RESEARCH ARTICLE | Translational Physiology

Upregulation of circulating myomiR following short-term energy restriction is inversely associated with whole body protein synthesis

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Margolis LM, Rivas DA, Pasiakos SM, McClung JP, Ceglia L, Fielding RA. Upregulation of circulating myomiR following short-term energy restriction is inversely associated with whole body protein synthesis. Am J Physiol Regul Integr Comp Physiol 313: R298–R304, 2017. First published June 28, 2017; doi:10.1152/ajpregu.00054.2017.—The objective of the present investigation was to determine whether energy restriction (ER) influences expression of skeletal muscle–specific microRNA (miRNA) in circulation (c-myomiR) and whether changes in c-myomiR are associated with rates of whole body protein synthesis. Sixteen older (64 ± 2 yr) overweight (28.5 ± 1.2 kg/m²) men enrolled in this 35-day controlled feeding trial. A 7-day weight maintenance (WM) period was followed by 28 days of 30% ER. Whole body protein turnover was determined from [15N]glycine enrichments in 24-h urine collections, and c-myomiR (miR-1-3p, miR-133a-3p, miR-133b, and miR-206) expression was assessed from serum samples by RT-quantitative PCR upon completion of the WM and ER periods. Participants lost 4.4 ± 0.3 kg body mass during ER (P < 0.05). After 28 days of ER, miR-133a and miR-133b expression was upregulated (P < 0.05) compared with WM. When all four c-myomiR were grouped as c-myomiR score (sum of the median fold change of all myomiR), overall expression of c-myomiR was higher (P < 0.05) at ER than WM. Backward linear regression analysis of whole body protein synthesis and breakdown and carbohydrate, fat, and protein oxidation determined protein synthesis to be the strongest predictor of c-myomiR score. An inverse association (P < 0.05) was observed with ER c-myomiR score and whole body protein synthesis (r = −0.729, r² = −0.530). Findings from the present investigation provide evidence that upregulation of c-myomiR expression profiles in response to short-term ER is associated with lower rates of whole body protein synthesis.

energy deficit; weight loss; whole body protein turnover; microRNA

THE NEED FOR ADULTS ≥60 yr old to participate in weight loss interventions has become increasingly more common, as 35% of this population is considered obese (16). Although weight loss improves metabolic and cardiovascular health, over time it can also result in undesirable reductions in skeletal muscle mass (53). The loss of muscle mass during energy restriction (ER; e.g., weight loss) is likely attributed, in part, to a dimin-ished rate of whole body and skeletal muscle protein synthesis (3, 35, 48). Reductions in protein synthetic rate during ER are due to the use of endogenous protein stores in energy-yielding processes and gluconeogenesis, rather than anabolism, resulting in a net negative protein balance (12). For an older population that may already be experiencing age-associated losses in muscle mass (e.g., sarcopenia), lower rates of protein synthesis with ER may further compromise skeletal muscle mass and physical function (52).

Circulating microRNA (c-miRNA), small noncoding RNA present in blood, have been identified as potential noninvasive markers of physiological adaptations within skeletal muscle (2, 32). Biogenesis of miRNA occurs in the nucleus of cells, where pre-miRNA is exported into the cytoplasm to become mature miRNA (30). From the cytoplasm, mature miRNA can be released into the circulation either actively in membrane-derived vesicles or passively in apoptotic bodies (49). Although the exact mechanism by which c-miRNA influence cellular processes remains elusive, it has been observed that alterations in c-miRNA profiles reflect the underlying physiological condition of the tissue (1, 32). Upregulation of circulating skeletal muscle–specific miRNA (c-miRNA; miR-1-3p, miR-133a-3p, miR-133b, and miR-206) has been consistently reported in disease states that are known to blunt the rate of protein synthesis and diminish skeletal muscle mass, such as metabolic acidosis and Duchenne muscular dystrophy (9, 43), compared with healthy controls (7, 13, 25, 31). Within skeletal muscle, miyomiR are critical regulators of the transcription factors MyoD and myogenin, which are necessary for regeneration of myofibers (11), as well as the central pathway, mechanistic target of rapamycin complex 1 (mTORC1), which controls protein synthesis (33, 34, 45). While c-nyomiR expression increases under pathophysiological conditions that negatively impact skeletal muscle protein synthesis and mass, whether expression of c-nyomiR reflects rates of protein synthesis has not been examined.

