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Regulation of miRNAs in human skeletal muscle following acute endurance exercise and short-term endurance training

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Key points

- The discovery of microRNAs (miRNAs) has established new mechanisms that control health, but little is known about the regulation of skeletal muscle miRNAs in response to exercise.
- This study investigated components of the miRNA biogenesis pathway (Drosha, Dicer and Exportin-5), muscle enriched miRNAs, (miR-1, -133a, -133b and 206), and several miRNAs dysregulated in muscle myopathies, and showed that 3 h following an acute exercise bout, *Drosha*, *Dicer* and *Exportin-5*, as well as miR-1, -133a, -133-b and miR-181a were all increased, while miR-9, -23a, -23b and -31 were decreased.
- Short-term training increased miR-1 and miR-29b, while miR-31 remained decreased.
- Negative correlations were observed between miR-9 and HDAC4 protein, miR-31 and HDAC4 protein and between miR-31 and NRF1 protein, 3 h after exercise.
- miR-31 binding to the HDAC4 and NRF1 3′ untranslated region (UTR) reduced luciferase reporter activity.
- Exercise rapidly and transiently regulates several miRNA species potentially involved in the regulation of skeletal muscle regeneration, gene transcription and mitochondrial biogenesis.

Abstract The identification of microRNAs (miRNAs) has established new mechanisms that control skeletal muscle adaptation to exercise. The present study investigated the mRNA regulation of components of the miRNA biogenesis pathway (Drosha, Dicer and Exportin-5), muscle enriched miRNAs, (miR-1, -133a, -133b and -206), and several miRNAs dysregulated in muscle myopathies (miR-9, -23, -29, -31 and -181). Measurements were made in muscle biopsies from nine healthy untrained males at rest, 3 h following an acute bout of moderate-intensity endurance cycling and following 10 days of endurance training. Bioinformatics analysis was used to predict potential miRNA targets. In the 3 h period following the acute exercise bout, *Drosha*, *Dicer* and *Exportin-5*, as well as miR-1, -133a, -133-b and -181a were all increased. In contrast miR-9, -23a, -23b and -31 were decreased. Short-term training increased miR-1 and -29b, while miR-31 remained decreased. Negative correlations were observed between miR-9 and HDAC4 protein ($r = -0.71$; $P = 0.04$), miR-31 and HDAC4 protein ($r = -0.87$; $P = 0.026$) and miR-31 and NRF1 protein ($r = -0.77$; $P = 0.01$) 3 h following exercise. miR-31 binding to the HDAC4

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and NRF1 3′ untranslated region (UTR) reduced luciferase reporter activity. Exercise rapidly and transiently regulates several miRNA species in muscle. Several of these miRNAs may be involved in the regulation of skeletal muscle regeneration, gene transcription and mitochondrial biogenesis. Identifying endurance exercise-mediated stress signals regulating skeletal muscle miRNAs, as well as validating their targets and regulatory pathways post exercise, will advance our understanding of their potential role/s in human health.

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Introduction

Endurance exercise elicits important cellular stress signals responsible for skeletal muscle adaptation. Common adaptations, such as improved mechanical, metabolic, neuromuscular and contractile function, are influenced by the transcriptional and translational regulation of genes that encode the proteins controlling these processes (Dela *et al.* 1994; Russell *et al.* 2003, 2005; Short *et al.* 2003; Wadley *et al.* 2007). Control of exercise-mediated skeletal muscle gene transcription and translation is regulated by transcription factor activation (Keller *et al.* 2001; McGee *et al.* 2006), histone modification (McGee *et al.* 2009) and DNA methylation (Nakajima *et al.* 2010; Barres *et al.* 2012). However, the discovery of microRNAs (miRNAs) (Lee *et al.* 1993; Reinhart *et al.* 2000) has revealed another level of complexity in transcriptional and translational regulation (Bartel, 2004).

miRNAs, small (∼20–30 nucleotides) non-coding ribonucleic acids (RNAs), inhibit protein translation or enhance messenger RNA (mRNA) degradation (Hamilton & Baulcombe, 1999; Reinhart *et al.* 2000). miRNA biogenesis occurs in the nucleus with the transcription of primary miRNA (pri-miRNA) transcripts (Bartel, 2004). The pri-miRNA is then cleaved into a ∼60–70 nt miRNA precursor (pre-miRNA) by the RNase-III type endonuclease Drosha associated to Pasha (also known as DGCR8), exported to the cytoplasm by Exportin-5 (XPO5) and cut into a ∼22 nt miRNA duplex by Dicer (Lee *et al.* 2003; Lund *et al.* 2004). One of the duplex strands is degraded, while the other confers the mature miRNA; the latter is incorporated into the RNA-induced silencing complex (RISC). This mature miRNA allows the RISC to identify and bind to the 3′ or 5′ untranslated regions (UTR) of the target mRNAs, resulting in its degradation or repression of protein translation (Bartel, 2004; Lee *et al.* 2009). It has been suggested that destabilisation of target mRNAs is the predominant reason for reduced protein output (Guo *et al.* 2010).

Skeletal and cardiac muscle show an enrichment of miR-1, -133a, -133b, -206, -208a, -208b, -486 and -499 (McCarthy & Esser, 2007; Callis *et al.* 2008; van Rooij *et al.* 2008, 2009; Small *et al.* 2010). Muscle-enriched miRNAs (commonly referred to as myomiRs) influence multiple facets of muscle development and function through their regulation of key genes controlling myogenesis (Chen *et al.* 2006; Kim *et al.* 2006; Rao *et al.* 2006), myosin heavy chain expression (McCarthy *et al.* 2009; van Rooij *et al.* 2009) and growth-promoting signalling pathways (Small *et al.* 2010). These miRNAs can be regulated by MyoD, MEF2, SRF (Liu *et al.* 2007; van Rooij *et al.* 2008; Liu & Olson, 2010) and myocardin-related transcription factor-A (MRTF-A; Small *et al.* 2010). These transcription factors and transcriptional co-activators are upregulated in human skeletal muscle following exercise (Yang *et al.* 2005; McGee *et al.* 2006; Lamon *et al.* 2009). The regulation of muscle enriched miRNAs following endurance exercise is underexplored. However, miR-1 and miR-133a are sensitive to muscle contraction in response to endurance (Safdar *et al.* 2009; Nielsen *et al.* 2010; Aoi *et al.* 2010) and resistance (Drummond *et al.* 2008) exercise.

The muscle enriched miRNAs-1, -133a and -206, as well as several other miRNAs including miR-9, -23, -29, -31 and -181, show dysregulation in several human myopathies and dystrophies (Eisenberg *et al.* 2009; Greco *et al.* 2009; Gambardella *et al.* 2010). Several of these miRNA species are also dysregulated in skeletal muscle of chronic disease conditions characterised by impaired exercise capacity, such as chronic obstructive pulmonary disease (COPD; Lewis *et al.* 2012), chronic kidney disease (CKD; Wang *et al.* 2011) and amyotrophic lateral sclerosis (ALS; Williams *et al.* 2009; Russell *et al.* 2012), as well as following bed-rest (Ringholm *et al.* 2011). Reduced skeletal muscle activity caused by cessation of training also regulates miRNA levels (Nielsen *et al.* 2010). Whether an increase in skeletal muscle activity stimulated via acute endurance exercise or endurance training also regulates miR-9, -23, -29, -31 and -181 in human skeletal muscle is unknown.

Given the fundamental role of miRNAs in transcription and transcriptional regulation, we measured (1) expression levels of members of the miRNA biogenesis complex, (2) muscle-enriched miRNAs and (3) miRNAs known to be dysregulated in muscle wasting and chronic disease conditions. Measurements were made in human skeletal muscle biopsy samples following a single bout

of moderate-intensity endurance cycling exercise, as well as after 10 days of combined moderate- or high-intensity endurance cycling training.

Methods

Subjects

The nine male subjects participating in this study were healthy but exercised less than 2 h per week (peak pulmonary oxygen consumption ($\dot{V}_{O_2peak}$) of 44.1 ± 7.2 ml min^{-1} kg^{-1}; age, 23 ± 5 years; height, 178 ± 8 cm; weight, 79 ± 8 kg; Benziane *et al.* 2008). The subjects were fully informed of the possible risks involved in the study before providing written consent. The study was approved by the Monash University Standing Committee on Ethics in Research Involving Humans and Karolinska Institutet Ethics Committee. The study was conducted according to the Helsinki Declaration.

Peak pulmonary oxygen consumption

$\dot{V}_{O_2peak}$ tests were completed on an electromagnetically braked cycle ergometer (Lode B.V medical technology, Groningen, The Netherlands). Participants cycled at an initial workload of 100 W, which increased to 150 W after 2.5 min, followed by a 25 W increase every 2.5 min until volitional exhaustion. Volitional fatigue was defined as the point at which the subjects' cadence decreased below 60 rpm and/or a respiratory exchange ratio (RER) of 1.12. $\dot{V}_{O_2peak}$ was determined as the average over the last minute from samples measured every 10 s (MOXUS metabolic system, AEI Technologies, Pittsburgh, PA, USA) corresponding to the highest $\dot{V}_{O_2}$ that occurred in the last minute of the test. Peak power output (PPO) was also measured. These parameters were used to determine the exercise intensities of 70, 75% and 90% of individual $\dot{V}_{O_2peak}$ for the relevant exercise tests and training sessions.

