all detected genes using the WGCNA R package and correlations that reached a statistical significance of 0.02 were used for gene set enrichment analysis. The gene set most negatively associated with miR-542 was mitochondrial genes, and this observation was true whether the analysis was performed using the patients only or the whole cohort (Table 4). The gene sets positively correlated with miR-542 included sets associated with the inflammatory response raising the possibility that some aspect of inflammatory signaling contributes to the expression of miR-542 (Table 4). However, there was no simple association of miR-542 expression with inflammatory cytokines (IL-2, IL-6, IL-8, or TNF-α) or with global DNA bound NF-κB p50 or p65 indicating that these cytokines alone are not sufficient to explain the association of miR-542 with disease severity (data not shown). The positively correlated gene sets also included the gene set for P53 activity. As mitochondrial ribosomal stress has been shown to promote P53 activation this observation is consistent with miR-542-driven mitochondrial ribosomal stress activating P53.

Does miR-542-3p Inhibit Protein Synthesis?

miR-542-3p inhibits the expression of a number of proteins that make up the cytoplasmic small ribosomal subunit promoting cytoplasmic ribosomal stress and reducing 18S rRNA expression (39). Consequently, this miRNA should suppress maximal protein synthesis. We therefore confirmed the reduction in RPS23 and 18S rRNA in muscle cells (Fig. 5, A and B) and determined the effect of miR-542-3p on leucine- and IGF-1-stimulated proteins synthesis by quantifying puromycin incorporation. Transfection of LHCN-M2 muscle myoblasts with miR-542-3p
decreased puromycin incorporation (Fig. 5C) suggesting that miR-542-3p inhibited the formation of the small ribosomal subunit and inhibited protein synthesis.

DISCUSSION

The data presented here show that increased expression of miR-542-3p and miR-542-5p is associated with muscle dysfunction in COPD patients. Quadriceps expression of these miRNAs is associated with lung function measured as either TLCO% predicted or FEV1% predicted. Consistent with promotion of mitochondrial ribosome stress by miR-542-3p, expression of the 12S rRNA was reduced. Furthermore, the expression of miR-542-3p/5p was inversely associated with the expression of mitochondrial genes and positively associated with the genes associated with activation of the p53 pathway, consistent with an association with mitochondrial dysfunction and ribosomal stress. We also show that miR-542-3p can reduce RPS23 and 18S rRNA expression and inhibit protein synthesis in muscle cells. Taken together, the data suggest that disease situated in the lung promotes the muscle expression of miR-542-3p/5p where it promotes mitochondrial dysfunction and inhibits protein synthesis. The association of miR-542-3p/5p with inflammatory signaling raises the possibility that in this context inflammatory mediators released as part of, or in response to, lung disease promote miR-542-3p/5p expression but the lack of correlation of miRNA expression with the classical mediators of inflammatory signaling mean that the factors underlying this association remain to be identified. The promoter that controls the expression of miR-542 has yet to be confirmed but the close proximity of this miRNA to the miR-424-503 host gene suggests that these genes are coexpressed from the same promoter. This suggestion is supported by relatively tight correlation of the expression of miR-542 expression with miR-424 in all the studies we have performed. This region of DNA contains multiple binding sites for STAT, NF-κB, and glucocorticoid receptors suggesting that it functions as part of a stress response system. An alternative possibility for the elevation of these miRNAs in muscle is delivery from another tissue via exosomes. However, this route would require sufficient production of these miRNAs and exosomes in an organ to increase the levels in muscle sixfold and where skeletal muscle accounts for ~40% of the body mass.

Table 4. Gene set enrichment for genes associated with miR-542

| Hallmark                              | NES   | NOM P Value | FDR Q Value |
|---------------------------------------|-------|-------------|-------------|
| Positive                              |       |             |             |
| Epithelial mesenchymal transition     | 4.212 | 0.000       | 0.000       |
| Interferon gamma response             | 2.879 | 0.000       | 0.000       |
| IL2 STAT5 signaling                   | 2.716 | 0.000       | 0.000       |
| Inflammatory response                 | 2.621 | 0.000       | 0.000       |
| Apoptosis                             | 2.616 | 0.000       | 0.001       |
| Coagulation                           | 2.605 | 0.000       | 0.001       |
| Complement                            | 2.421 | 0.000       | 0.002       |
| TNFα signaling via NF-κB              | 2.087 | 0.006       | 0.013       |
| UV response Dn                        | 2.054 | 0.002       | 0.014       |
| Hypoxia                               | 1.913 | 0.008       | 0.022       |
| Apical junction                       | 1.775 | 0.020       | 0.042       |
| P53 pathway                           | 1.767 | 0.028       | 0.040       |
| KRAS signaling up                     | 1.762 | 0.015       | 0.038       |
| Negative                              |       |             |             |
| Oxidative phosphorylation             | −5.046| 0.000       | 0.000       |
| Fatty acid metabolism                 | −3.058| 0.000       | 0.000       |
| Adipogenesis                          | −2.231| 0.000       | 0.002       |