For older individuals engaging in weight loss interventions, understanding the physiological consequences of ER is critical to identify appropriate strategies to minimize muscle loss. Determination of noninvasive markers that reflect skeletal muscle adaptation could potentially allow for understanding of physiological adaptations to nutrient interventions. The pri-
primary objective of this investigation was to determine the effect of short-term ER on c-miR expression and to examine their relationship to measurements of whole body protein turnover. We hypothesized that ER would upregulate c-miR expression and be inversely associated with whole body protein synthesis.

**METHODS**

Participants. Participants were sedentary (<2 exercise sessions per week), overweight (25–35 kg/m²) men between 60 and 75 yr of age. Participants were in good health and free of any chronic disease as determined by medical screening. For enrollment in this investigation, participants were willing to consume only foods and beverages provided by the Human Nutrition Research Center on Aging (HNRCa) metabolic kitchen, abstain from alcohol, tobacco, and dietary supplement use, and maintain their level of physical activity. The Tufts University Health Sciences Campus Institutional Review Board approved this investigation, with informed written consent obtained from all participants.

Study design. For this 35-day controlled feeding intervention, subjects were placed on a eucaloric [weight maintenance (WM)] diet for the first 7 days. Nitrogen balance (NBAL), whole body protein turnover (WBPTO), and resting metabolic rate (RMR) were measured at the end of the WM period. Starting on study day 8, energy intake was reduced by 30% of total energy needs for the remaining 28 days. Dietary protein intake was set at 1.0 g kg⁻¹ day⁻¹, with dietary fat and carbohydrate manipulated to reduce energy intake. WBPTO, NBAL, and RMR were measured again at the end of the ER period (NBAL was also measured on day 13 to determine the initial adaptations to ER). In an additional arm of this investigation, participants received 90 mmol/day KHCO₃ or a matched placebo for the 28 days of ER. Because no effect of KHCO₃ was observed on outcome variables, data were collapsed for the effect of energy status on independent variables. Participants reported to the HNRCa two to three times per week to pick up study food and beverages and to be weighed to assess compliance.

Anthropometrics. Height was measured in duplicate to the nearest 0.1 cm using a stadiometer. Body mass was measured using a calibrated digital scale (Seca, Los Angeles, CA) to the nearest 0.1 kg at baseline to confirm study eligibility and twice a week during the WM and ER periods.

Diet. Energy needs were individualized for each participant using the Harris Benedict equation with a fixed factor of 1.2 to account for dietary thermogenesis and daily living activities (e.g., walking and brushing teeth). The 7-day WM period allowed time for adaptation to the diet prescription. Dietary protein was set at 1.0 g kg⁻¹ day⁻¹ to be consistent with recommendations from the PROT-AGE Study Group that older individuals’ protein requirements are above the current Recommended Dietary Allowance of 0.8 g kg⁻¹ day⁻¹ and that they should consume ≥1.0 g kg⁻¹ day⁻¹ (4). Dietary fat accounted for 25–30% of total energy, and carbohydrate provided the remainder of the prescribed energy. Energy intake was reduced by 30% of total energy needs at the end of the WM period. Dietary protein intake remained constant at 1.0 g kg⁻¹ day⁻¹, fat accounted for 25–30% of total energy, and carbohydrate provided the remainder of the prescribed energy. Meals were provided in 3-day menu cycles and prepared in the HNRCa metabolic kitchen. Each meal was prepared in advance and checked for accuracy by study staff. A multivitamin/mineral supplement was provided to ensure that micronutrient requirements were met. Energy and macronutrient intakes were recorded and analyzed using Nutrition Data System for Research (University of Minnesota; Table 1).

Nitrogen balance. Total nitrogen content of the urine was determined on days 6, 13, and 34 from a single pooled 24-h urine sample using pyrocheluminescence. Urinary creatinine was measured using the Jaffe reaction to verify complete urine collections.

Apparent nitrogen balance was calculated as nitrogen intake – urinary nitrogen excretion + miscellaneous (estimated at 5 mg/kg) and fecal (estimated at 2 g/day) losses (17). Urinary creatinine was measured on an automated clinical chemistry analyzer (model AU400, Olympus America, Melville, NY). Total nitrogen excretion was measured using a nitrogen/protein determinator (model FP-2000, LECO, St. Joseph, MI).