Exercise tests and training

One week after completing the $\dot{V}_{O_2peak}$ test, subjects completed a pre-training acute exercise trial which consisted of cycling for 60 min at ∼70% of their $\dot{V}_{O_2peak}$. The cycle training regimen commenced ∼7 days later. Training consisted of 10 days of endurance training on the Lode cycle ergometer, including 4 days of high-intensity interval training as described previously (Benziane *et al.* 2008). This protocol improves exercise performance and activates molecular signalling pathways controlling metabolism in humans (Benziane *et al.* 2008). Each subject performed the same relative level of exercise. Subjects rode at ∼75% of their $\dot{V}_{O_2peak}$ for 45 min on days 1, 5, 6, and 10, for 60 min on day 3, and 90 min on day 8. High-intensity training took place on days 2, 4, 7, and 9, consisting of 6×5 min intervals at ∼90–100% of subjects' $\dot{V}_{O_2peak}$ with 2 min recovery at or below 40% $\dot{V}_{O_2peak}$ between exercise bouts. A second $\dot{V}_{O_2peak}$ test was performed 4 days after the training period. All tests were performed in the fasted state. Subjects were instructed to abstain from caffeinated products and alcoholic beverages 24 h before the exercise trials while consuming their normal diet, which they recorded in daily food diaries during the 3 days before the exercise trials. They were instructed to consume the same food or record any changes from the initial diet. Food diaries were analysed for total energy consumption and the relative energy sources were 52% CHO, 20% fat and 28% protein. This dietary control was effective, since diet and energy consumption were unchanged.

Muscle biopsy

Three muscle biopsies were taken from the same thigh but from three separate incisions 20–50 mm apart. An area on the thigh over the vastus lateralis was cleaned and sterilised. The skin was anaesthetised with lignocaine (5% xylocaine; AstraZeneca, North Ryde, Australia) and an incision was made through the skin and muscle fascia. The first muscle biopsy (150—250 mg) was taken at rest using the percutaneous needle biopsy technique with suction applied. Following the initial biopsy, subjects completed the acute 1 h exercise cycling test. Thereafter, the subjects rested in a supine position and were instructed to keep as still as possible for 3 h after which a second biopsy was taken. A third muscle biopsy was taken 2 days after the last training session which also occurred 2 days before the last $\dot{V}_{O_2peak}$ test was performed. Muscle biopsies were immediately frozen in liquid nitrogen and stored at −80°C until analysis.

RNA extraction and reverse transcription

RNA was extracted from ∼30 mg of skeletal muscle samples using Tri-Reagent® Solution (Ambion Inc., Austin, TX, USA) according to the manufacturer's protocol. The RNA concentration was assessed using the Nanodrop 1000 Spectrophotometer (Thermo Fisher Scientific, MA, USA). The ratio between A260/A280 was 1.75 to 1.95 for all samples. RNA quality was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA). The RNA integrity number (RIN) was between 7.5 and 9.0 for all samples. One microgram of total RNA was DNAse treated for 15 min at room temperature using Sigma DNase treatment kit (Sigma-Aldrich, St Louis, MO, USA). The reaction was inactivated for 10 min at 70°C. First-strand cDNA was generated from 1 μg RNA in 20 μl reaction buffer using the High Capacity RT-kit (Applied Biosystems, Carlsbad, CA, USA); 1 × RT

Table 1. Details for the primers used in the present study

Gene symbol	Sequence accession number	Primer name	Sequence	Length	Primer location	Annealing temp.	Dynamic range (cycles)	Slope	Efficiency (%)
DROSHA	NM_013235	h Drosha For	ACTGTGGCTGTTTATTTCAAGGG	100	Exon 34	60	23.80–28.89	$y = -3.38$	97.8
		h Drosha Rev	ATTTTTCAAGCGCATCCATTG		Exon 35/36				
XPO5	NM_020750	h Exportin 5 For	GCGCCGAAAGGACCA	98	Exon 31	60	26.82–31.78	$y = -3.32$	99.9
		h Exportin 5 Rev	AGGGAAGATTCTTAATGTGAACTTCTT		Exon 32				
DICER1	NM_177438	h Dicer For	ATCCCATGATGCGGCC	100	Exon 28/29	60	21.93–26.84	$y = -3.29$	101.4
		h Dicer Rev	CTAAATTTGGCAGTTTCTGGTTCC		Exon 29/ 30a/b				
NRF1	NM_005011	h NRF1 For	GGTGCAGCACCTTTGGAGAA	73	Exon 5	60	25.67–30.95	$y = -3.53$	91.9
		h NRF1 Rev	CCAGAGCAGACTCCAGGTCTTC		Exon 6				
MFN1	NM_033540	h MFN1 For	TGTTTTGGTCGCAAACTCTG	160	Exon 6	60	24.0–28.86	$y = 3.23$	103.8
		h MFN1 Rev	CTGTCTGCGTACGTCTTCCA		Exon 7 / 8				
MFN2	NM_014874	h MFN2 For	ATGCATCCCCACTTAAGCAC	301	Exon 3	60	20.24–25.31	$y = -3.39$	97
		h MFN2 Rev	CCAGAGGGCAGAACTTTGTC		Exon 5				
EIF4E	NM_001134651	h EIF4e For	GCAGCAGATGATGAAGTAATAGGAGTT	69	Exon 5/6	60	25.86–29.87	$y = -3.32$	100
		h eIF4e Rev	CCAGACTTGGACGACGTCTTCT		Exon 6				
RPS6KB1	NM_003161	h p70s6k For	GGACACTGGAGAAGTTCAAG	276	Exon 11/12	60	25.21–29.26	$y = -3.365$	98.2
		h p70s6k Rev	CGGATTTTTGGTTCAAAGGA		Exon 14				
HDAC4	NM_006037	h HDAC4 For	CACAGACTCCGCGTGCAGCA	94	Exon 8/ 9	60	25.81–30.71	$y = -3.23$	104
		h HDAC4 Rev	GGGCGCGATACCGTTCTCCG		Exon 9				
FBXO32	NM_058229	h Atrogin-1 For	GCAGCTGAACAACATTCAGATCAC	97	Exon 6	60	22.88–26.91	$y = -3.38$	97.5
		h Atrogin-1 Rev	CAGCCTCTGCATGATGTTCAGT		Exon 7				
FOXO1	NM_002015	h FoXO1 For	AAGAGCGTGCCCTACTTCAA	209	Exon 1	60	23.79–28.98	$y = -3.45$	94.6
		h FoXO1 Rev	CTGTTGTTGTCCATGGATGC		Exon 2				
FOXO3a	NM_001455	h FoXO3a For	CTTCAAGGATAAGGGCGACA	113	Exon 2a/b	60	25.88–31.08	$y = -3.39$	97.4
	NM_013235	h FoXO3a rev	TCTTGCCAGTTCCCTCATT		Exon 3				

buffer and random primers, 8 mM dNTP and 2.5 U μl^{-1} MultiScribe^{TM} RT enzyme. The RT protocol consisted of 10 min at 25°C, 120 min at 37°C, 5 min at 85°C then cooled to 4°C. Before diluting cDNA, 1 μl ribonuclease H (RNase H; Life Technologies, Mulgrave, VIC, Australia) was added to each sample and incubated at 37°C for 30 min. The cDNA was stored at −20°C until further analysis.

mRNA and miRNA analyses using real-time PCR

Real-time PCR was carried out using a Stratagene MX3000 thermal cycler to measure mRNA and miRNA levels. mRNA levels for Drosha, Dicer and Exportin-5, NRF1, Mfn1, Mfn2, atrogin-1/MAFbx, eIF4e, p70^{s6k}, HDAC4, FoXO1 and FoxO3a were measured using 1 × SYBR® Green PCR Master Mix (Agilent Technologies, Santa Clara, CA, USA; no. 4309155) and 5 ng of cDNA. All primers were used at a final concentration of 300 nM. Primer details are presented in Table 1. The PCR condition conditions were 1 cycle of 10 min at 95°C; 40 cycles of 30 s at 95°C, 60 s at 60°C, 60 s at 72°C; 1 cycle (melting curve) 60 s at 90°C, 30 s at 55°C, 30 s at 95°C. To compensate for variations in input RNA amounts and efficiency of the reverse transcription, data were normalised to cDNA input as quantified using Oligreen (Invitrogen, Carlsbad, CA, USA) as published by our group (Cannata *et al.* 2010). Primers were designed using Primer3 software, verified using the Basic Local Alignment Search Tool (BLAST) and