NES, normalized enrichment score; NOM, nominal; FDR, false discovery rate.
Significance of the Findings

A significant proportion of COPD patients report leg fatigue as a more important locus of exercise limitation than breathlessness (16, 28). A well-recognized cause of this early fatigue is a reduction in mitochondrial function and oxidative capacity. One intriguing component of this reduction in oxidative capacity is a greater reduction in the activity of mitochondrial complex I and IV, which require translation to occur both in the cytoplasm and mitochondria, compared with complex II which only requires translation in the cytoplasm (37). Consequently, a relative reduction in translation in mitochondria would lead to a greater reduction in the activity of complexes I and IV. This reduction in activity of electron transfer components requiring mitochondrial translation is also seen in sarcopenia where an accumulation of mutations in the mitochondrial genome has been suggested to contribute (4, 38). Our previous study showing that miR-542-3p suppressed the expression of mitochondrial ribosomal proteins and the 12S rRNA provided an additional mechanism by which mitochondrial translation can be seen via the occasional gene mutations in humans causing an inability to synthesize the small subunit of the mitochondrial ribosome (21, 32). These mutations have a similar, but more marked, effect on the electron transfer chain and on 12S rRNA levels to that observed here and ICUAW.

This mechanism does not appear to be restricted to COPD patients as we have also demonstrated similar effects in patients with ICUAW (11), in whom there is also preferential loss of complex I and complex IV activity. Taken together, these data suggest that changes mediated by miR-542-3p are not disease specific but a more general pathophysiologic process. In its most extreme form, the effects of inhibition of mitochondrial translation can be seen via the occasional gene mutations in humans causing an inability to synthesize the small subunit of the mitochondrial ribosome (21, 32). These mutations have a similar, but more marked, effect on the electron transfer chain and on 12S rRNA levels to that observed here and ICUAW.

The observation that miR-542-3p-mediated muscle dysfunction can complicate ICUAW, COPD, and indeed, “normal” aging suggests a currently unknown mechanism by which diverse etiologies can trigger a single underlying molecular mechanism. Immobility (or more specifically contractile history) would be one candidate since it is present to some extent in all three conditions. However, we did not see any association of miR-542-3p expression with daily physical activity. Other possible candidates might include inflammation that is present in the intensive care unit and at least episodically in COPD (at time of acute exacerbation), and as demonstrated in this study, there is an association of miR-542 expression with markers of the activation of inflammatory signaling in the muscle but no association with classic inflammatory cytokines. Identification of the mechanisms leading to increased expression of miR-542-3p would be insightful and could offer a novel therapeutic approach to this condition.
**Critique of the Method**

Even though we showed that miR-542-3p can suppress maximal protein synthesis in vitro, expression of this miRNA was not strongly associated with FFMI. There are a number of likely reasons for this lack of association. First, FFMI is not a good tool for detecting quadriiceps weakness in COPD (22), presumably because the muscle wasting observed in COPD is regional in nature. Second, there are multiple inputs into the regulation of protein synthesis and the increase of miR-542-3p would have to be one of the predominant regulators for a significant association to be observed. For example, we have already shown that an miRNA from the same locus as miR-542 (miR-424-5p) suppresses the expression of rRNAs by targeting PolR1A. The data are also consistent with mitochondrial dysfunction. The data are also consistent with mitochondrial dysfunction. The data are also consistent with mitochondrial dysfunction.

In conclusion, our data identify targeting of both cytoplasmic and mitochondrial ribosomal proteins by miR-542-3p as contributing to the loss of physical performance in patients with COPD. This reduction in ribosomal proteins reduces the availability of small ribosomal subunits inhibiting protein synthesis in each compartment. The expression of these miRNAs is elevated in response to components of the disease process that are known to cause tissue hypoxia, and in vitro data reported here confirm this as a plausible mechanism.

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In conclusion, our data identify targeting of both cytoplasmic and mitochondrial ribosomal proteins by miR-542-3p as contributing to the loss of physical performance in patients with COPD. This reduction in ribosomal proteins reduces the availability of small ribosomal subunits inhibiting protein synthesis in each compartment. The expression of these miRNAs is elevated in response to components of the disease process that are known to cause tissue hypoxia, and in vitro data reported here confirm this as a plausible mechanism.
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