Resting metabolic rate. RMR was measured using open-circuit indirect calorimetry (TrueOne 2400, Parvo Medics, Sandy, UT) at the end of the WM (study day 7) and ER (study day 35) periods. Measurements were conducted between 0600 and 0800 after a 10-h overnight fast. Participants rested in the supine position for ~30 min before each measurement in a quiet, dim, temperature-regulated room. An acrylic hood was placed over the participant’s head for collection of all expired air during the test. Participants were instructed to remain still, stay awake, and breathe normally during the collection period. The test was discontinued when 20 min of steady-state O₂ consumption (V˙O₂) and CO₂ production (V˙CO₂) were recorded.

Substrate oxidation. Nitrogen values determined by the 24-h urine collections coincided with RMR measurements to estimate substrate oxidation according to Ferrarini (15); protein oxidation (g/day) = 6.25 × urinary nitrogen (g); glucose oxidation (g/day) = 4.12 × V˙CO₂ – 2.91 × V˙O₂ – 2.56 × urinary nitrogen (g); fat oxidation (g/day) = 1.69 × V˙CO₂ – 1.69 × V˙O₂ – 1.92 × urinary nitrogen (g).

Blood sampling. Blood samples were obtained at the end of the WM and ER periods after an overnight fast. Blood samples were allowed to clot at room temperature and then centrifuged at 2,135 × g for 10 min at 4°C. Serum was derived and stored at −80°C until analyzed.

**Table 1. Dietary intake**

<table>
<thead>
<tr>
<th></th>
<th>Weight Maintenance</th>
<th>Energy Restriction</th>
<th>Δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy intake, kcal/day</td>
<td>2,611 ± 153</td>
<td>1,810 ± 51</td>
<td>−800 ± 34</td>
</tr>
<tr>
<td>Carbohydrate, g/day</td>
<td>404 ± 11</td>
<td>257 ± 9</td>
<td>−147 ± 7</td>
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<tr>
<td>Fat, g/day</td>
<td>77 ± 2</td>
<td>51 ± 1</td>
<td>−27 ± 1</td>
</tr>
<tr>
<td>Protein, g/day</td>
<td>89 ± 2</td>
<td>89 ± 2</td>
<td>−0.2 ± 0.1</td>
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</table>

<table>
<thead>
<tr>
<th></th>
<th>Absolute intake</th>
<th>Relative intake</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy intake, kcal-kg⁻¹-day⁻¹</td>
<td>29.46 ± 0.52</td>
<td>21.5 ± 0.5</td>
</tr>
<tr>
<td>Carbohydrate, g-kg⁻¹-day⁻¹</td>
<td>4.56 ± 0.09</td>
<td>3.05 ± 0.09</td>
</tr>
<tr>
<td>Fat, g-kg⁻¹-day⁻¹</td>
<td>0.87 ± 0.02</td>
<td>0.60 ± 0.01</td>
</tr>
<tr>
<td>Protein, g-kg⁻¹-day⁻¹</td>
<td>1.00 ± 0.01</td>
<td>1.05 ± 0.01</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 16.

Whole body protein turnover. WBPTO was assessed for a 24-h period starting on study days 6 and 34 using the “end-product” method (14). Stable isotope [¹⁵N]glycine was administered in the morning following a 12-h overnight fast. Immediately before each isotope dosing, subjects provided a baseline urine sample for determination of background [¹⁵N]nitrogen and completely emptied their bladders. A single oral dose of [¹⁵N]glycine (300 mg; Cambridge Isotope Laboratories, Andover, MA) was dissolved in bottled water and then administered (21). Urine was collected for 24 h after dosing. The [¹⁵N] enrichment of urinary nitrogen [ratio of tracer to tracee (t/r)] was determined using isotope ratio mass spectroscopy (Metabolic Solutions, Nashua, NH). The t/r ratio for the cumulative sample was corrected for the background [¹⁵N]nitrogen enrichments. Nitrogen intake (I) was determined from analysis of 24-h food consumption. Nitrogen flux (Q), protein synthesis (PS), protein breakdown (PB), apparent nitrogen balance was calculated as nitrogen intake – urinary nitrogen excretion + miscellaneous (estimated at 5 mg/kg) and fecal (estimated at 2 g/day) losses (17). Urinary creatinine was measured on an automated clinical chemistry analyzer (model AU400, Olympus America, Melville, NY). Total nitrogen excretion was measured using a nitrogen/protein determinator (model FP-2000, LECO, St. Joseph, MI).