Table 2. miRNA probe sequence and assay number

MicroRNA	Sequence	Assay ID
miR-1	UGGAAUGUAAAGAAGUAUGUAU	002222
miR-23a	AUCACAUUGCCAGGGAUUUCC	000399
miR-23b	AUCACAUUGCCAGGGAUUACC	000400
miR-29a	UAGCACCAUCUGAAAUCGGUUA	002112
miR-29b	UAGCACCAUUUGAAAUCAGUGUU	000413
miR-29c	UAGCACCAUUUGAAAUCGGUUA	000587
miR-31	AGGCAAGAUGCUGGCAUAGCU	002279
miR-133a	UUUGGUCCCCUUCAACCAGCUG	002246
miR-133b	UUUGGUCCCCUUCAACCAGCUA	002247
miR-181a	AACAUUCAACGCUGUCGGUGAGU	000480
miR-206	UGGAAUGUAAGGAAGUGUGUGG	000510
miR-455	UAUGUGCCUUUGGACUACAUCG	001280

synthesised by Geneworks (Hindmarsh, SA, Australia). miRNA levels were measured using specific primer and probes sets as per the manufacturer's instructions (Applied Biosystems, Carlsbad, CA, USA; Russell *et al.* 2012; Table 2) using the 1 × Taqman Universal Master Mix II, no UNG kit (Applied Biosystems; no. 4440047). For miRNA analyses, RNA (10 ng) was reverse transcribed using the Taqman microRNA Reverse Transcription (RT) kit (Applied Biosystems). The RT reaction consisted of 1 mM dNTP, 0.27 U μl^{-1} RNase inhibitor, 3.3 U μl^{-1} MultiScribe^{TM} enzyme, 1 × buffer and 7.5 × diluted primers. The RT conditions consisted of 30 min at 16°C, 30 min at 42°C,

5 min at 85°C then cooled to 4°C. PCR plates and films were purchased from Axygen Scientific, Inc. (Union City, CA, USA). miRNA primers were diluted 40×. PCR conditions consisted of 1 cycle of 10 min at 95°C; 40 cycles of 15 s at 95°C, 60 s at 60°C. RNU48 was used as an endogenous control. All samples were run in triplicate for all mRNA and miRNA targets measured. All analysis was performed using the Stratagene MX3000 thermal cycler dedicated software and employed the DdCt method.

Protein extraction and Western blot analysis

Frozen muscle (50 mg) was freeze-dried and dissected free of blood and connective tissue. Skeletal muscle protein was extracted in ice-cold buffer (20 mM Tris, pH 7.8, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 0.5 mM Na_3VO_4, 1% Triton X-100, 10% glycerol, 10 mM NaF, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 1 mM dithiothreitol, 5 mM $Na_4O_7P_2$, 1 mM benzamidine, 1 μg ml^{-1} aprotinin, 1 μg ml^{-1} leupeptin, and 1 μM microcystin). Homogenates were rotated for 60 min at 4°C and centrifuged at 12,000 *g* for 10 min at 4°C, and protein concentration of the resulting supernatant was determined using a commercial kit (Pierce BCA Protein Assay Kit, Thermo Scientific, Rockford, IL, USA). Aliquots of muscle lysate were mixed with Laemmli sample buffer, and 40 μg of total protein/sample were separated by SDS–PAGE electrophoresis. Following electrophoresis, proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). For the measurement of eIF4e, $p70^{s6k}$ and HDAC4 proteins the membranes were blocked in Tris-buffered saline and 0.1% (vol/vol) Tween 20 (TBST) containing 7.5% non-fat dried milk for 1–2 h at room temperature, washed 3× with TBST for 10 min, and then incubated at 4°C overnight with the following primary antibodies diluted in 1% BSA in TBS: eIF4e 1:1000 (Cell Signalling Technology, Inc., Beverly, MA, USA, no. 9742), $p70^{s6k}$ 1:1000 (Santa Cruz Biotechnology; SC-230) and HDAC4 1:1000 (Cell Signalling Technology, Inc., Beverly, USA, no. 2072). Blots were normalised against the GAPDH protein (Santa Cruz no. sc 25778; 1:2000). Immunoreactive proteins were detected using enhanced chemiluminescence (Standard ECL, GE Healthcare, Little Chalfont, Buckinghamshire, UK) and quantified by densitometric scanning.

For the measurement of NRF1, MNF1, MFN2, Atrogin-1, FoxO1 and FoxO3 proteins, electrophoresis was performed using a 4–12% NuPAGE® Novex Bis-Tris Gel (Invitrogen) in NuPAGE® SDS Mops Running Buffer (Invitrogen). Protein transfer was performed in a Bjerrum buffer containing 50 mM Tris, 17 mM glycine and 10% methanol using PVDF membranes. Membranes were blocked with 5% BSA in PBS, after which they were incubated at 4°C with the following primary antibodies diluted in 3% BSA in PBS: atrogin-1 (Lot-1; ECM Biosciences, Versailles, USA); FoxO3 (FKHRL1; Santa Cruz Biotechnology, no. sc-11351); FoxO1 (Cell Signalling Technology, Inc., no. 2880); NRF1 (Rockland Immunochemicals Inc. Gilbertsville, PA, USA); MFN-1 and MFN-2 (Abnova GmBH, Heidelberg, Germany). Primary antibodies were diluted 1:500 except for atrogin-1 and NRF1, which were diluted 1:1000. Following overnight or 1 h incubation (NRF1), the membranes were washed and incubated for 1 h with a goat anti-rabbit IgG antibody labelled with an infrared-fluorescent 800 nm dye (Alexa Fluor® 800, LI-COR Biosciences, Lincoln, NE, USA) diluted 1:5000 in PBS containing 50% Odyssey® blocking buffer (LI-COR Biosciences) and 0.01% SDS. Membranes exposed to the anti-MFN-1 and MFN-2 antibodies were incubated for 1 h with a goat anti-mouse IgG antibody labelled with an infrared-fluorescent 680 nm dye (Alexa Fluor® 680, Invitrogen) diluted 1:5000 in PBS containing 50% Odyssey® blocking buffer (LI-COR Biosciences) and 0.01% SDS. After washing, proteins were exposed on an Odyssey® Infrared Imaging System (LI-COR Biosciences) and individual protein band optical densities were determined using the Odyssey® Infrared Imaging System software. Blots were normalised against the GAPDH protein (Santa Cruz no. sc-25778; 1:2000) which remained stable following the single bout and training periods ($P = 0.82$). In Fig. 5 the black vertical lines in the densitometry images indicate where one earlier time point between the Pre and Post 3 hrs time points was removed from the blot. This time point was not analysed for other targets due to a lack of tissue and removed from these blots for consistency with the other results. All samples for each subject were run on the same gel.

Prediction of miRNA targets

For miRNAs that were regulated following acute endurance exercise, we predicted the potential mRNA/protein targets based on 3′UTR sequence homology using miRWalk (Dweep *et al.* 2011). The miRWalk program enables the prediction of miRNA targets by incorporating several known prediction software programs. miRanda, miRDB, miRWalk, RNA22 and Targetscan were chosen within the miRWalk program.

Reporter assay experiments

Human HDAC4 and NRF1 200–300 bp 3′UTRs, including the putative miR-31 binding sites, were amplified from human cDNA by PCR using Ex Taq HS (TaKaRa, Osaka, Japan). The amplified fragment was cloned downstream of luciferase under the SV40 promoter using the *Xba*I (HDAC4) or *Eco*RI (NRF1) of the pLuc2EXN plasmid (pLuc2-HDAC4; pLuc-NRF1. Primer sequences

for amplifying the HDAC4 3′UTR were: Forward 5′ AAT CTAGATGTTGCTGTCAGATTCTATTTTCAG 3′; Reverse 5′ AATCTAGATGACTGTCAGTTACTGTTGAAGAGA 3′. Primer sequences for amplifying the NRF1 3′UTR were: Forward 5′ GTGGAAACAATAATTCACCCAGTT 3′; Reverse 5′ ATCCTGGTCTAGGACGATTTTTC 3′. For overexpression of miR-31, pCXbG-miR-31 was generated by amplifying miR-31 from human genomic DNA by PCR using following primers: 5′ AAAGTCGA CCCTCCCTCAGGTGAAAGGAA and 3′ AAGATATC TCGAGAAGGGCGCACATACACAG. The PCR product was digested with *Sal*I and *Eco*RV and introduced into the pCXbG plasmid at the *Xho*I and *Eco*RV sites. Reporter assay was performed as described (Wada *et al.* 2011; Russell *et al.* 2012). Briefly, C2C12 myoblasts (ATCC) were seeded in 6-well plates 18 h before transfection. Cells were transfected with 0.2 ug pLuc2EXN, pLuc2EXN-HDAC4 3′UTR or pLuc2EXN-NRF1 3′UTR, 0.2 ug of pRL-Tk (Promega, Madison, WI, USA) and 0.6 ug of pCXbG-miR-31 or pCXbG-sΔ (mock vector) with Lipofectamine 2000 (Invitrogen). Cells were lysed 24 h after transfection and luciferase activity was measured using the dual luciferase reporter assay system (Promega Co). Firefly luciferase activity was normalised to *Renilla* luciferase activity.