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Substrate oxidation. Nitrogen values determined by the 24-h urine collections coincided with RMR measurements to estimate substrate oxidation according to Ferrarini (15); protein oxidation (g/day) = 6.25 × urinary nitrogen (g); glucose oxidation (g/day) = 4.12 × V˙CO₂ – 2.91 × V˙O₂ – 2.56 × urinary nitrogen (g); fat oxidation (g/day) = 1.69 × V˙CO₂ – 1.69 × V˙O₂ – 1.92 × urinary nitrogen (g).

Blood sampling. Blood samples were obtained at the end of the WM and ER periods after an overnight fast. Blood samples were allowed to clot at room temperature and then centrifuged at 2,135 × g for 10 min at 4°C. Serum was derived and stored at −80°C until analyzed.

Substrate and hormone analysis. Serum glucose and triglyceride concentrations were assessed using a chemistry analyzer (model AU400e, Beckman Coulter, Brea, CA). Insulin concentrations were determined using an automatic gamma counter (2470 Wizard², PerkinElmer, Cambridge, MA) and human-specific RIA insulin kits (Millipore, Billerica, MA).

Whole body protein turnover. WBPTO was assessed for a 24-h period starting on study days 6 and 34 using the “end-product” method (14). Stable isotope [¹⁵N]glycine was administered in the morning following a 12-h overnight fast. Immediately before each isotope dosing, subjects provided a baseline urine sample for determination of background [¹⁵N]nitrogen and completely emptied their bladders. A single oral dose of [¹⁵N]glycine (300 mg; Cambridge Isotope Laboratories, Andover, MA) was dissolved in bottled water and then administered (21). Urine was collected for 24 h after dosing. The [¹⁵N] enrichment of urinary nitrogen [ratio of tracer to tracee (t/r)] was determined using isotope ratio mass spectroscopy (Metabolic Solutions, Nashua, NH). The t/r ratio for the cumulative sample was corrected for the background [¹⁵N]nitrogen enrichments. Nitrogen intake (I) was determined from analysis of 24-h food consumption. Nitrogen flux (Q), protein synthesis (PS), protein breakdown (PB),
and net protein balance (NET) were calculated using the following equations, where \( D \) denotes the oral dose of \( ^{15} \text{N} \) (\( D = g \text{ glycine} \times 0.1972 \)):

\[
\text{Q}(\text{g} \cdot \text{N} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}) = [D \div (\text{corrected} \ \text{t} / 24) \times \text{body weight}] \\
\text{PS}(\text{g} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}) = [\text{Q} - (\text{E} / 24 \times \text{body weight})] \times 6.25 \\
\text{PB}(\text{g} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}) = [\text{Q} - (1 / 24 \times \text{body weight})] \times 6.25 \\
\text{NET}(\text{g} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}) = \text{PS} - \text{PB}
\]

Circulating miRNA extraction and expression. Circulating miRNA were extracted from 200 \( \mu \)l of serum using a miRNAeasy serum/plasma kit, which allows for extraction and purification of small (<200-nt) cell-free RNA (catalog no. 217184, Qiagen, Valencia, CA). Before RNA extraction, samples were centrifuged for 10 min at 4°C to remove potential contaminating cellular debris. Because of the small amount of RNA in the serum, 3.5 \( \mu \)l of a spike-in control (Caenorhabditis elegans miR-39; catalog no. 219610, Qiagen) were added to all samples before RNA extraction to determine the yield of template recovered.

The c-myomiR of interest (miR-1, miR-133a-3p, miR133b, and miR-206) were analyzed using TaqMan miRNA assays (catalog no. 4427975, Applied Biosystems) and a multiplex RT and preamplification protocol (29). miRNA were reverse-transcribed using the Taq-Man miRNA RT kit (catalog no. 4366596, Applied Biosystems) with the miRNA-specific stem-loop RT primers pooled in 1\( \times \)Tris-EDTA (TE) buffer for a final dilution of 0.05\( \times \)for each miRNA RT primer. The RT primer buffer (6 \( \mu \)l) was added to the RT reaction mix (0.3 \( \mu \)l of 100 mM dNTP, 3 \( \mu \)l of enzyme, 1.5 \( \mu \)l of 10\( \times \)RT buffer, and 0.19 \( \mu \)l of RNase inhibitor) and 3 \( \mu \)l of serum RNA. A preamplification step was performed to increase cDNA template using a primer pool of 20\( \times \)TaqMan small RNA assay for the miRNA of interest at 0.05\( \times \)concentration in 0.1\( \times \)TE buffer. Preamplification reaction mix consisted of 3.75 \( \mu \)l of primer pool, 2.5 \( \mu \)l of cDNA, 12.5 \( \mu \)l of TaqMan Universal PCR master mix (2\( \times \)) without UNG (catalog no. 4440040, Applied Biosystems), and 6.25 \( \mu \)l of nuclease-free H\(_2\)O. RT and preamplification were conducted using a thermal cycler (model T100, Bio-Rad, Hercules, CA). RT-qPCR amplifications were conducted using a real-time PCR detection system (CFX96 Touch, Bio-Rad). After preamplification, sufficient template was present to quantify cDNA using a spectrophotometer (model ND-1000, Nanodrop Technologies, Wilmington, DE) to ensure that equal amounts were loaded per sample.

All myomiR were normalized to the geometric mean of spike-in control miR-39 (external control) and U6 (internal control). Normalization of miRNA to the geometric mean of external and internal controls allows correction of both technical and interindividual variations. Three potential internal controls (U6, U44, and U48) were assessed for this analysis. U6 was determined to be the most stable and least variable internal control in the present investigation. The geometric mean of spike-in control miR-39 and U6 was determined to be a homogenously and stably expressed housekeeper with the geometric mean of cycle thresholds (\( C_T \)) of 28.14 \( \pm \)0.04 at WM and 28.18 \( \pm \)0.04 at ER and coefficients of variation of 3.5% (22). Fold changes were expressed relative to WM and calculated using the \( \Delta C_T \) method (41).

Circulating miRNA sample quality control. miRNA released from red blood cells due to hemolysis can dramatically alter expression of specific miRNA, potentially leading to misinterpretation of results (5, 37). To assess potential contamination, serum samples were analyzed spectrophotometrically (ND-1000 spectrophotometer, Thermo Scientific) for the presence of free hemoglobin at an absorbance of 414 nm. Additionally, the ratio of miR-451a (miRNA that is highly enriched in erythrocytes and has been shown to cross the C\(_T\) earlier with hemolysis) to miR-23a-3p (miRNA that is not present in erythrocytes and is unaffected by hemolysis) was calculated (miR ratio = \( \Delta C_T \) miR-23a-3p/miR-451a) (5, 47). This analysis revealed likely hemolysis of two WM samples (miR ratio > 6), resulting in exclusion of these two participants from the c-myomiR analysis (n = 14). All remaining samples had the same miR ratio at WM (4.79 \( \pm \)0.20) and ER (4.79 \( \pm \)0.20), indicating that hemolysis did not factor into differences observed between the two periods of the investigation.

Bioinformatics analysis. miR-1-3p, miR-133a, miR-133b, and miR-206 were uploaded to DNA Intelligent Analysis (DIANA)-miRPath 3.0 (Alexander Fleming Biological Sciences Research Center, Athens, Greece; http://diana.imis.athena-innovations.gr) to determine potential biological processes and genes potentially regulated by these miyomiR (50). Predictive gene ontology categories were determined using microT-CDS probing for biological processes targeted by all four myomiR.

myomiR score. To group the four myomiR (miR-1, miR-133a-3p, miR-133b, and miR-206) assessed in circulation, a myomiR score was calculated as the sum median fold change for all myomiR (20).

Statistical analysis. Normality was determined using Shapiro-Wilk tests for dependent variables. Expression of c-myomiR was not normally distributed. Fold change data for c-myomiR were log-transformed (log\(_2\)) for statistical analysis. Student’s paired t-test was used to assess differences between WM and ER values for RMR, substrate oxidation, metabolic profile, WBPTO, and c-myomiR expression. Repeated-measures ANOVA was used to assess NBAL. Backward linear regression analysis of whole body protein synthesis and breakdown and carbohydrate, fat, and protein oxidation was conducted to determine the strongest predictor of c-myomiR score. Spearman’s rank correlation coefficient was used to determine the correlation of c-myomiR scores to measurements of whole body protein synthesis. Significance was set at \( P < 0.05 \). Data were analyzed using IBM SPSS Statistics for Windows (version 22.0; IBM, Armonk, NY).

RESULTS

Participant characteristics and weight loss. Sixteen men (64 \( \pm \)2 yr) participated in the study. Baseline body mass, height, and body mass index were 89.3 \( \pm \)2.2 kg, 1.8 \( \pm \)0.4 m, and 28.5 \( \pm \)1.2 kg/m\(^2\), respectively. Body mass was stable during the 7-day ER period with a change of \( <1\% \) (88.8 kg) from study day 1 to 7. The mean energy deficit during the 28-day ER period was 31 \( \pm \)1%, resulting in declines (\( P < 0.05 \)) of body mass (4.4 \( \pm \)0.8 kg) and body mass index (1.4 \( \pm \)0.3 kg/m\(^2\); Fig. 1).