Statistics

A one-way analysis of variance with repeated measures was used to determine the effect of acute exercise and short-term training on the dependent variables. Significance was set at $P < 0.05$. A *post hoc* analysis was performed using multiple *t* tests with the Bonferroni adjustment. Linear regression was performed to establish the correlation between miRNAs and their predicted protein targets pre exercise, following acute exercise and following short-term training.

Results

Following 7 days of endurance training, mean (± SEM) $\dot{V}_{O_2peak}$ increased by 10% from 44.1 ± 7.2 to 48.5 ± 5.3 mlkg^{-1} min^{-1}. Peak power output (PPO) increased by 15% from 248 ± 41 to 286 ± 31 W, as reported previously ($P < 0.05$; Benziane *et al.* 2008).

Following 60 min of acute endurance exercise, mRNA expression levels of several key members of the miRNA processing complex, *Drosha*, *Dicer* and *Exportin-5*, were significantly upregulated by 35, 35 and 30%, respectively, during the first 3 h post exercise. Short-term endurance training did not affect the expression of these genes (Fig. 1).

The myomiRs miR-1, miR-133a and miR-133b, but not miR-206, were significantly increased by 60%, 35% and 40% following acute endurance exercise (Fig. 2). Only

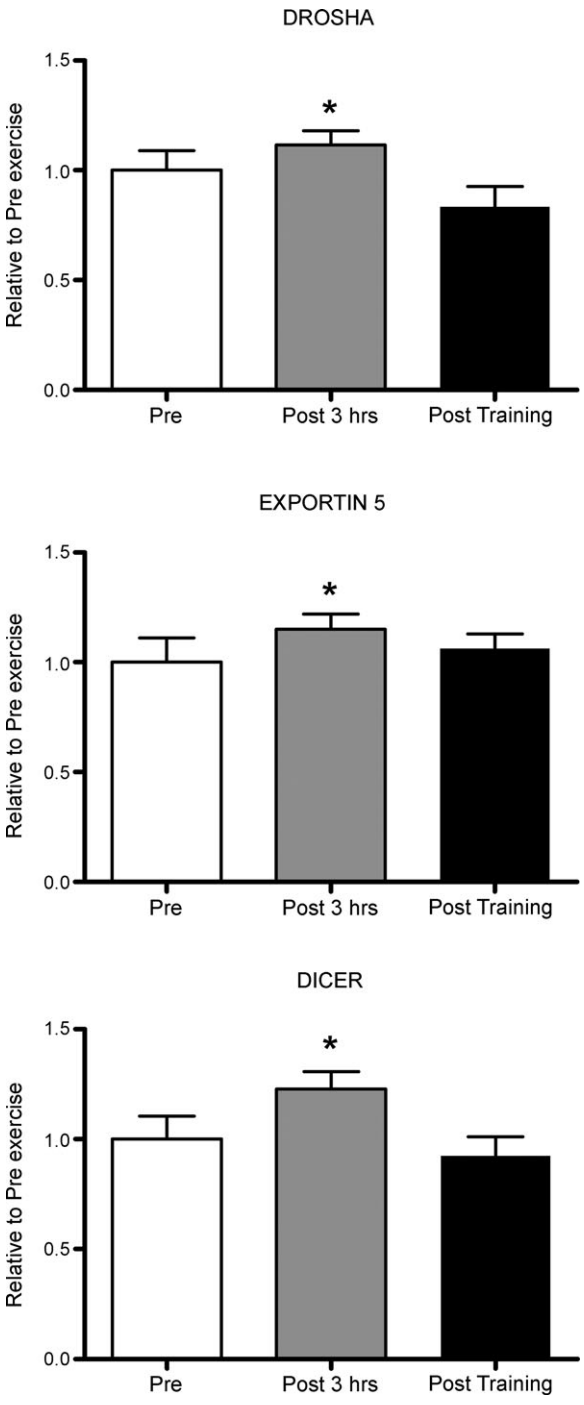

Figure 1. Components of the miRNA biogenesis machinery are regulated by a single bout of moderate-intensity endurance exercise
Pre, sample taken before the acute exercise bout; Post 3 hrs, sample taken 3 h after the acute exercise bout; Post training, sample taken 2 days after the 10 day training programme. *Significantly different from other groups, $P < 0.05$, $n = 9$ subjects per group with data presented as means ± SEM.

miR-1 levels remained elevated following short-term endurance training (Fig. 2).

Several other miRNA species, previously shown to be dysregulated in muscle wasting and chronic disease conditions were also investigated. Acute endurance exercise reduced miR-9, -23a, -23b and -31 by 50%, 24%, 27% and 28%, respectively. miR-181 was significantly increased by 35%, with no change observed for miR-29a, -29b and -29c (Fig. 3). Short-term training increased miR-29b by 210% and decreased in miR-31 by 35% (Fig. 3).

As miRNAs can regulate multiple genes, and a gene can be regulated by multiple miRNAs, (Lewis *et al.* 2003) we used the miRWALK prediction tool to identify potential mRNA/protein targets (http://www.ma.uni-heidelberg.de/apps/zmf/mirwalk). The miRNAs that were regulated by an acute bout of endurance cycling exercise were screened using miRWALK to predict their potential gene targets. As miRNAs can regulate both mRNA and protein, mRNA and protein levels of several of these targets were measured. The targets selected were identified by at least two or more of the five prediction software programs used within the miRWalk program for an individual miRNA. Finally, the predicted targets selected for mRNA and protein analysis were those that have been shown to be regulated by exercise and which are known to influence mitochondrial biogenesis and metabolism, including NRF1 (Short *et al.* 2003; Kelly & Scarpulla, 2004), mitofusin-1 (MFN1) and MFN2 (Bach *et al.* 2003; Cartoni *et al.* 2005), as well as targets regulating muscle growth such as eIF4e (Koshiba *et al.* 2004), $p70^{s6K}$ (Camera *et al.* 2010), HDAC4 (McGee *et al.* 2009), atrogin-1/MAFbx, FoXO1 and FoXO3a (Russell *et al.* 2005; Louis *et al.* 2007). Table 3 shows the predicted targets for each miRNA.

Following acute exercise, NRF1 protein, but not mRNA, was increased by 54%. *MFN2 mRNA*, but not protein, was increased by 32%, while *MFN1 mRNA* and protein levels were unaltered (Fig. 4). A significant 50% reduction in eIF4e protein levels, with no changes in its *mRNA* levels was observed. *$p70^{s6K}$ mRNA* and protein was unaltered (Fig. 5). *HDAC4 mRNA* and protein were increased by 2- and 19-fold, respectively. Post training, HDAC4 protein remained elevated 4-fold above pre-training levels, but was lower than the increase observed 3 h post the acute exercise bout (Fig. 5). Following acute exercise *Atrogin-1/MAFbx*,

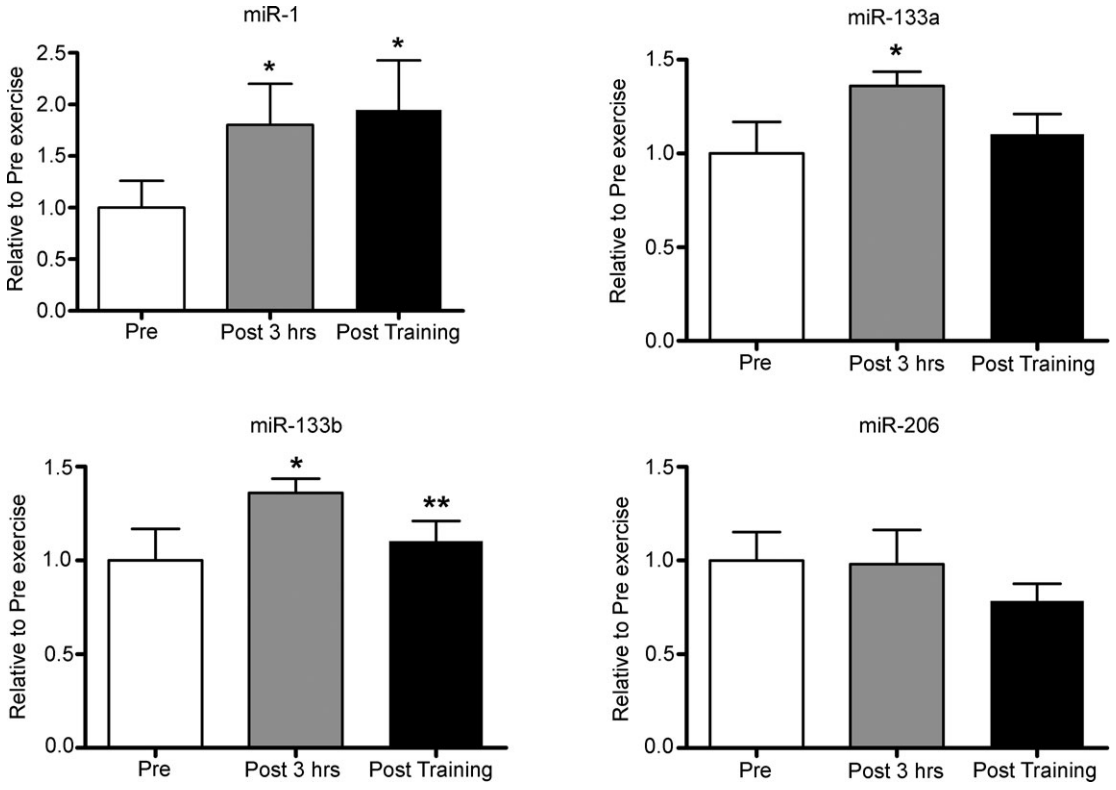

Figure 2. Influence of a single bout of moderate-intensity endurance exercise and short-term training on muscle-enriched miRNAs
Pre, sample taken before the acute exercise bout; Post 3 hrs, sample taken 3 h after the acute exercise bout; Post training, sample taken 2 days after the 10 day training programme. *Significantly different from Pre, $P < 0.05$; **significantly different from the Pre 3 h time point, $P < 0.01$, $n = 9$ subjects per group with data presented as means ± SEM.

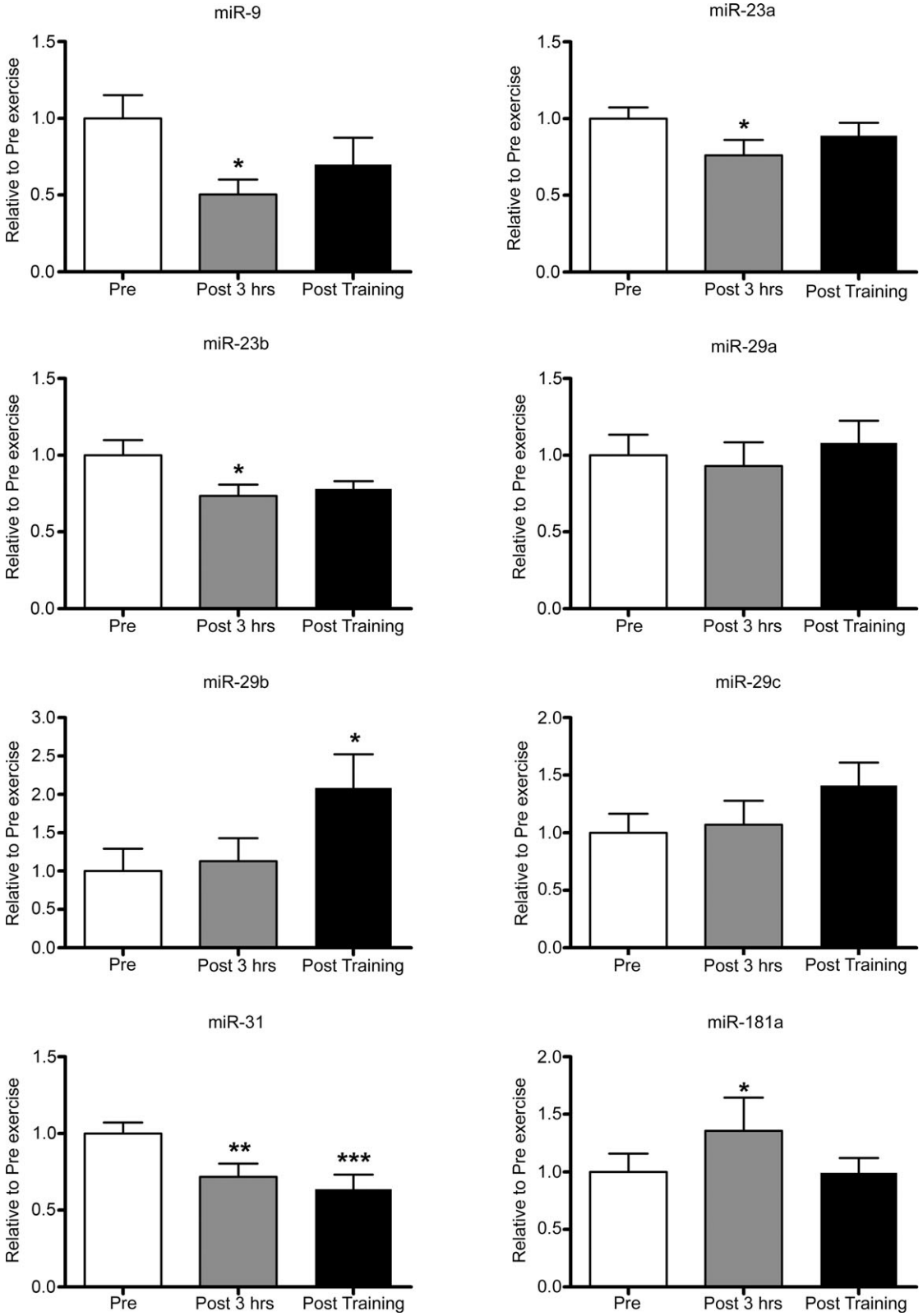

Figure 3. Influence of a single bout of moderate-intensity endurance exercise and short-term training on miRNAs known to be dysregulated in myopathies and neuromuscular disorders
Pre, sample taken before the acute exercise bout; Post 3 hrs, sample taken 3 h after the acute exercise bout; Post training, sample taken 2 days after the 10 day training programme. *Significantly different from other groups, $P < 0.05$, $n = 9$ subjects per group with data presented as mean ± SEM; **significant different from pre, $P < 0.01$; ***significant different from pre, $P < 0.001$.

Table 3. Predicted miRNA targets using miRWalk

miRNA	NRF1	MFN1	MFN2	eIF4e	P70s6k	HDAC4	Atrogin-1	FoXO1	FoXO3a
miR-1									
miR-133a									
miR-133b									
miR-206									
miR-9						*a*			
miR-23a							*b*		
miR-23b									
miR-23b									
miR-31									
miR-181									

Grey shaded box depicts a possible target. *a*, Roccaro *et al.* (2010); *b*, Wada *et al.* (2011); previously validated targets.

FoXO1 and *FoXO3a mRNA* levels increased by 1.0-, 4.7- and 2.4-fold, respectively, without any changes observed in their protein levels. Short term training did not affect *atrogin-1/MAFbx*, *FoXO1* and *FoXO3a mRNA* or protein levels (Fig. 6).

The correlations between miRNA species and their predicted protein targets were only observed following the single bout of moderate-intensity exercise (Fig. 7). Based on the predicted miRNA/protein targets listed in Table 3, inverse correlations were observed between miR-9 and HDAC4 protein ($r = -0.71$; $P = 0.04$), miR-31 and HDAC4 protein ($r = -0.87$; $P = 0.026$) and miR-31 and NRF1 protein ($r = -0.77$; $P = 0.01$). Whether these correlations could have a potential cause and effect was of interest to establish. Since miR-9 regulates HDAC4 (Roccaro *et al.* 2010), we performed a luciferase reporter assay in C2C12 myotubes to determine if miR-31 can regulate HDAC4 and NRF1 *in vitro*. The luciferase reporter assay showed a 30% and 18% reduction in luciferase/renilla activity for HDAC4 and NRF1 respectively (Fig. 8). Transfection of the miR-31 expression plasmid increased miR-31 miRNA levels by 2.2-fold in the C2C12 cells (Fig. 8); however, did this not affect either *HDAC4* or *NRF1 mRNA* or protein (Supplementary Fig. 1).

Correlations were also made between $\dot{V}_{O_2peak}$, PPO and the miRNAs, mRNAs and proteins measured. Pre-training $\dot{V}_{O_2peak}$, positively correlated with miR-181 ($r = 0.70$; $P = 0.03$) while pre-training PPO was inversely correlated with miR-23a ($r = -0.79$; $P = 0.012$). Post-training PPO was negatively correlated with miR-31 ($r = -0.74$; $P = 0.042$). No other correlations were observed.