Metabolic adaptations. At the conclusion of ER, RMR declined (\( P < 0.05 \)) 73 \( \pm \)31 kcal compared with WM (Table 4).
2). Similarly, respiratory quotient (\(\dot{V}CO_2/\dot{V}O_2\)) was reduced (\(P < 0.05\)) 0.05 ± 0.0, resulting in an 18 ± 2% decrease (\(P < 0.05\)) in whole body carbohydrate oxidation and an 18 ± 3% increase (\(P < 0.05\)) in whole body fat oxidation during ER compared with WM. ER did not alter whole body protein oxidation. Insulin concentrations were 15.0 ± 6.1 pmol/l lower (\(P < 0.05\)) during ER than WM, which resulted in a decline of homeostatic model assessment of insulin resistance (HOMA-IR) of 0.6 ± 0.2 (Table 2). Triglyceride concentrations were 30.9 ± 10.7 mg/dl lower (\(P < 0.05\)) during ER than WM. No effect of energy status on circulating glucose concentrations was observed.

**NBAL and WBPTO.** At the conclusion of the WM period, participants were in NBAL (−0.10 ± 0.50 g/day), suggesting that they had adequately adapted to the study diet before initiating the ER period of the investigation. Compared with WM, NBAL was most negative (\(P < 0.05\), −1.07 ± 0.74 g/day) during the initial phase of ER (study day 13) but returned to values similar to WM (−0.04 ± 0.62 g/day) at the end of ER. Similarly, compared with WM, change in whole body protein flux (0.04 ± 0.02 g N·kg\(^{-1}\)·day\(^{-1}\)), synthesis (0.26 ± 0.15 g protein kg\(^{-1}\)·day\(^{-1}\)), breakdown (0.22 ± 0.16 g protein kg\(^{-1}\)·day\(^{-1}\)), and net (0.04 ± 0.2 g protein kg\(^{-1}\)·day\(^{-1}\)) whole body protein synthesis and breakdown and carbohydrate, fat, and protein oxidation determined protein synthesis to be the strongest predictor of c-mymiR. An inverse association (\(P < 0.05\)) was observed with ER c-mymiR scores and whole body protein synthesis (\(r = -0.729, r^2 = -0.530\); Fig. 2B). Additionally, \(\Delta (ER - WM)\) whole body protein synthesis was inversely associated (\(r = -0.544, r^2 = -0.294, P < 0.05\)) with c-mymiR score (Fig. 2C).

**Bioinformatics analysis.** A total of 40 gene ontology biological processes were identified as significant predictive targets for miR-1-3p, miR-133a, miR-133b, and miR-206. The 15 most significant categories are displayed in Table 3. Relevant predicted biological processes captured by bioinformatics analysis indicating an influence on myomiR at the skeletal muscle level included skeletal muscle tissue regeneration and skeletal muscle satellite cell commitment.

**DISCUSSION**

The primary finding of the current study was an overall increase in c-mymiR expression following short-term (28-day) ER (30% of total energy requirement) compared with WM in older men. This increase in c-mymiR expression was

### Table 2. Metabolic adaptations

<table>
<thead>
<tr>
<th></th>
<th>Weight Maintenance</th>
<th>Energy Restriction</th>
<th>(P) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>RMR, kcal</td>
<td>1,586 ± 23</td>
<td>1,513 ± 22</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>(RQ (\dot{V}CO_2/\dot{V}O_2))</td>
<td>0.85 ± 0.02</td>
<td>0.80 ± 0.02</td>
<td>&lt;0.01</td>
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<tr>
<td>Substrate oxidation, %</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>45 ± 2</td>
<td>27 ± 3</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Fat</td>
<td>40 ± 2</td>
<td>58 ± 2</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Protein</td>
<td>15 ± 1</td>
<td>15 ± 1</td>
<td>0.88</td>
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<tr>
<td>Metabolic profile</td>
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<tr>
<td>Glucose, mmol/l</td>
<td>5.2 ± 0.1</td>
<td>5.2 ± 0.1</td>
<td>0.78</td>
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<tr>
<td>Insulin, pmol/l</td>
<td>77.3 ± 7.8</td>
<td>62.2 ± 7.7</td>
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<tr>
<td>HOMA IR</td>
<td>2.6 ± 0.3</td>
<td>2.1 ± 0.3</td>
<td>0.04</td>
</tr>
<tr>
<td>Triglyceride, mg/dl</td>
<td>132.6 ± 13.4</td>
<td>101.7 ± 13.4</td>
<td>&lt;0.01</td>
</tr>
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</table>