Discussion

Skeletal muscle miRNAs are responsive to skeletal muscle contractile activity and disuse and play a key role in muscle adaptation/maladaptation. Endurance exercise is a key intervention used to maintain skeletal muscle health and prevent chronic disease. The present study provides evidence that in the 3 h period following an acute bout of endurance cycling exercise in untrained men, gene expression of components of the miRNA biogenesis pathway were elevated, as were several muscle enriched miRNAs including miR-1, -133a and 133-b, as well as miR-181a. miR-9, -23a, -23b and -31 were decreased following acute exercise. Short-term training increased miR-1 and miR-29b, while miR-31 remained decreased.

miRNA biogenesis is a complex process requiring co-ordination of pri-miRNA transcription, and its cleavage by endonucleases, exportation from nucleus to cytoplasm, additional cleavage and then incorporation into the RISC complex (Bartel, 2004). Acute endurance exercise in untrained males increases mRNA for two key endonuclease III enyzmes, Drosha and Dicer, as well as the miRNA export protein, Exportin-5, 3 h post exercise. Following acute resistance exercise in healthy men Exportin-5 increases at 3 h post exercise (Drummond *et al.* 2008). Exportin-5 exports pre-miRNA from the nucleus to the cytoplasm, and thus its upregulation following exercise most likely assists in the processing of new pre-miRNAs required for exercise-induced adaptations. Exportin-5 also mediates nuclear export of unspliced mRNA, suggesting a novel gene-regulatory mechanism involving Exportin-5 (Lund & Dahlberg, 2006). Mechanisms regulating the miRNA biogenesis machinery following exercise in skeletal muscle are unknown and this should be a focus for future investigation.

In our study, the myomiRs, miR-1, -133a and -133b, but not -206, were increased 3 h post exercise. In another study, the same myomiRs increased 1 h post endurance cycling, but returned to pre-exercise levels when measured 4 h post exercise (Nielsen *et al.* 2010). The major difference between the studies is that the subjects in the present study were considerably less trained, with $\dot{V}_{O_2peak}$ and PPO approximately 20% and 30% lower, respectively, than in the earlier report (Nielsen *et al.* 2010). While exercise intensity and durations were similar between studies,

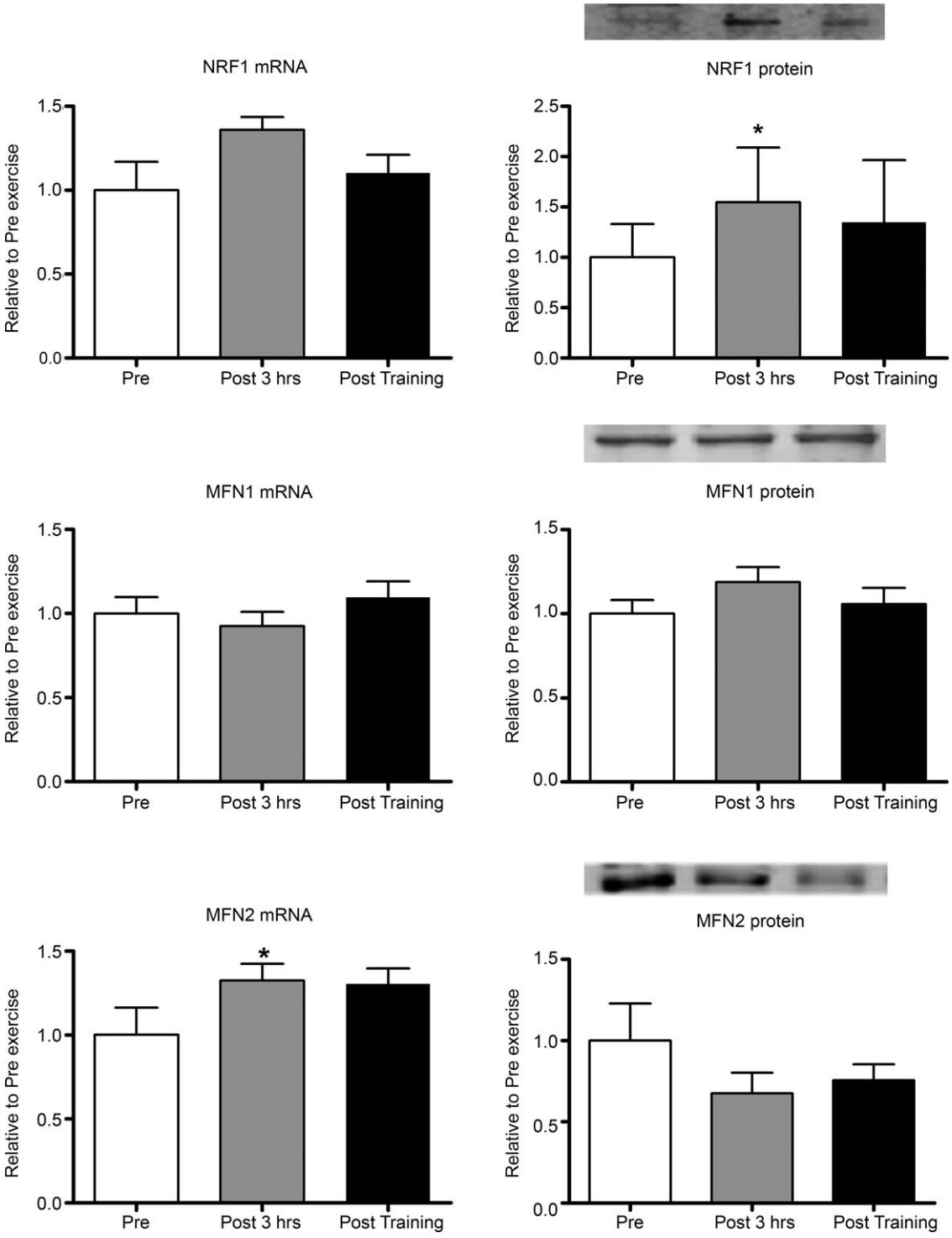

Figure 4. Regulation of targets involved in mitochondrial biogenesis and function following a single bout of moderate-intensity endurance exercise and short-term training
Pre, sample taken before the acute exercise bout; Post 3 hrs, sample taken 3 h after the acute exercise bout; Post training, sample taken 2 days after the 10 day training programme. *Significantly different from other groups, $P < 0.05$, $n = 9$ subjects per group with data presented as means ± SEM.

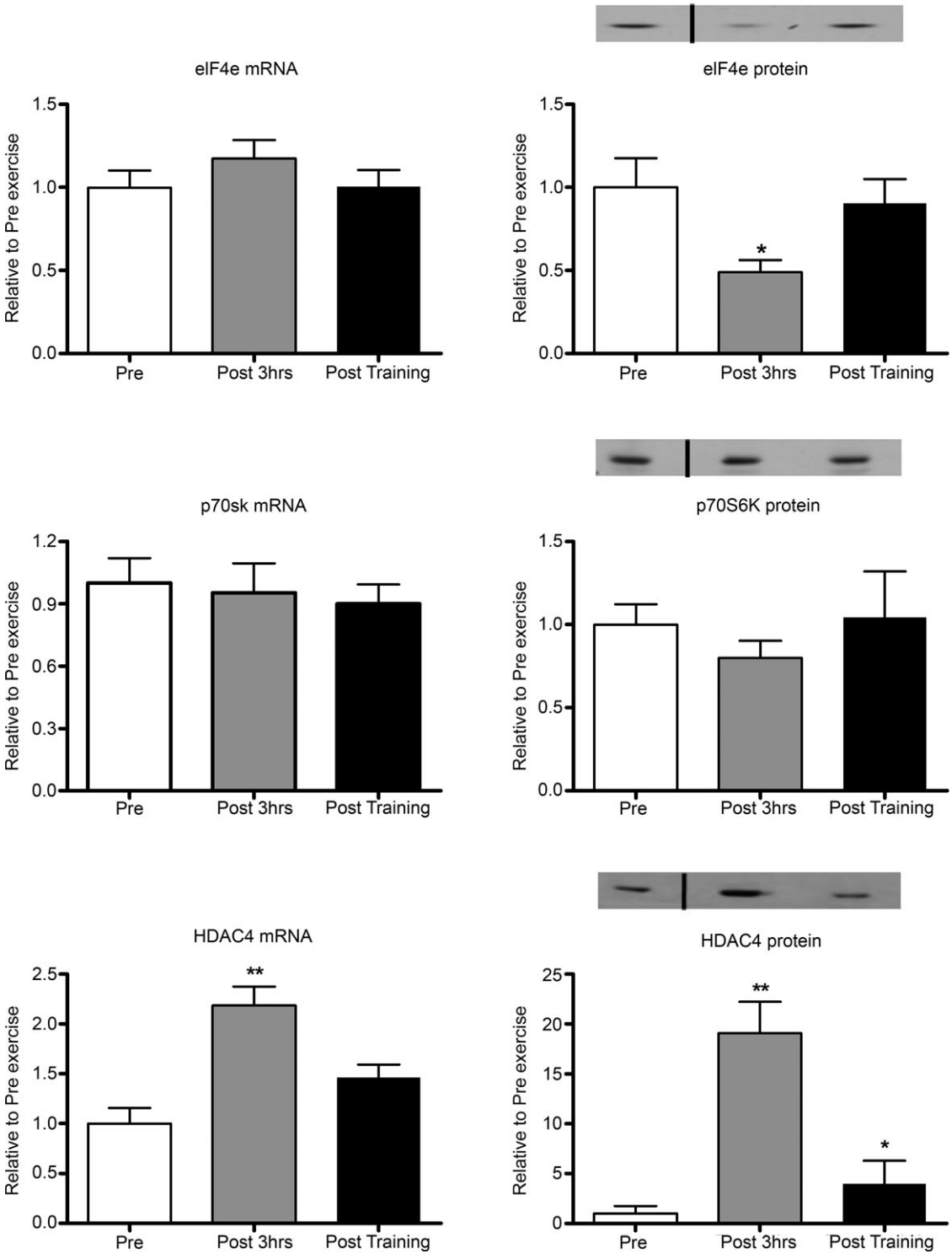

Figure 5. Regulation of targets involved in protein synthesis and transcriptional regulation following a single bout of moderate-intensity endurance exercise and short-term training
Pre, sample taken before the acute exercise bout; Post 3 hrs, sample taken 3 h after the acute exercise bout; Post training, sample taken 2 days after the 10 day training programme. *Significantly different from other groups, $P < 0.05$; **significantly different from other groups, $P < 0.01$. The black vertical lines in the densitometry images indicate where one earlier time point between the pre and Post 3 hrs time points was removed from the blot. This time point was not analysed for other targets due to a lack of tissue and removed from these blots for consistency with the other results. All samples for each subject were run on the same gel. $n = 9$ subjects per group with data presented as means ± SEM.