Values are means ± SE; \(n = 16\). Serum samples for metabolic profiles were analyzed in triplicates. RMR, resting metabolic rate; \(RQ\), respiratory quotient; \(\dot{V}CO_2\), CO\(_2\) production; \(\dot{V}O_2\), O\(_2\) consumption; HOMA-IR, homeostatic model assessment of insulin resistance.
Table 3. Gene ontology analysis

<table>
<thead>
<tr>
<th>Biological Process</th>
<th>P Value</th>
</tr>
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<tbody>
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<td>Vascular smooth muscle contraction</td>
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<tr>
<td>Skeletal muscle tissue regeneration</td>
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<td>Fcε-receptor signaling pathway</td>
<td>0.023</td>
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<td>Protein complex assembly</td>
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<td>Positive regulation of transcription from RNA to polymerase</td>
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<td>II promoter</td>
<td>0.036</td>
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<tr>
<td>Phosphatidylinositol-mediated signaling</td>
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<tr>
<td>Relaxation of smooth muscle</td>
<td>0.029</td>
</tr>
<tr>
<td>Macromolecular complex assembly</td>
<td>0.029</td>
</tr>
<tr>
<td>Positive regulation of macromolecular biosynthetic process</td>
<td>0.036</td>
</tr>
<tr>
<td>Eye photoreceptor cell fate commitment</td>
<td>0.036</td>
</tr>
<tr>
<td>Negative regulation of transcription from RNA to polymerase</td>
<td></td>
</tr>
<tr>
<td>II promoter</td>
<td>0.036</td>
</tr>
<tr>
<td>Skeletal muscle satellite cell commitment</td>
<td>0.036</td>
</tr>
<tr>
<td>Transmission of virus</td>
<td>0.036</td>
</tr>
<tr>
<td>Pteridine-containing compound biosynthetic process</td>
<td>0.036</td>
</tr>
<tr>
<td>Development in symbiotic interaction</td>
<td>0.036</td>
</tr>
</tbody>
</table>

Top 15 gene ontology categories with common genes targeted by all 4 muscle-specific microRNA (miR-1-3p, miR-133a-3p, miR-133b, and miR-206). Italics denote relevant categories.

Inversely associated with 24-h measures of whole body protein synthesis. Bioinformatics analysis of common biological processes regulated by the four myomiR (miR-1–3p, miR-133a-3p, miR-133b, and miR-206) of interest in the current investigation identified skeletal muscle regeneration as a top predictive target. Several previous investigations (10, 11, 28, 42) using cell culture and small animal models have reported that myomiR are critical for myogenic regulatory factors to shuttle satellite cells through proliferation of myoblasts to differentiation into myotubes. Specifically, manipulation of miR-1 and miR-206 expression has identified that these two myomiR promote myogenic differentiation (11, 28). Repression of miR-1 and miR-206 expression in cell culture models results in an elevated expression of paired box protein Pax7, promoting proliferation while diminishing differentiation and, thus, delaying myogenesis (7, 11). When miR-1 and miR-206 are overexpressed, Pax7 is downregulated, resulting in a reduction in proliferation and an upregulation in muscle progenitor cell differentiation (11). Progression from proliferation to differentiation is dependent on an altered ratio of Pax7 to MyoD, where Pax7 expression decreases and MyoD expression increases (38). MyoD and myogenin are the transcription factors regulating increased expression of miR-1 and miR-206 during differentiation, allowing MyoD and myogenin to promote their own expression through inhibition of Pax7 (42, 46). While miR-1 and miR-206 repress proliferation and promote differentiation, miR-133 increases proliferation of myoblasts and inhibits differentiation (19, 26, 51). Expression of miR-133a is upregulated by serum response factor, a transcription factor that regulates cell cycling, apoptosis, cell growth, and cell differentiation. Increased miR-133a expression initiates a negative-feedback loop, as miR-133a targets serum response factor to repress its expression, allowing for enhanced proliferation (10). Together, data from past investigations show that myomiR are critical to maintain myogenesis and skeletal muscle regeneration to optimize skeletal muscle health.

Commitment of satellite cells to proliferate and differentiate in support of skeletal muscle regeneration is an important factor contributing to the maintenance of skeletal muscle protein turnover and mass (6, 18). Regeneration is characterized by elevated rates of protein synthesis due to the generation of newly formed myofibers (27). Given the influence of myomiR on skeletal muscle regeneration, upregulation of c-myomiR expression following 28 days of 30% ER compared with WM in the present investigation may indicate that modulation of their expression could potentially contribute to reductions in rates of protein synthesis. In support of this hypothesis, we observed an inverse association between c-myomiR score and whole body protein synthesis: elevated expression of c-myomiR was associated with lower rates of protein synthesis. It is important to note that while associations are not indicative of causality, we are the first to show that weight loss alters c-myomiR and that those changes might influence whole body protein synthesis. Our study design, however, which did not include muscle biopsies, does not provide an opportunity to further examine the potential mechanism by which c-myomiR may impact protein synthesis. To determine a mechanism whereby alterations of c-myomiR change the rates of protein synthesis and, potentially, have long-term impact on skeletal muscle mass, more in-depth analyses are required. As skeletal muscle only accounts for as much as 40% of WBPTO (44), follow-up investigations using stable isotope infusions and muscle biopsies are warranted to determine potential mechanistic functions of ER-induced alterations in c-myomiR on skeletal muscle.

Despite inverse correlations between c-myomiR and whole body protein synthesis, no effect of energy restriction was observed on whole body protein synthesis. Present findings conflict with previous reports that short-term ER results in diminished rates of protein synthesis compared with WM values (40, 48, 54). Discordant results in the present investigation can likely be explained by methodological discrepancies. Specifically, past studies were primarily conducted in the fasted state under resting conditions, while the current investigation assessed whole body protein synthesis over a 24-h period. The inclusion of dietary intake, particularly protein, and activities of daily living is known to influence the rate of protein synthesis during ER (36). It is not surprising that addition of these factors would result in findings that differ from those of past investigations conducted under fasted, resting conditions. Additionally, NBAL results indicated that, overall, participants adapted to the study diets at the conclusion of ER, as NBAL values at ER did not differ from those at WM. These findings are in agreement with previous reports (8, 23, 24, 39) of a steady decline in nitrogenous losses with more sustained (>14-day) periods of ER. This adaptive response can be enhanced when dietary protein is fed at an optimal amount (3, 35, 36, 39). In the present study, dietary protein intake of 1.0 g·kg⁻¹·day⁻¹ was maintained during WM and ER. This amount of protein was selected on the basis of a recent consensus report (4) that this level of protein intake is appropriate for individuals >65 yr old. Our results appear to support this claim, as consumption of 1.0 g·kg⁻¹·day⁻¹ was sufficient for adaptation to ER. Despite the lack of an energy effect on whole body protein synthesis, our findings are the first to show that elevations in c-myomiR expression were associated with a lower rate of whole body protein synthesis following ER.

Although results from the present investigations provide new insight into the relationship between c-myomiR expression and whole body protein synthesis, they are not without...
limitations. The lack of assessment of changes in body composition limits our ability to determine whether increased expression of c-myomiR was associated not only with lower rates of whole body protein synthesis, but also reductions in fat-free mass. Additionally, use of a targeted approach to assess the four original myomiR (miR-1-3p, miR-133a, miR-133b, and miR-206) in the current study limited our ability to capture altered expression of other potentially relevant miRNA in response to ER. Use of a microarray or miRNA sequencing would have provided a more global view of changes in circulating miRNA profiles. However, the intent of this investigation was to determine whether ER upregulated miR-1-3p, miR-133a, miR-133b, and miR-206, similar to disease states that negatively impact skeletal muscle protein synthesis and muscle mass (13, 25, 31).

Perspectives and Significance

In conclusion, findings from the present study show that restricting energy intake by 30% for 28 days upregulated expression of c-myomiR, which was associated with lower rates of whole body protein synthesis in older men. Results from our bioinformatics analysis indicated that the potential functional effects of those changes on skeletal muscle may be through dysregulation of skeletal muscle regeneration. While these results establish a relationship, not a causality, between c-myomiR and whole body protein synthesis, further investigations are warranted to clearly define the role of c-myomiR in protein turnover and skeletal muscle mass and function in response to ER.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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