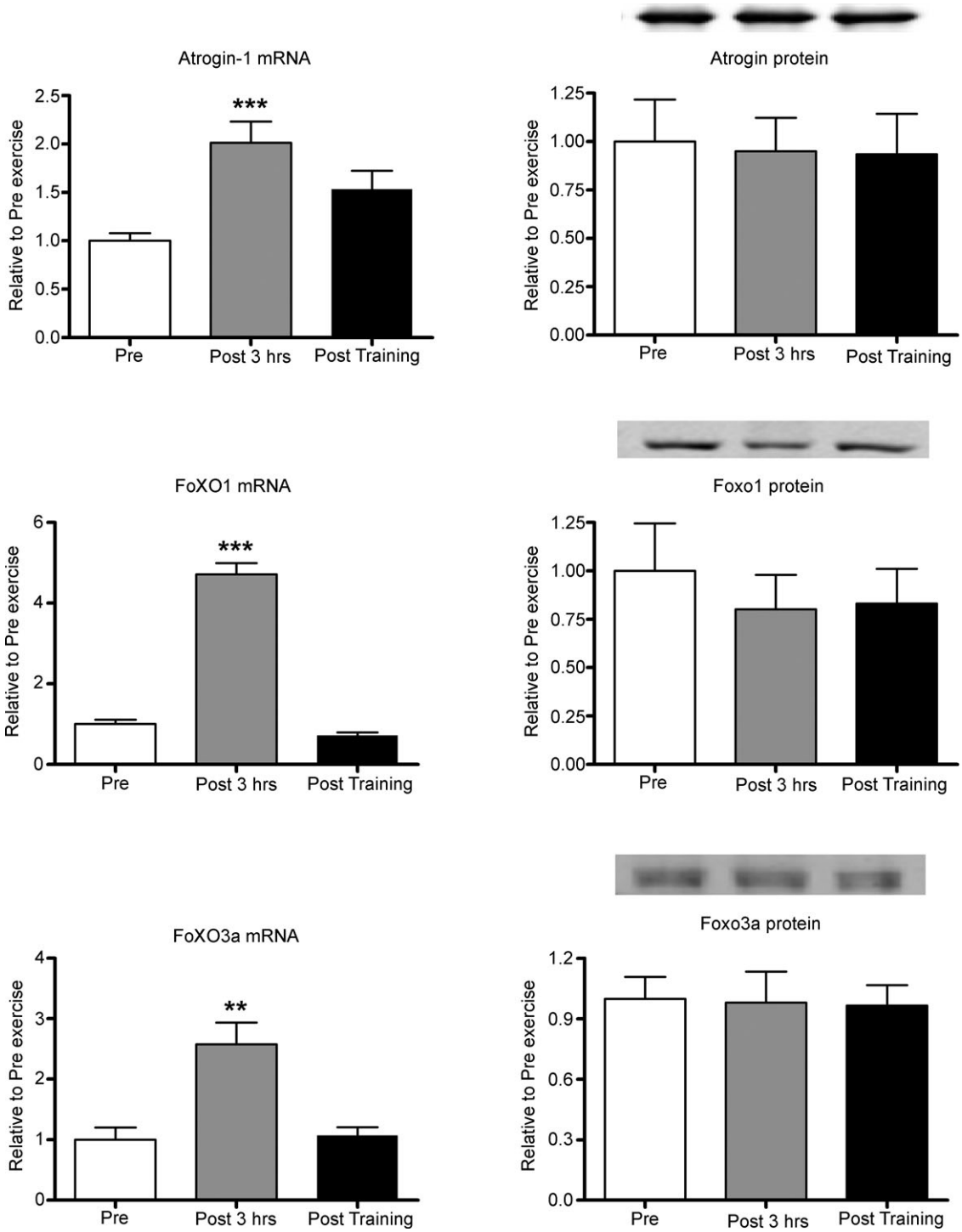

Figure 6. Regulation of targets involved in protein breakdown following a single bout of moderate-intensity endurance exercise and short-term training
Pre, sample taken before the acute exercise bout; Post 3 hrs, sample taken 3 h after the acute exercise bout; Post training, sample taken 2 days after the 10 day training programme. **Significantly different from the other groups, $P < 0.001$; ***significantly different from other groups, $P < 0.001$. Only 8 of the 9 subjects are represented due to insufficient sample remaining for 1 subject. Data presented as means ± SEM.

subjects in the present study may have had more muscular stress due to lack of familiarity with cycling exercise. This may account for the prolonged response of the miRNAs measured here. Of the myomiRs measured, only miR-1 remained elevated following 10 days of training. However, it has been observed that after 6 weeks of moderate-intensity endurance training miR-1 and -133a are reduced (Keller *et al.* 2011) and after 12 weeks of high-intensity endurance training, miR-1, -133a, -133b, and -206 are reduced (Nielsen *et al.* 2010). The differences between studies may arise from the training status of the subjects and the duration of the training period or may be a reflection of temporal changes in muscle adaptations that occur with endurance exercise training. The effect of the acute upregulation of these myomiRs following an endurance exercise bout is unknown. miR-1 and -206 increase muscle cell differentiation and inhibit proliferation, while miR-133a/b has the opposite effect

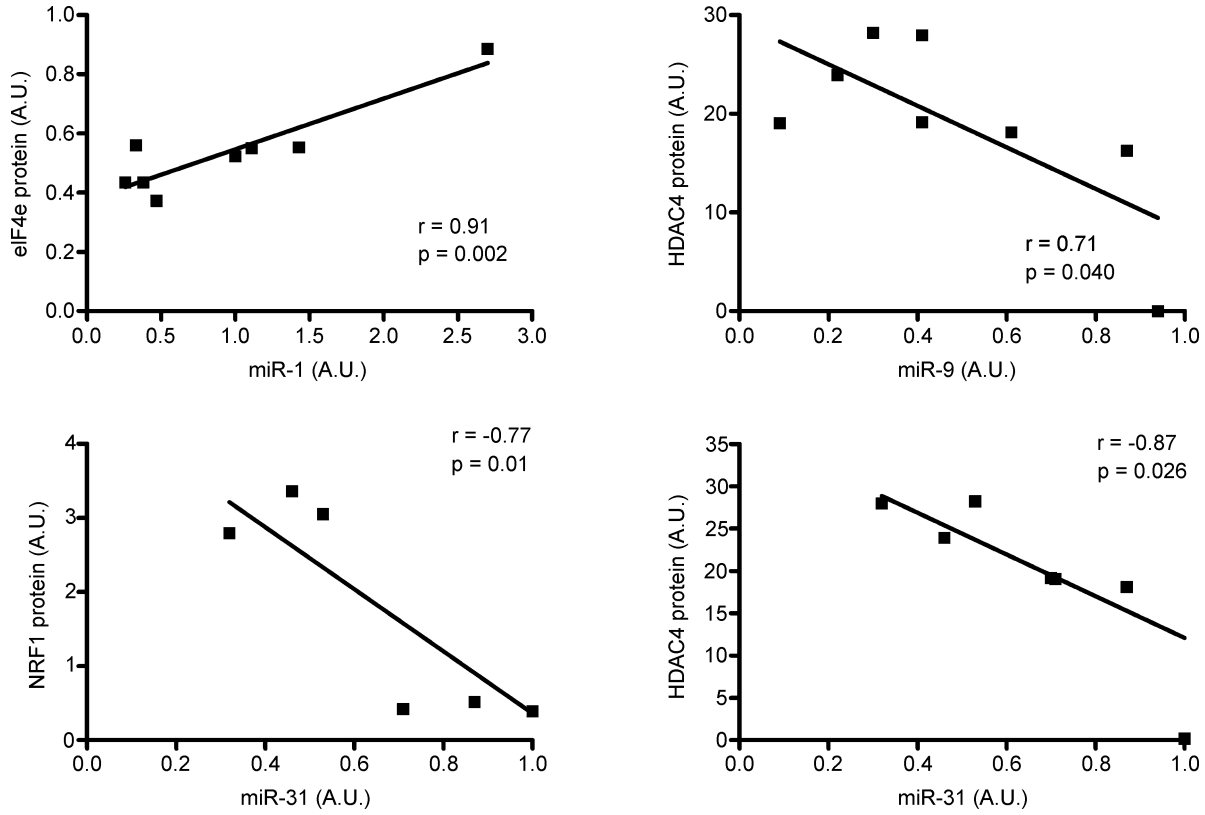

Figure 7. Correlation analysis between miRNAs and predicted protein targets 3 h following the single bout of moderate-intensity endurance exercise
Only 8 of the 9 subjects are represented in these correlations due to insufficient sample remaining for 1 subject. Data presented as means ± SEM.

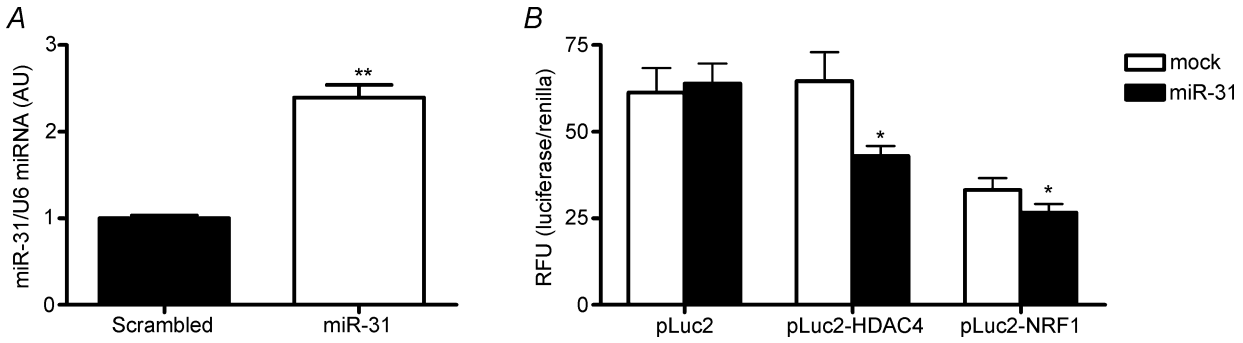

Figure 8. Luciferase reporter assay to test the relationship between miR-31 and HDAC4 and miR-31 and NRF1in C2C12 myoblasts
$N = 3$ independent experiments with 3 replicates per experiment. pLuc2, empty luciferase control vector; p Luc2-HDAC4, luciferase vector contacting the HDAC4 3-UTR; p Luc2-NRF1, luciferase vector contacting the NRF1 3-UTR; mock, control miRNA.

(Chen *et al.* 2006; van Rooij *et al.* 2008). Acute endurance cycling exercise increases myogenin (Kadi *et al.* 2004), a positive regulator of myogenesis. Additionally, endurance training remodels skeletal muscle as an adaptive response to enhance metabolic, mitochondrial and contractile function and to repair damage to myofibres (Seene *et al.* 2009; Egan *et al.* 2011). The regulation of these miRNAs should play a positive role in regulating skeletal muscle myogenesis as the muscle adapts to the stress of exercise.

miR-9, -23, -29, -31 and -181 are dysregulated in skeletal muscle of patients with various neuromuscular disorders and dystrophies (Wang *et al.* 2008; Eisenberg *et al.* 2009; Greco *et al.* 2009; Russell *et al.* 2012). Following acute endurance exercise miR-9, -23a, -23b, and -31 were downregulated, with lower miR-31 levels following 10 days of training. In contrast miR-181 and miR-29b were elevated after acute exercise. The genes targeted by these miRNAs in skeletal muscle following acute endurance exercise are unknown. miR-9 regulates *Sirt*1 in pancreatic β-cells with a reduction in miR-9 resulting in an increase in *Sirt*1 (Ramachandran *et al.* 2011). Endurance exercise increases *Sirt*1 (Dumke *et al.* 2009). Whether this exercise-induced adaptation requires the reduction in miR-9 is unknown.

Loss-of-function/gain-of-function studies in proliferating vascular smooth muscle cells confirm that increases in miR-31 have a positive effect on proliferation (Liu *et al.* 2011). miR-31 is increased in regenerating muscle (Greco *et al.* 2009) and in muscle samples from patients with diseases such as ALS, neurogenic disease and Duchene muscular dystrophy (Greco *et al.* 2009; Russell *et al.* 2012). This increase in miR-31 may be an attempt to regenerate and delay skeletal muscle atrophy in these conditions. As acute endurance exercise increases muscle cell proliferative capacity (Choi *et al.* 2005), the decrease in miR-31 after acute endurance exercise reported here suggests that miR-31 may not play a role in regeneration of muscle cells, if this process was active. We also observed an increase in miR181a, supporting observations in mice following treadmill running (Safdar *et al.* 2009). miR-181 is suggested to inhibit Hox-A11 expression; the latter a repressor of MyoD (Naguibneva *et al.* 2006). The role of miR-181a in skeletal muscle following endurance exercise is unknown; however, it may promote skeletal muscle remodelling by negatively regulating repressors of myogenesis (Safdar *et al.* 2009). The only other non-myomiR that was increased post endurance exercise was miR-29b. miR-29b levels are increased during human myotube differentiation and in regenerating mouse muscle (Wang *et al.* 2008). Rescuing miR-29 in myoblasts isolated from muscles of mice with chronic kidney disease improves differentiation into myotubes (Wang *et al.* 2011). The upregulation of both miR181a and miR-29b in muscle following endurance exercise may be a response to assist with muscle repair, regeneration and remodelling. It is worth noting that after 6 weeks of moderate-intensity endurance training miR-29b, as well as a suite of other miRNAs, was downregulated (Keller *et al.* 2011), further suggesting that a temporal regulation, at least for miR-29b, may be a reflection of different adaptations controlled by miRNAs.

A causal relationship between the regulation of miRNAs and potential protein targets in human skeletal muscle following exercise is beyond the scope of the present study. However, the use of bioinformatics to predict potential targets of miRNAs, combined with linear regression analysis, provides correlational evidence for potential regulation. Within the limitations associated with prediction tools and correlational assessment, the exercise-induced regulation of HDAC4 proteins may involve miR-9 and miR-31, while the regulation of the NRF1 protein may involve miR-31. In support of this prediction, the miR-9 regulation of HDAC4 has been validated in Waldenström macroglobulinemia cells (Roccaro *et al.* 2010). Using a reporter assay, we were unable to validate a direct relationship between miR-31 and HDAC4 or miR-31 and NRF1 *in vitro*. This negative *in vitro* result obtained under non-physiological conditions, does not exclude the possibility that such interactions are possible in human skeletal muscle following endurance exercise.

The expression levels of several miRNA species are sensitive to muscle contraction stimulated by endurance exercise. However, the exact stress signals initiating these changes in miRNA expression are unknown. During exercise, numerous stress signals are initiated by changes in motor nerve activation and calcium concentrations, mechanical and contractile stress, increased muscle blood flow and shear stress, hormonal and metabolic stress, which activate intracellular signalling pathways controlling skeletal muscle gene transcription and translation (Bassel-Duby & Olson, 2006; Koulmann & Bigard, 2006; Favier *et al.* 2008; Russell, 2010). Identification of the primary stressor/s activated by endurance exercise that regulate miRNA expression should be investigated in the future.

In conclusion, the stress associated with a single bout of moderate intensity cycling, performed by untrained males, causes an increase in muscle-enriched miRNAs, including miR-1, -133a and 133-b, as well as miR-181a. It also decreases miR-9, -23a, -23b and -31; species upregulated in several muscle wasting disorders. miR-1 remained elevated, while miR-29b and -31 remaining decreased, following short-term training of 10 days. miRNAs play important roles in human health and their rapid, yet transient regulation with endurance exercise suggests that they contribute to the positive adaptations of endurance exercise. Identifying the exercise-mediated intracellular stress signals regulating miRNAs will be an important

progression in our understanding of their potential role/s in human health.

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Additional information

Competing interests

The authors declare no conflict of interest.

Author contributions

A.P.R. was responsible for the development of the project, data analysis, interpretation and prepared the manuscript. S.L. and H.B. performed and analysed Western blotting; S.W. and T.A. completed the in vitro miRNA, mRNA and protein analysis; I.G. and E.L.B. performed the bioinformatics and miRNA analysis; A.V.C., J.R.Z., R.J.S., N.S. were involved in the development of the project; G.D.W. completed gene expression analysis and assisted with the development of the project. All authors were responsible for revising the manuscript throughout the review process.

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